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The Effect of Shear on Aqueous Alkaline Protein Solutions

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THE EFFECT OF SHEAR ON AQUEOUS ALKALINE PROTEIN SOLUTIONS

by

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ABSTRACT

The effect of shear on aqueous alkaline protein solutions of casein and a medium viscosity soybean was studied. A Haake Rotovisco viscometer and an Eppenboch agitator were used as shear sources. The light scattering technique was employed to note the changes in molecular weight.

The protein solution was sheared at 20% solids, and clarified by centrifugation and filtration. The solutions were investigated with the light scattering photometer, at pH 10.3, 13% ammonium hydroxide (based on weight of protein), 23°C. and between 0.2 and 2.0% concentration.

Results showed that denaturization, causing aggregation, resulted from pressure filtration during clarification of the protein solutions. Light scattering measurements on concentrations less than 0.5%, deviated from light-scattering theory. Casein aggregates decreased in molecular weight with shear. Shearing stresses apparently dissociate or disperse the protein molecule.

THE ORIGIN AND THE DEVELOPMENT OF THE PROBLEM

Recent developments in the paper industry have brought about sharp increases in coating application speeds. Today, most paper companies are faced with the problem of increasing production speeds to meet the demands of the market and to keep abreast with competition. Today's high speed coaters call for high solids coatings. High solids are needed so dryer capacities do not have to be enlarged and to insure sufficient coat weight during single application.

The property requirements for adhesives used in high solids coatings are critical. They must possess desired rheological properties and high adhesive and cohesive strength properties. The uniformity of the adhesive, as supplied by a supplier, also must be considered.

During the application and metering of coatings to paper shearing stresses of high magnitude are developed. The increase in coating speeds and solids content magnifies the shear effects. The shearing stresses developed during coating applications could possibly alter the adhesive used in the coating.

The purpose of this study was to determine the effect of shear on aqueous alkaline protein solutions. Because of the complexity of most paper coating dispersions, a simple aqueous alkaline protein system was chosen for investigation. This reduced the number of variables and rendered study feasible. The proteins

selected for study were a medium viscosity soybean protein and casein. The effect of shear on these solutions was to be observed by changes in the molecular weight of the protein molecule.

SOURCE AND MODIFICATION OF CASEIN AND SOYBEAN PROTEINS

Industrial soybean protein used in the paper industry is defined as alkaline soluble and acid precipitatable. The protein fractions are obtained from solvent extracted soybean flakes. The oil-free flakes are slurried in water, to which small amounts of alkali are added to increase the solubility of the protein. The undissolved flakes are removed and the solution is clarified. The clarified solution is brought to its isoelectric point by acid addition, precipitating the protein curd which is dewatered and dried (1).

Casein is prepared from skim milk by adding acid to precipitate the casein at its isoelectric point (2).

The soybean protein isolated by the above method is termed unmodified and is not suitable as a coating adhesive. Modification of the protein must be performed to reduce its solution viscosity. During modification the structure of the raw protein is radically altered.

Modification of proteins can be accomplished by physical, chemical, and biological action. Physical actions which alter proteins include heat, freezing, pressure, irradiation, ultrasonic waves, and surface forces. Chemical agents which alter proteins

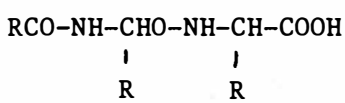
include, hydrogen and hydroxyl ions, cations and anions, organic solvents and solutes, and oxidizing and reducing agents. Biological actions altering proteins are enzymes which are catalytically active proteins (3).

Proteins for use in paper coatings are normally modified by alkaline hydrolysis to reduce their molecular weight and viscosity. Generally, sodium hydroxide is added to a protein slurry and the temperature is elevated where hydrolysis takes place, decreasing the molecular weight and viscosity.

CHEMICAL STRUCTURE OF SOYBEAN AND CASEIN PROTEINS

Protein chemists state that casein and soybean proteins are polymers containing peptide linkages between amino acids formed by the elimination of water. Proteins are described as having primary, secondary, and tertiary structures. The primary structure is the structure imposed by the order in which different amino acids are linked together. Interaction between polar groups within the same and adjacent chains stabilizing configurations is known as the secondary structure. The tertiary structure refers to the specific folding of chains as in globular proteins found in nature (3).

The basic structure of casein is as follows:



where R may be -H, $-CH_3$, $-C_6H_5$, $-(CH_2)_4$, $-NH_2$, $-CH_2$ COOH, etc. The

type and arrangement of the reactive side groups, determine to a major extent the properties of casein.

MOLECULAR WEIGHT AND SHAPE OF CASEIN AND SOYBEAN PROTEIN

Unmodified industrial soybean protein has a globular shape. Calvert (4) describes unmodified industrial soybean protein as having a weight average molecular weight of over 200,000 and possessing a flattened spherical shape in solution. Modified, medium and low viscosity soybean protein grades are characterized as having weight average molecular weights from 90,000 to 111,000 and possessing shapes of football-like rods.

The number average molecular weight of whole casein ranges from 33,600 to 375,000 (5) (2). The shape of the molecule was felt to be approximately globular. The molecule is described as highly hydrated in solution by Hostettler (6). Sullivan (7) el at describes the whole protein as having a weight average 70% of the molecular weight at 24,000.

