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A SEDIMENTATION EQUILIBRIUM STUDY OF OVOMUCIN

by

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ABSTRACT

Sedimentation equilibrium ultracentrifugation was used to detect heterogeneous protein-protein assocations in interacting mixtures of lysozyme and ovomucin and to determine the change in molecular weight of ovomucin during egg white thinning. Molecular weight distribution (MWD) patterns calculated from sedimentation equilibrium data using multiple regression analysis revealed the presence of a temperature-sensitive interaction at pH 6.9 between lysozyme and ovomucin at ionic strengths of 0.13 and below. The extent of interaction differed depending on the method of preparation of ovomucin. The interaction of lysozyme was stronger with β-ovomucin than with α-ovomucin and removal of sialic acid residues from β-ovomucin did not decrease this interaction but acetylation of lysozyme did. Self-association of α-ovomucin at low ionic strength was also observed.

The apparent molecular weight of native ovomucin isolated from blended fresh egg white by gel filtration on Sepharose 4B was 5.64×10^6 at pH 6.95 and ionic strength 0.13. Detailed ultracentrifugal analysis indicated a remarkable dependence of molecular weight on protein concentration. The apparent molecular weight, amino acid and carbohydrate compositions of native ovomucin were similar to those of ovomucin isolated from egg white that had been stored for 166 h at $30^{\circ}C$. The molecular weights of ovomucin, isolated by gel filtration on Sepharose 4B of fresh egg white reduced with 0.02% 2-mercaptoethanol, were 309,500 and 726,200. It is thus considered that disulfide cleavage of ovomucin does not occur during natural thinning.

The relation of the results obtained in the present study of ovomucin to the mechanism of egg white thinning was also discussed.

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A	absorbance
A_r	area under peak in molecular weight distribution
C	concentration, g/l
E	molar extinction coefficient
$M_{w\ app}$	apparent weight average molecular weight
MOL WT	molecular weight
MWD	molecular weight distribution
R	universal gas constant, 8.315×10^7 ergs/degree mole
RA	reduced and alkylated
T	absolute temperature, ^oK
UV	ultra violet
$\Gamma/2$	ionic strength
ln	natural logarithm
r	radial distance from centre of rotation, cm.
rpm	revolutions per minute
ρ	density of solution, g/ml
$\bar{\nu}$	partial specific volume of solute, ml/g
ω	angular velocity, radians per second

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INTRODUCTION

Egg white thinning, a well known phenomenon, is a decrease in the viscosity of thick white. As fresh eggs are stored at room temperature the gel structure of the thick white, composed mainly of ovomucin, is gradually destroyed with the increase in pH of egg white and changes into thin white. Many workers have been concerned with elucidating the mechanism of this non-microbial deterioration and it is generally assumed that the polydisperse glycosulfoprotein ovomucin is directly involved. Thus characterization of the physical and chemical properties of ovomucin is essential. Many studies have been carried out on ovomucin which has been chemically modified with 2-mercaptoethanol because of the poor solubility of ovomucin in non-denaturing solvents. However, information on the properties of native ovomucin, that is ovomucin soluble in non-denaturing solvents without chemical modification, is necessary to understand the in vivo process of egg white thinning. The preparation of a soluble ovomucin by gel filtration of blended egg white has been reported, allowing soluble ovomucin to be prepared without chemical modification and cleavage of covalent bonds.

Of several hypotheses proposed to explain the role of ovomucin in egg white thinning, one that has received much support states that the interaction of ovomucin with lysozyme is responsible for the gelatinous structure of thick white and the decrease in this interaction during storage results in thinning. Evidence for this suggestion has been obtained using turbidimetric measurements at 450 nm and 550 nm of mixtures of lysozyme and reduced or alkylated ovomucin. Since not all interaction products could cause turbidity, these data may not be reliable. Molecular weight distributions of interacting

proteins calculated by multiple regression analysis of sedimentation equilibrium data can be used to provide direct evidence of a lysozyme-ovomucin interaction. An advantage of this technique over turbidimetric measurements is that soluble protein-protein interaction products can be detected. Moreover use of a UV scanning system provides a direct measure of protein concentration, since proteins absorb strongly at 280 nm. To this end it would be interesting to compare results obtained using sedimentation equilibrium ultracentrifugation to those obtained using turbidimetric methods, particularly under those conditions (pH 7.0, ionic strength 0.13) which approximate the in vivo condition, where no lysozyme-ovomucin interaction can be detected by turbidimetric measurement. It would also be interesting to see if there is any difference in the extent of interaction of lysozyme with chemically modified versus unmodified ovomucin as all previous measurements of the lysozyme-ovomucin have been conducted using chemically modified ovomucin.