AGGREGATION AND DISSOCIATION

Halwer (8) reports that light scattering molecular weight determinations of native proteins are essentially independent of electrolyte concentration, but scattering determinations on α- and β - casein fractions show increased aggregation with increased electrolyte concentration. α- and β - casein are defined as two protein fractions found in whole casein. Appendix I

(9) contains a flow diagram showing the procedure for the isolation of α- and β - casein protein fractions from whole casein protein fractions from whole casein. Various studies performed on α- and β - casein fractions indicate that proteins altered or fractionated from their native globular state tend to aggregate at low ionic strength, below 0.3 and low protein concentrations. Ionic strength is defined as $1/2 \sum (c z^2)$ where c is concentration of ions and z is the charge on the ions (10). It was also found that high pH values favor less aggregation and less dependence on electrolyte concentration.

Dreigen, Noble, and Waugh (11) state; pH of 12, ionic strengths of 0.3 to 1.2 and room temperature are conditions that casein fractions are completely dissociated to subunits. Subunits are described as the smallest molecular unit of a protein that can exist in solution and still maintain its identity as the specific protein. Unpredictable aggregation, usually irreversible, occurred (1) at ionic strengths below 0.3/pH 12 (2) when a rapid pH-ionic strength increase was imposed at pH 7 and (3) on surface denaturization caused by bubbling or foaming.

Calvert (4) suggested that because of soybean proteins molecular size true solutions are not formed, but rather colloidal solutions. Aqueous colloidal solutions of protein consist of molecular aggregates above their isoelectric point, enveloped with tightly bound water.

From the information cited above, protein molecules appear to be easily altered and radically changed from their natural state. The conditions favorable for light scattering measurement appear to be ionic strengths of 0.3 to 1.2, room temperature, pH 10 to 12, and concentrations of .004 to .015 g./ml. Under these conditions protein aggregates should be dissociated to subunits. Thus, any change in the molecular weight of the subunit under shear can be noted.

EXPERIMENTAL

EXPERIMENTAL DESIGN

The approach followed in investigating the effects of shear on an aqueous alkaline protein solutions was (1) the selection of a shearing device, and (2) the selection of a method to detect any changes. A Haake Rotovisco viscometer and an Eppenboch high shear agitator were chosen as shearing devices.

The variable chosen for study was the change in weight average molecular weight. Molecular weights were to be determined using a photometer and applying light scattering technique. The experimental work consisted of taking molecular weight measurements before and after shearing stresses were applied to protein solutions.

Since light scattering measurements are sensitive to extraneous materials such as dust particles, clarification of the protein solutions was necessary. The protein solutions were clarified by centrifugation and pressure filtration through membrane filters.

EXPERIMENTAL EQUIPMENT

Haake Rotovisco and Eppenboch

A Haake Rotovisco viscometer (series 67356, with measuring head 500/50 and temperature control to $\pm$ .1^{o}C.) was used as a shearing source. This model has nine speeds and will give shearing rates using beaker and bob assembly up to 1370 sec.$^{-1}$.

An Eppenboch is a high shear dispersing device. It consists of a rotator-stator arrangement. The rotator is a sharp edged impeller and rotates in close clearance with the stator. Although the shearing rate of the Eppenboch has not been recorded it is thought to be around 500,000 $sec.^{-1}$.

Light Scattering Photometer

A Brice Phoenix Model 2000 light scattering instrument was used for weight average, molecular weight determinations. The cells used were D-101 and D-104 semi-octagonal cells with 40 ml. and 15 ml. minimum capacities, and a C-105 cylindrical cell with 15 ml. minimum capacity. The semi-octagonal cells were used for molecular weight determinations by the dissymmetry method. These cells allowed scattering measurements at 45^{o}, 90^{o} and 135^{o}. The dissymmetry method of data handling involved the ratio of light scattered at 45^{o} and 135^{o} along with the light scattered at 90^{o} for molecular weight calculations.

The cylindrical cell was used for molecular weight determinations by the Zimm method. This cell allows light scattering measurements at all angles between 45^{o} and 135^{o}.

Filter Assembly and Centrifuge

A Millipore filter was used for clarification of solutions for light scattering measurements. The Millipore assembly used was a pressure filter. The filters used were polyesters with pore sizes of .45 micron.

An International Centrifuge (model 2) was used for centrifugation. The centrifuge was run at 2700 R.P.M. which corresponds to 1550 g.

PRINCIPLE OF LIGHT SCATTERING

Light scattering is based on the fact that when an oscillating electric field of light transverses any real medium, it induces oscillating electric moments to the particles composing the medium. The induced particles act as secondary sources of radiation of the same wavelength, thus scattered light results. Scattered light of the same wavelength, thus scattered light results. Scattered light of the same wavelength results provided the frequency of the induced oscillations is far from the natural frequency of the electrons.

The amount of light scattered and the pattern in which it is scattered depends on the molecular weight, shape, and size of the particles being observed. For a full treatment of the theory of light scattering and the derivation of equations for determining molecular weight, shape and size, K. A. Stacey's Light-Scattering in Physical Chemistry (12) can be consulted. The final derived equation for molecular weight, along with the working equations used for the Brice-Phoenix Instrument are given below.

BASIC WORKING EQUATION

The basic equation, which applies to the proteins solutions being studied, was set forth by Debye (13) and Zimm (14). The relation they derived is as follows:

$$\frac{Hc}{\tau} = \frac{1}{M_w P(\theta)} + 2Ac + \ldots\ldots \qquad (1)$$

where $H = 32\pi^3 n_o^2 (dn/dc)^2 / 3\lambda^4 N$

n.= refractive index of the solvent,

dn/dc = specific refractive index gradient,

λ = wavelength of light used,

N = Avagadro's number,

c = concentration g./ml.,

P (θ) = particle scattering factor, which is a function of the scattering angle θ,

M_w = weight average molecular weight,

A = second virial coefficient which is a measure of the polymer-solvent interaction,

τ = turbidity.

The molecular weight, shape, and size of molecules in solution and the polymer-solvent interaction coefficient are found by plotting values of Hc/τ as a function of $\sin^2(\theta)$ and concentration as described by Zimm (14). For the Brice-Phoenix model 2000, the working equation for Hc/τ is as follows:

$$\frac{Hc}{\tau} = \frac{15.2\times10^{5}\,(dn/dc)^{2}\,c\,(1+\cos^{2}\theta)}{1.22\,(R_w/R_c)\,a\,K\,(r/r')\,(\sin\theta)\left[\frac{F_\theta\,G_\theta}{F_o\,G_o}_{\text{solution}} - \frac{F_\theta\,G_\theta}{F_o\,G_o}_{\text{solvent}}\right]} \quad (2)$$

where K = correction factor for cell alignment depending on the angle of viewing

R_w/R_c = correction factor for incomplete compensation of refractive effects,

r/r' = calibration factor for narrow slit system and circular cell,

a = working standard constant,

Sin θ = correction factor for the volume change on viewing the solution at different angles,

1/(1+cosθ) = correction factor applied when unpolarized light is used,

F_o = filter factor at zero degree angle,

F_θ = filter factor at angle θ

G_o = galvanometer reading at zero degree

G_θ = galvanometer reading at angle θ

The Zimm method requires that measurements be taken at a series of angles and concentrations followed by extrapolation to zero angle and concentration. An alternative method which requires an assumption of the characteristic molecular shape was chosen because of the time element. This method involves a determination of the particle

scattering factor, $1/P(\theta)$, by dissymmetry measurements. The dissymmetry of various molecules is determined by comparing the scattering at 45^{o} and 135^{o}. Stacey (12) lists tables giving $1/P(\theta)$ values associated with dissymmetry values. Using the dissymmetry method, molecular weights and polymer-solvent interaction measurements can be determined with angular measurements at 0^{o}, 45^{o}, 90^{o}, and 135^{o} at a series of concentrations.

By plotting Hc/τ versus c and extrapolating to zero concentration a value of Hc/τ at zero concentration can be obtained. Using this value along with $1/P(\theta)$, the scattering factors; the molecular weight can be found. The particle scattering factor is found by consulting dissymmetry tables (12) after the intrinsic dissymmetry is determined. The intrinsic dissymmetry is the dissymmetry at zero concentration. Dissymmetry values are plotted against concentrations. By extrapolating to zero concentration the intrinsic dissymmetry is found. At zero concentration

$$Hc/\tau = 1/M_{w} P(\theta) . \qquad (3)$$

Hc/τ is given by the intercept at zero concentration. $1/P(\theta)$ is obtained by consulting the dissymmetry table after the intrinsic dissymmetry is obtained at zero concentration. A measure of polymer-solvent interaction is given by the slope of the plot of Hc/τ versus concentration.

CLARIFICATION OF SOLUTIONS

The solution to be observed using light scattering technique must be optically clear. The scattering of the solvent is subtracted from the scattering of samples being observed. Dust and other extraneous highly scattering impurities such as polysaccharides must be free from the solution. Conditions of denaturization should be avoided in protein studies.

EXPERIMENTAL PROCEDURE

1. 40 g. of protein were slurried with 162 ml. of distilled water.

2. The sample was placed in a water bath at 60 $\pm$ 1^{o}C. for 15 minutes.

3. 13% NH_4 OH based on dry protein was added.

4. The samples were cooked with slow stirring for 30 minutes in the water bath, followed by a pH check for control at 10.3.

5. 40 ml. samples were placed in a Haake MV beaker and MV II rotor and sheared at 529 sec.$^{-1}$ for periods of 30 and 90 minutes at 60 $\pm$ 1^{o}C.

6. 10 ml. of the sheared protein were diluted to 100 ml. with a distilled water NaOH solution at pH 11.2. The resulting protein solution had a pH of 10.3 and about 2.0% concentration.

7. The 2.0% stock solution was centrifuged for ten minutes at 1550g's to remove extraneous large particles.

8. The stock solution was clarified by filtering under 20 PSI under a CO_2 atmosphere through a .45 micron Millipore filter.