It is known that ovomucin is a complex of at least two distinct glycoproteins, α- and β-ovomucin, and the interaction of lysozyme with β-ovomucin has been reported to be stronger than that with α-ovomucin. Moreover, the binding sites in the ovomucin-lysozyme interaction have been studied. Since these determinations were done using turbidimetric methods it seems necessary to repeat the experiments using sedimentation equilibrium ultracentrifugation as a direct measure of protein-protein interactions.

It has been proposed that during egg white thinning, ovomucin is depolymerized by the reduction or alkaline hydrolysis of disulfide bonds. Thus to elucidate more clearly the mechanism of egg white thinning it is important to determine the molecular weight of native

ovomucin and the change in molecular weight of ovomucin during thinning. Sedimentation equilibrium ultracentrifugation is a classic technique for determination of molecular weight of macromolecules in solution and has an advantage over other techniques in that the determinations can be done under conditions of pH and ionic strength approximating the physiological condition. If cleavage of disulfide bonds in ovomucin by reduction or alkaline hydrolysis does occur during natural thinning it should be possible to detect a relatively low molecular weight ovomucin in stored and thinned egg white.

The specific objectives, therefore, of the present investigation were:

(1) To study the interaction of lysozyme with modified and unmodified ovomucin under conditions of pH and ionic strength close to the in vivo condition and to determine the binding groups in the interaction.

(2) To study the change in chemical and physical properties of native ovomucin during natural thinning.

LITERATURE REVIEW

Ovomucin, a high molecular weight glycoprotein present mainly in the thick white fraction of avian eggs (McNally, 1933) is the essential component in maintaining the firm gel-like consistency of thick egg white (Hill et al., 1949). Since Lanni and Beard (1948) and Gottschalk and Lind (1949 a, b) showed that the inhibitory activity of egg white against hemagglutination by influenza viruses was contained in the ovomucin fraction, much effort has been made to characterize ovomucin. Lanni et al. (1949) studied the properties of the semipurified inhibitor and found it to have a molecular weight of 7.6×10^6 using sedimentation velocity ultracentrifugation. Detailed electron microscopic examination of inhibitor preparations revealed the presence of structures of highly asymmetric shape in the form of filaments or fibres (Sharp et al., 1950), and the asymmetry of particle shape was in accord with previously demonstrated high viscosity of inhibitor preparations. Sharp et al. (1951) reported that these preparations consisted essentially of ovomucin and that ovomucin is a mixture of exceedingly large mucoproteins, not all of which possess inhibitory activity.

Chemical analysis of ovomucin by Young (1937) showed the presence of hexose and hexosamines, a high content of cysteine, and that ovomucin was similar in composition to the chalazae. Feeney et al. (1960) reported that ovomucin contained 40 per cent of the total sialic acid of egg white. Brooks and Hale (1961) also reported on the presence and amount of hexosamine in ovomucin preparations. A preliminary report on the chemical composition of ovomucin was given by Robinson and Monsey (1966) who reported that ovomucin was a

glycoprotein cross-linked by disulfide bonds and contained 33 per cent (w/w) carbohydrate composed of galactose, mannose, glucosamine, galactosamine, and N-acetylneuraminic acid. Kato et al. (1970 a, b) reported on the difference in hexose, hexosamine, and sialic acid between ovomucin isolated from thick versus thin egg white and on the change in chemical composition of ovomucin during storage. The latter study also reported on the amino acid composition of ovomucin isolated from fresh thick white. A detailed chemical and physical characterization of an ovomucin preparation free of other recognized egg white proteins was given by Donovan et al. (1970) who reported the presence of ester sulfate groups that accounted for all of the sulfur in ovomucin. Robinson and Monsey (1971) and Robinson (1972) reported on the detailed chemical composition of ovomucin, including amino acids, N-acetylglucosamine, N-acetylgalactosamine, galactose, mannose and sialic acid. They also reported the presence of ester sulfate and phosphate. Further studies on the changes in chemical composition of ovomucin during liquefaction of thick egg white have been reported (Robinson and Monsey, 1972 a, b). Young and Gardner (1972) reported on the chemical composition of ovomucin prepared by gel filtration of egg white and found general agreement with previous reports. The chemical composition of ovomucin prepared without chemical modification was reported by Adachi et al. (1973) and that of an ovomucin fraction isolated by high speed centrifugation was reported by Sleigh et al. (1973). In both reports, the yield and purity of ovomucin compared favourably with others. A detailed study on the carbohydrate of egg white ovomucin preparations was reported by Smith et al. (1974). These authors found that the proportions of hexose,

hexosamine and sialic acid were widely variable in different preparations and that this heterogeneity may be due to the presence of several different glycoproteins and to variations in the size and composition of the carbohydrate groups.