9. Light scattering readings were taken at 0^o, 45^o, 90^o, and 135^o on the solvent and for five or more protein concentrations. Standardization of the Brice-Phoenix light scattering instrument was done using a polystyrene standard from the ArRo Laboratories.

10. A solids determination on the final concentration of scattering solution was taken for back calculation of all solutions.

11. Step five of the above procedure was omitted and measurements were taken on the unsheared protein.

PRESENTATION AND DISCUSSION OF RESULTS

Table I summarizes the experimental results. The table gives the shearing rate and time, the intrinsic dissymmetry, the particle scattering factor and the weight average molecular weight.

Figures 1 and 2 are dissymmetry versus concentration plots. The intrinsic dissymmetry of the protein being investigated is given by the $[\mathcal{Z}]$ intercept of figure 1 and 2. The data points of both figures 1 and 2 lead to easy extrapolation to zero concentration for intrinsic dissymmetric determinations.

Figures 3 and 4 are $Hc/\mathcal{T}$ versus c plots. The data of both figures 3 and 4 show scattering of points at concentrations below .5%. The data points above .5% lead to easy extrapolation to zero concentration for molecular weight values.

The experimental results listed in Table I show that high weight average molecular weights were obtained for all proteins investigated as compared to the literature values cited. The high molecular weight values obtained indicate that large molecules and or molecular aggregates are present in the protein solutions. Since literature values cited state that a medium viscosity soybean protein has a weight average molecular weight of from 90,000 to 111,000, it is apparent that the molecules observed may consist of molecular aggregates or possibly polymerized structures. Large molecules appear to be in both sheared and unsheared protein solutions.

Soybean protein increased in molecular weight about four fold as a result of thirty minutes shearing. Molecular weight rose from 2,000,000 for the unsheared protein to 7,600,000 following shearing. A possible explanation for the large increase in molecular weight following shearing is that denaturation may have taken place causing extreme aggregation.

Casein was found to have a molecular weight of 4,100,000 in contrast to soybean protein; it decreased in molecular weight with shearing. Casein's molecular weight decreased to 3.8 million with shear by the Haake and 2.6 million with shear by the Eppenboch. Native casein solutions appear to contain some large molecular aggregates. It is proposed that these aggregates became somewhat dispersed or dissociated with shear. This fact may account for the drop in molecular weight of casein.

Since unsheared soybean and casein proteins appeared to be highly aggregated under solution conditions which favor dissociation, the experimental procedure must be considered as a possible cause of aggregation. Neurath et al. (15) reported heat, pressure and surface forces could alter or modify proteins. Dreizen et al. (11) as earlier cited, stated aggregation can result because of denaturization caused by surface forces. Surface forces developed during pressure filtration of the solutions could be the cause of aggregation. This explains why high molecular weight aggregates appeared to be present in unsheared protein, as well as, in sheared protein. Two theories on aggregation

were put forth by Bain et al. (16) in 1961. The first being cross-linking by a sulfhydryl-disulfide reaction. The second being the air oxidation of sulfhydryl groups in a precipitated state.

The type of bonding causing aggregation is very hard to determine. This arises from the fact that the basic units which make up a protein contain many reactive side groups. Casein, for instance, contains by weight .15% anionic groups, .11% cationic groups, .25% nonionic polar groups and .363% nonpolar groups (17). Taking into account the molecular weight of casein and the above cited percentages, the number of reactive groups which can possibly interact or react with each other are numerous. With this enormous number of reactive groups, the chief forces involved in aggregate formation can only be postulated to include both ionic and secondary bonds of the dispersion, orientation and hydrogen bonding types.

TABLE I

SUMMARY OF WEIGHT AVERAGE MOLECULAR WEIGHTS
ON PROTEIN AND CASEIN SOLUTIONS

Sample Number	Protein Type	Shearing Rate Sec.$^{-1}$	Type of Shear and Time Sheared	Intrinsic Dissymmetry,	Particle Scattering Factor $\frac{1}{\overline{P}(\theta)}$	Weight Average Molecular Weight
1	Soybean Medium Viscosity	None	None	3.58	2.20	2,000,000
2	Soybean Medium Viscosity	529	Haake Rotovisco 30 minutes	3.07	2.10	7,600,000
3	Casein	None	None	4.10	2.44	4,100,000
4	Casein	529	Haake Rotovisco 90 minutes	3.75	2.35	3,840,000
5	Casein	5×10^5 Estimated	Eppenboch 30 Minutes	3.12	2.10	2,570,000

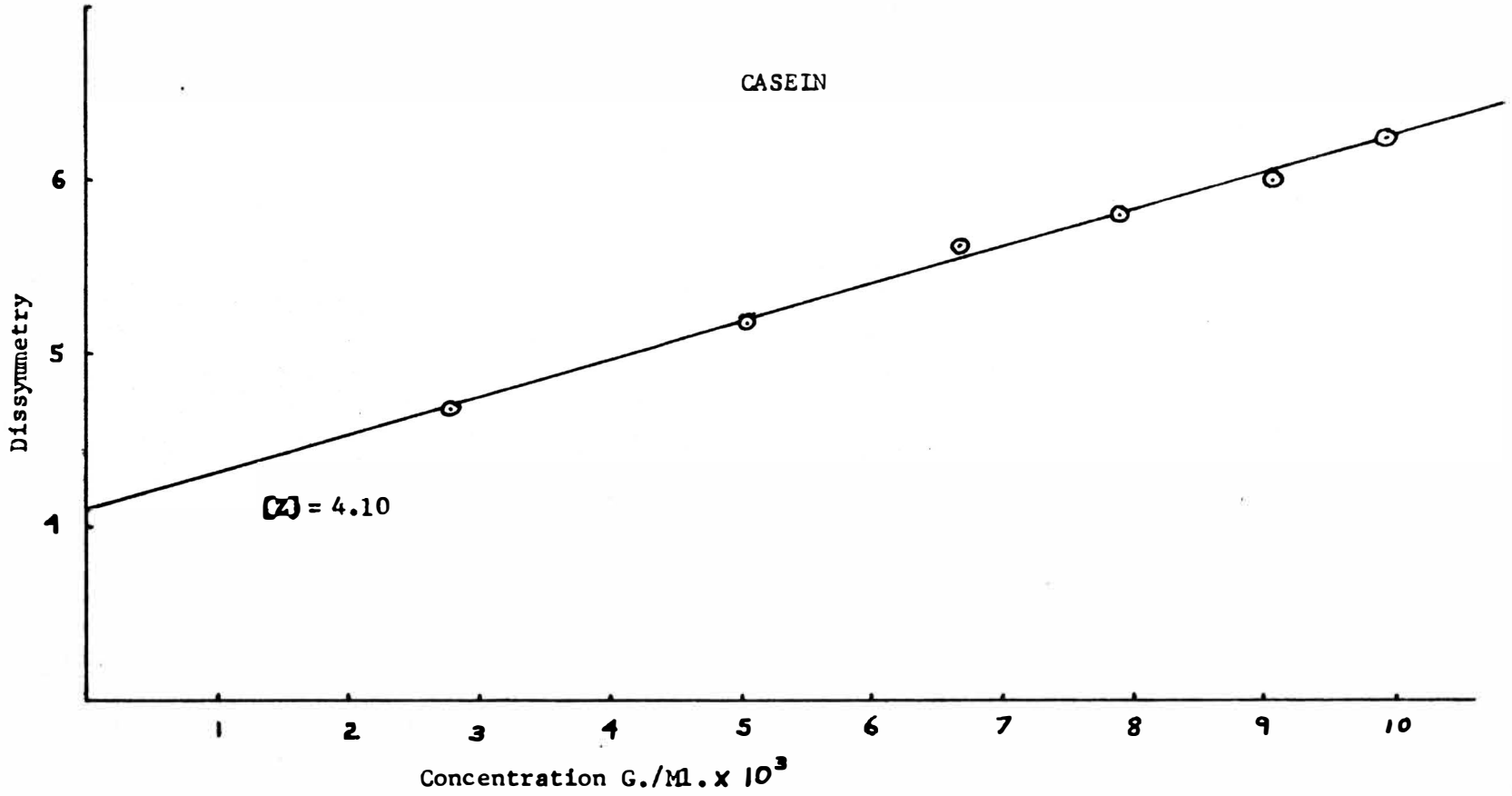


Figure 1. Values of Dissymmetry as a Function of Concentration for Determination of the Intrinsic Dissymmetry. Intercept is the Intrinsic Dissymmetry.