Kato et al. (1970 a) reported the presence of two components - a fast moving (peak F) and a slow moving (peak S) component, in the electrophoretic pattern of ovomucin from thick egg white. Further studies (Kato and Sato, 1971; Kato et al., 1971; Kato et al., 1977) showed the presence of two components by free boundary electrophoresis, density gradient column electrophoresis and chromatography on lysozyme-Sepharose 4B, respectively. Subsequent chemical analysis of the two components showed peak F to be a carbohydrate-rich and peak S a carbohydrate-poor component, consisting of about 60 per cent and 15 per cent carbohydrate, respectively. At the same time, Robinson and Monsey (1971, 1975) reported on the presence of a homogeneous carbohydrate-poor glycoprotein (α-ovomucin) and a heterogeneous carbohydrate-rich glycoprotein fraction (β-ovomucin) obtained from preparations of reduced ovomucin by sedimentation equilibrium ultracentrifugation in a density gradient of cesium chloride in the presence of 4 M guanidine hydrochloride. The oligosaccharide moiety of α-ovomucin was found to contain mainly N-acetylglucosamine and mannose while the β-ovomucin fraction was distinguished from all other egg white proteins by its high carbohydrate content and significant amounts of N-acetylgalactosamine, ester sulfate and large amounts of sialic acid. Hayakawa and Sato (1976) reported on dissociation of soluble ovomucin by sonication and the separation into two components, peak I and peak II, by DEAE-cellulose column chromatography. The chemical composition of these subunits were compared to those reported by

Kato et al. (1971) and Robinson and Monsey (1971). The carbohydrate and amino acid compositions of peak I, α-ovomucin and peak S are almost the same as are those of peak II, β-ovomucin and peak F.

The nature of the carbohydrate side chains and their linkage to the protein of ovomucin was reported by Kato et al. (1973). It was shown by these authors that there are at least three types of carbohydrate side chains in ovomucin: a chain composed of galactose, galactosamine, sialic acid and sulfate in a molar ratio of about 1:1:1:1; a chain composed of mannose and glucosamine in a molar ratio of about 1:1; and a chain composed of galactose and glucosamine in a molar ratio of about 1:1. The carbohydrate-peptide linkage in carbohydrate-poor ovomucin (α-ovomucin) was identified as N-glycosidic linkage between N-acetylglucosamine and asparagine, but the structure of its carbohydrate sequence was not determined. The carbohydrate-peptide linkage in carbohydrate-rich (β-ovomucin) ovomucin was identified as O-glycosidic between threonine or serine and N-acetylgalactosamine. The structure of its carbohydrate sequence was determined (Kato et al., 1978 b, c) as N-acetylneuraminyl-(2-->3)-galactosyl-(1-->3)-N-acetylgalactosaminitol-6-sulfate.

The possible role of ovomucin in the deterioration of thick white during storage of shell eggs has received much attention. Brant et al. (1955) reported that egg white is essentially a solution of proteins containing a little salt and sugar with part of the solution enmeshed in a gelatinous structure, and that thick egg white is apparently gelatinous because it contains a high content of the ovomucin fraction. Moreover, Feeney et al. (1955) found that ovomucin solutions thinned at rates similar to those of blended egg white.