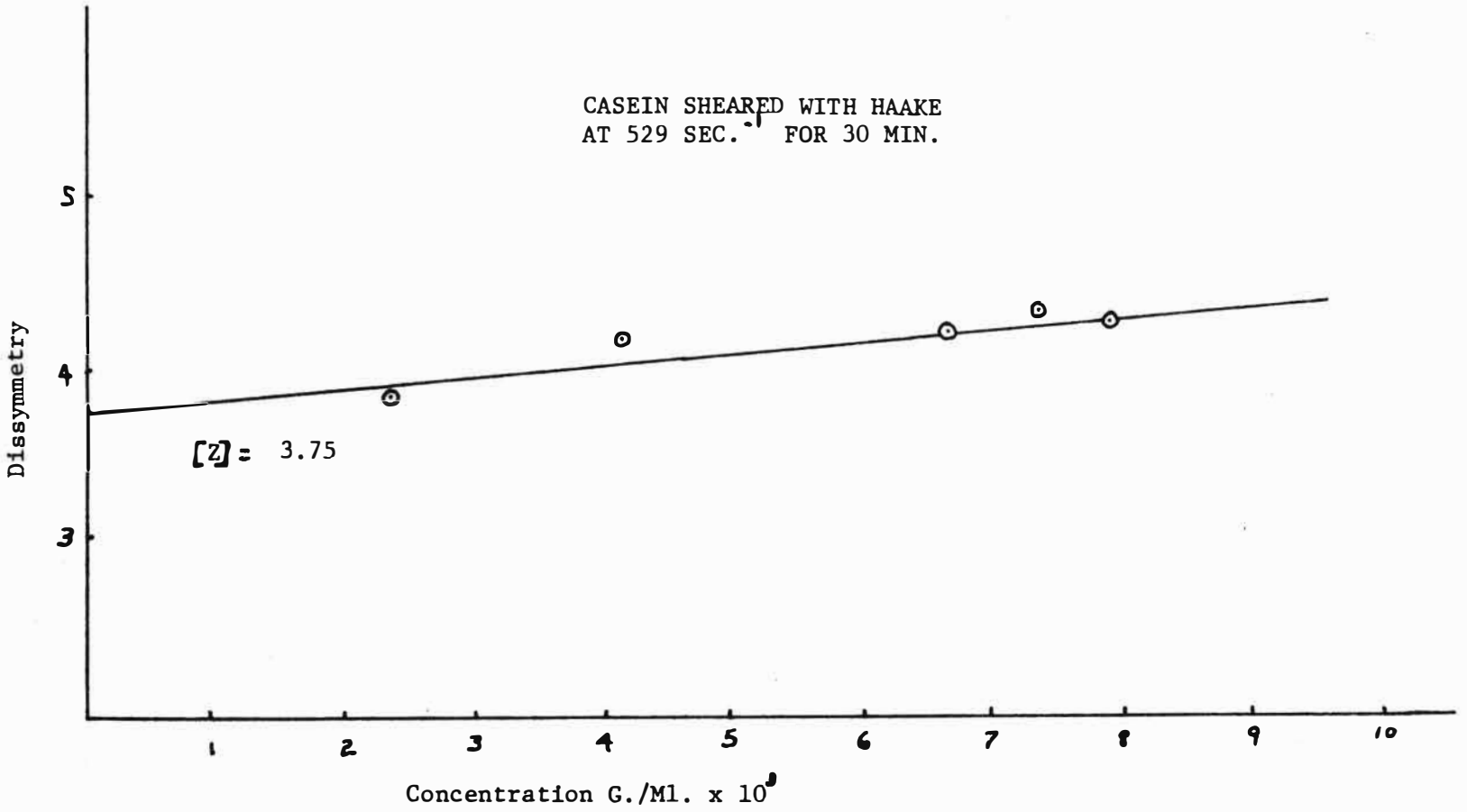


Figure 2. Values of Dissymmetry as a Function of Concentration for Determination of the Intrinsic Dissymmetry. Intercept is the Intrinsic Dissymmetry.

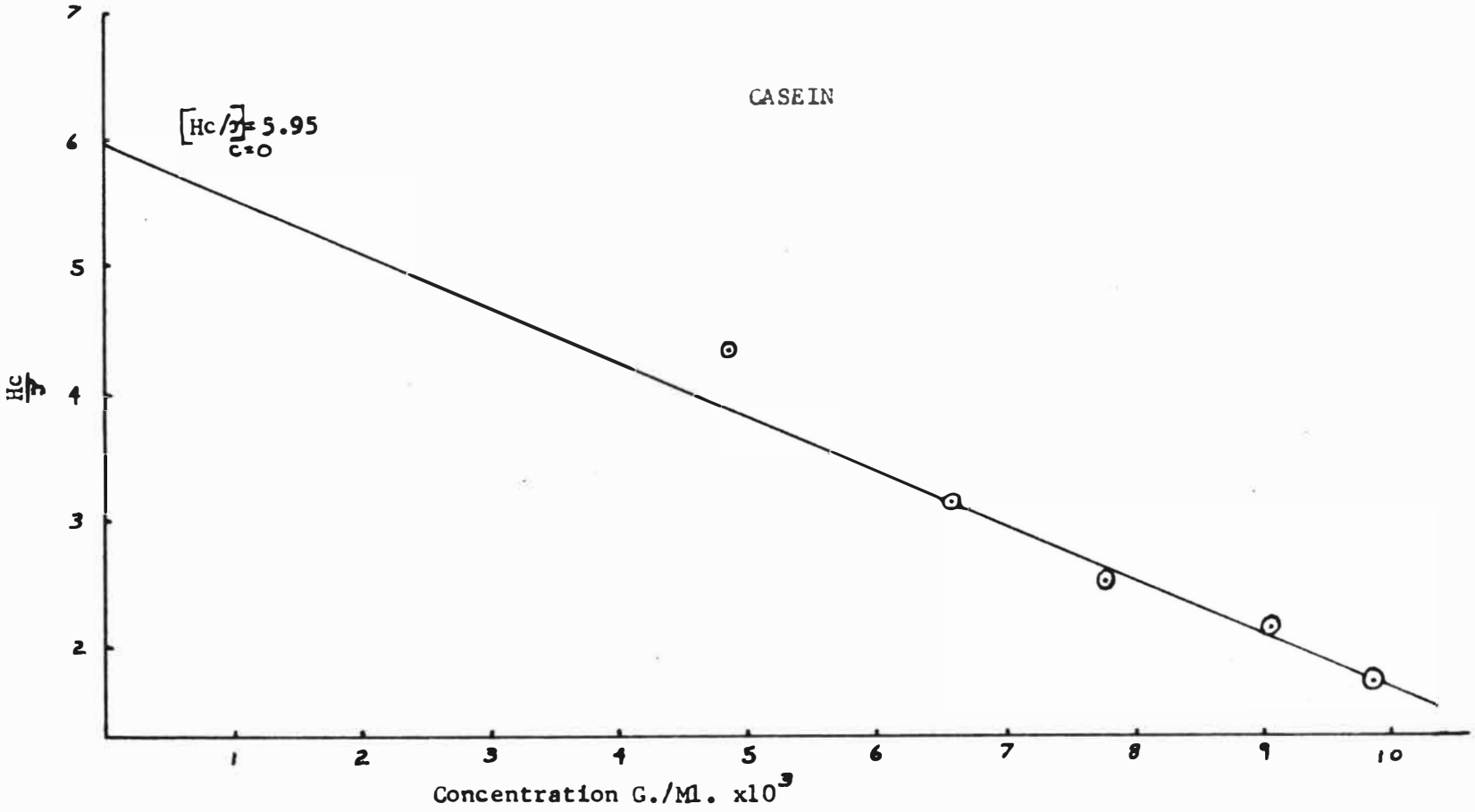


Figure 3. Light Scattering Data at 90°. Hc/τ are Plotted Aganst Concentration. The Value of Hc/τ at Zero Concentration is Given by the Intercept.

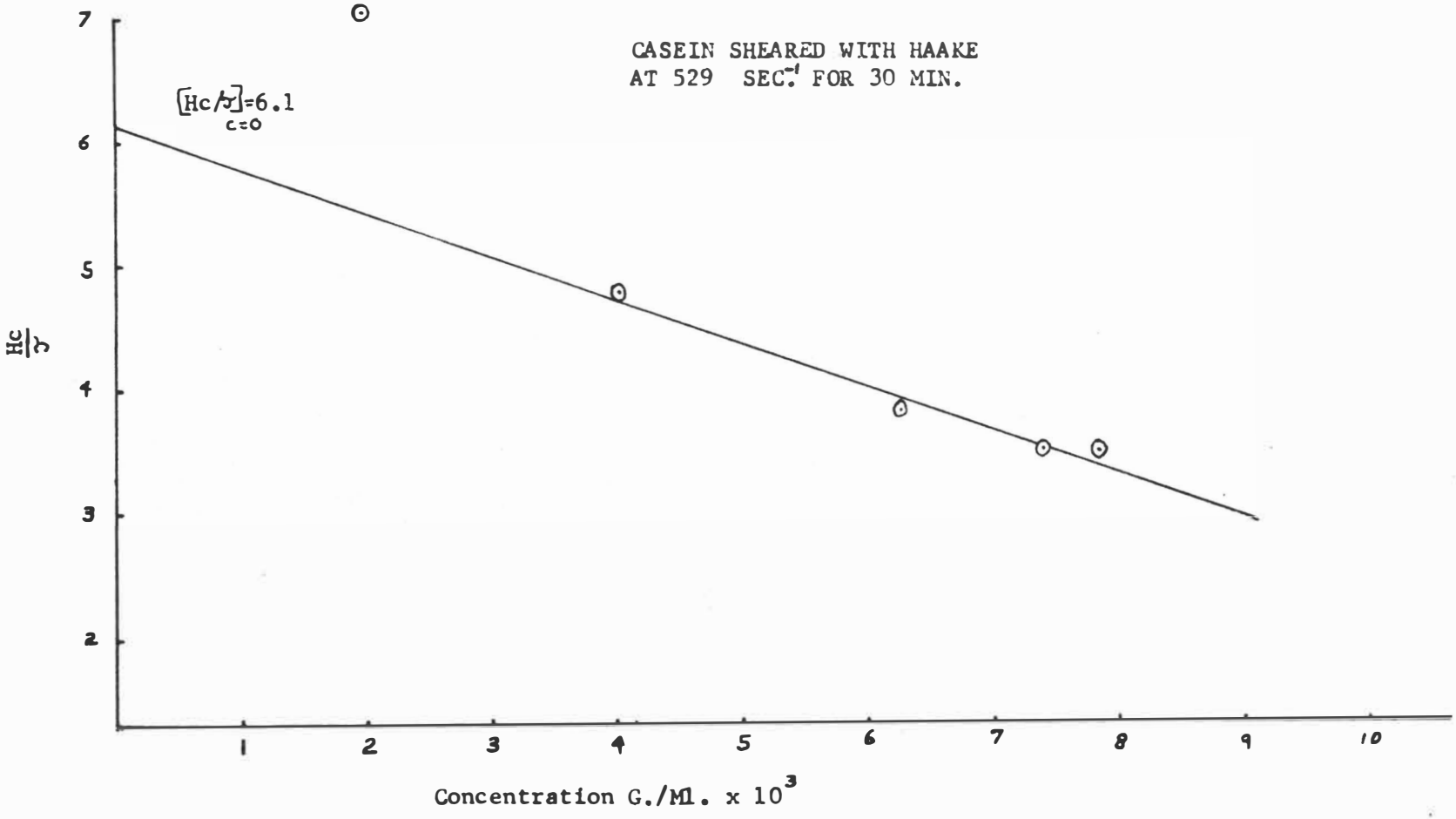


Figure 4. Light Scattering Data at 90° . Hc/τ are Plotted Aganst Concentration. The Value of Hc/τ at Zero Concentration is Given by the Intercept.