MacDonnell et al. (1950, 1951) found that small amounts of reducing agents simulate certain effects of prolonged storage of eggs, notably thinning of the thick white and suggested that thinning might be due to reduction of the disulfide bonds of ovomucin. Feeney et al. (1951) studied the deterioration of the separated components of eggs (whites and yolk) to try and identify the possible sources of reducing substances which cause natural thinning. Feeney et al. (1956) studied the kinetics and mechanisms of yolk deterioration in shell eggs and postulated that thinning may be due to solubility changes in ovomucin caused by the high pH of stored egg white, at which pH the action of sulfhydryl reducing agents is greatly accelerated. Robinson and Monsey (1964) described a method for preparing a soluble form of ovomucin by reducing ovomucin gel with 2-mercaptoethanol, suggesting that the insolubility of ovomucin gel is due to presence of disulfide bonds. Nakamura et al. (1969) reported that the intrinsic viscosity of ovomucin gel decreased markedly after treatment with the reducing agent sodium thioglycolate. Donovan et al. (1972) and Tomimatsu and Donovan (1972) reported a thinning or decrease in viscosity of the gel-like thick portion of chicken egg white produced by exposure of ovomucin to alkaline pH. They suggested that natural thinning was caused by alkaline hydrolysis of the disulfide bonds of ovomucin as opposed to reduction. This point of view was supported by the work of Sato et al. (1976) who studied the solubilization of insoluble ovomucin during thick white thinning. Beveridge and Nakai (1975) studied by viscosity and ultracentrifugal measurements, the effect of -SH blocking and oxidation on the thinning of egg white and suggested that their results support the hypothesis that thinning

is due to depolymerization of ovomucin by reduction of disulfide bonds.

A number of workers have proposed that the interaction of ovomucin with other proteins is important to explain the mechanism of egg white thinning. Hill et al. (1949) reported that the firmness of egg white appeared to result directly from the binding of ovomucin to other proteins. Hawthorne (1950) suggested that egg white thinning resulted from the slow insolubilization of ovomucin due to a structural change in ovomucin caused by a protein-protein interaction of ovomucin with lysozyme. Several investigators have supported the existence of an interaction between ovomucin and lysozyme that results in the formation of an insoluble complex (Feeney et al., 1952; Cotterill and Winter, 1955; Dam and Bennett, 1963; Garibaldi et al., 1968; Robinson and Monsey, 1969 a, b). Cotterill and Winter (1955) and Rhodes and Feeney (1957) found a maximum interaction between ovomucin and lysozyme at pH 7. Less ovomucin-lysozyme complex was formed as the egg white became more alkaline suggesting that dissociation of the ovomucin-lysozyme complex, rather than its formation, might cause thinning. Wilcox (1955) obtained evidence for an association between the lysozyme level and quality of egg white, supporting this view point. Brooks and Hale (1959, 1961) studied the mechanical properties of thick white and suggested that the rigidity of thick white could be best explained by assuming that chains of an ovomucin-lysozyme complex are cross-linked into a network. Gradual dissociation or hydrolysis of the complex could account for the changes in thick white during thinning.

To determine the effect of the interaction of lysozyme with ovomucin on the rigidity of thick white gel, Robinson and Monsey (1969 b) reported on the stoichiometric composition of a pure lysozyme-reduced ovomucin complex. Their results suggested that the interaction was electrostatic in nature and that the decreased interaction observed at high pH values was compatible with a decrease in the positive charge of the lysozyme molecules. Dam (1971) reported on the effect of pH and temperature on the lysozyme-ovomucin interaction and concluded that if lysozyme-ovomucin complexing exists *in vivo*, it is not intimately involved in the process of thinning of the thick white. Alternately it was proposed that ovomucin itself played a major role in the structure of the thick white. Kato *et al*. (1970 a) showed that the interaction of ovomucin with lysozyme in the thick white was stronger than that in the thin white. As it was subsequently shown that ovomucin consisted of carbohydrate-poor and carbohydrate-rich components (Kato and Sato, 1971; Robinson and Monsey, 1971) and that there was a gradual dissociation of the carbohydrate-rich component into the liquid part of thick white during natural thinning (Kato and Sato, 1972; Robinson and Monsey, 1972 a) it was proposed than an ovomucin complex cross-linked by lysozyme existed in thick white and that thinning of the thick white accompanying storage of whole eggs was due to dissociation of the complex as the pH of the white increased (Kato *et al*., 1971; Robinson, 1972). Robinson and Monsey (1972 b) suggested that electrostatic interaction between the negative charges (in particular those carried by the sialic acid residues) of the ovomucin molecules and the positive charges of the lysozyme molecules are

important for the lysozyme-ovomucin interaction. Kato et al. (1975) studied the binding groups in the lysozyme-ovomucin interaction and found that the negative charges of the terminal sialic acid in ovomucin and the positive charges of the lysyl ε-amino groups in lysozyme are essential for the electrostatic interaction of lysozyme with ovomucin. It was also reported by these authors that the extent of interaction of lysozyme with β-ovomucin was greater than that with α-ovomucin, reflecting on the difference in sialic acid content. Kato et al. (1978) studied the changes in lysozyme during egg white thinning and found that lysozyme dissociates from the gel into the liquid fraction in thick white during storage. It was suggested by these authors that this dissociation of lysozyme from the gel might be caused by dissociation of β-ovomucin from the thick white gel during storage as was shown in their previous report (Kato and Sato, 1972).