CONCLUSIONS

With the objective of this thesis in mind, some general conclusions can be drawn. First, because of the complexity of the protein system light scattering measurements are very difficult to reproduce. From the practical standpoint, extraneous materials are very hard to remove from the protein solutions. Optical clarity, which light scattering solutions must have, is very difficult to obtain by filtration because the surface forces may seriously alter the protein system to be observed. The variables involved in light scattering measurements on a aqueous alkaline protein solutions are numerous. Along with the variation found in the measuring instrument, many errors can arise during successive dilution of the low concentration solutions used for measurements.

Regardless of how the protein molecule has been altered, its observed molecular weight in solution is dependent on its state of aggregation or dissociation. Thus the molecular weights determined by light scattering technique are dependent on the solution conditions which affect aggregation and dissociation. The solution conditions which affect aggregation and dissociation are pH, ionic strength, temperature and concentration.

From the results of this study, the molecules of casein were shown to disperse or dissociate with shear. The more intense the shearing rate, the more the dispersion or dissociation of casein.

Although soybean protein appears to aggregate on shearing, more work is needed in this area. The clarification of the soybean protein solution is very difficult.

The state of protein appeared to be altered radically during pressure filtration in clarifying the protein solutions for light scattering studies. Concentrations below 0.5% g./ml. could not be used for soybean and casein protein light scattering measurements because of apparent deviation from light scattering theory. Extreme difficulty was encountered when these points were used in extrapolating to zero concentration.

RECOMMENDATIONS

In order to prove the existence of molecular aggregates in solution after pressure filtration an alternate method of clarification should be used. Alexander and Block (18) recommend ultracentrifugation of light scattering samples for four to six hours at 20,000 g's for satisfactory clarification. By using ultracentrifugation to clarify samples before and after pressure filtration, a comparison can be made of molecular weights to determine if higher weight average molecular weights are present after pressure filtration.

Another approach, to prove that aggregates are present, is to note changes in molecular weights when either the temperature, pH or ionic strength of the protein solution is varied. If a decrease in molecular weight is noted then the large molecules observed in the results of this experiment must be made up of aggregated subunits.

The solution concentration should be kept between 0.5% and 1.0% with pH values of around 12 and ionic strengths between 0.5 and 1.0. These conditions are most favorable for dissociation of the protein into subunits (the lowest molecule weight of a protein that can exist and still be identified as that protein). The pH and ionic strength conditions can be met using urea as described by Halwer (8).

LITERATURE CITED

1. Circle, S. J., "Soybeans and Soybean Products" (K. W. Markley, editor), Vol. I, Chapter 8, New York, N. Y., Interscience Publisher (1950).

2. Salzberg, H. K., Georgevits, L. E., and Cobb, R. M. K., Tappi Monograph 22 p. 106 (1961).

3. Jirgensons, B., "Natural Organic Macromolecules", Chapter 12, pp. 183-203 (1962).

4. Calvert, F. E., Tappi 38, pp. 145A-147A (1955).

5. Mellander, E., Biochem. Vol. 300 p. 240 (1939).

6. Hostettler, H., Rychener, E., and Kunzee, L., Lander, Jahrb Schweiz, Vol. 62, p. 31 (1949).

7. Sullivan, R. A., Fitzpatrick, M. M., Stanton, E. K., Annino, R., Kissel, G., and Palermito, F., Arch. Biochem. Biophys, Vol. 55, p. 455 (1955).

8. Halwer, M., "Light-Scattering Study of Effects of Electrolytes on α and β Casein Solutions", Eastern Regional Research Laboratory, Philadelphia, Pennsylvania, U. S. Department of Agriculture (1953).

9. McKenzie, H. A., "Advances in Protein Chemistry", Vol. 22 p. 93 (1967).

10. Castellan, G. W., "Physical Chemistry", p. 335 (1964).

11. Preizen, P., Noble, R. W. and Waugh, P. F., "Light Scattering Studies of $\alpha_s 1,2$ -Caseins", J. Chem. Soc. Vol. 84 p. 4938 (1962).

12. Stacey, K. A., "Light-Scattering in Physical Chemistry", London Butterworths Scientific Publication (1956).

13. Debye, P., J. Phys. and Colloid Chem. Vol. 51, pp. 18-32 (1947).

14. Zimm, B. H., J. Chem. Phys. Vol. 16 pp 1099-1116, (1948).

15. Neurath, H., Greenstein, J. P., Putman, F. W.and Erickson, J. O., Chem. Revs. Vol. 34 pp. 159-265 (1944).

16. Bain, W. M., Circle, S. J., and Olson, R. A., Tappi Monograph Series 22 (1961).

17. Gordon, W. G., Semmett, W. F., Goble, R. S., and Morris, M., J. Am. Chem. Soc. Vol. 71, p. 3293 (1949).

18. Alexander, P. and Block, J. E., "Analytical Methods of Protein Chemistry", (1961).

APPENDIX I

MILK PROTEINS

Isolation of α and β -Casein Fractions