Kato et al. (1979) reported on the degradation of the O-glycosidically linked carbohydrate units of ovomucin during egg white thinning in an attempt to explain why the specific solubilization of β-ovomucin occurs during egg white thinning. It was suggested that the O-glycosidically linked carbohydrate units of β-ovomucin were important in maintaining the swollen rigid gel structure of thick egg white and that gradual liberation of these carbohydrate units during storage of egg white caused dissociation of the β-ovomucin molecules from the thick white; hence thinning.

EXPERIMENTAL

Materials

Unless otherwise indicated, all chemicals were reagent grade or the highest quality provided by the supplier, and were used without further purification. Lysozyme, prepared from hen's egg white by the direct crystallization method (Alderton and Fevold, 1946) was recrystallized five times and lyophilized. The following materials were obtained commercially. Neuraminidase (Clostriduim perfringens, type IV), N-acetylneuraminic acid (Escherichia coli), N-acetylglucosamine and N-acetylgalactosamine were all purchased from Sigma Chemicals, St. Louis, MO. D-galactose and D-mannose were products of Eastman Organic Chemicals, Rochester, N.Y. Bovine serum albumen was from Calbiochem, Inc., La Jolla, CA. Sephadex G-25 and Sepharose 4B were the products of Pharmacia Fine Chemicals, Sweden. All eggs were collected from the University flock within 24 hours after laying and the shells sterilized with 70% ethanol.

Methods

A. Preparation of Ovomucin Complex

Thick egg white from 25 eggs was separated by straining egg white through an aluminum sieve according to the method of Kato et al. (1970 a). The egg white which remained on the sieve was the thick white and that which drained readily through the sieve was the thin white. The thick white (500 ml) was blended at the slowest speed in a Sorvall Omni-mixer and crude ovomucin complex was precipitated by addition of 500 ml of 4% KCl as described by Kato et al. (1971). The gelatinous precipitate (200 ml) was separated from the supernatant by centrifugation at 10,000 rpm for 20 min at 4^{o}C using a Sorvall RC-2B

refrigerated centrifuge and GSA rotor, and was washed with 2% KCl solution until the supernatant was free from proteins as judged by precipitation with 2% trichloroacetic acid. The resulting gel (30 ml) was exhaustively dialyzed at 4°C against distilled water, containing 0.02% sodium azide, to remove KCl and is hereafter referred to as purified ovomucin complex.

B. Reduction of Purified Ovomucin Complex

Purified ovomucin complex was reduced according to the method of Robinson and Monsey (1971). 10 ml of 1.0 M Tris - HCl buffer, pH 8.2, containing 0.6 M 2-mercaptoethanol and 12 M urea were added slowly with stirring to 10 ml of purified ovomucin complex and the mixture allowed to stand at 20°C for 16 h. The thiol groups were then alkylated by the addition of an equivolume (20 ml) of 2 M Tris-HCl buffer, pH 8.0, containing 0.72 M iodoacetic acid and 8 M urea. The resulting mixture was allowed to stand in the dark at 20°C for 2 h. Following alkylation the reduced ovomucin complex (50 ml) was exhaustively dialyzed at 4°C against distilled water containing 0.02% sodium azide and is hereafter referred to as reduced, alkylated ovomucin complex (RA ovomucin complex).

C. Preparation of Native Ovomucin

Native ovomucin was prepared using a modification of the method of Young and Gardner (1972). Fresh egg white (30 ml), pH 7.8, was blended for 1 min using a Sorvall Omni-mixer at the slowest speed. The blended white was then diluted to 100 ml with 0.05 M Tris-HCl buffer, pH 8.6, containing 0.85% sodium chloride, and then blended as before. 10 ml were applied to a Sepharose 4B column (2.5 x 40 cm), with a 1 cm layer of Sephadex G-25 on the bottom, which had been

previously equilibrated with 2 bed volumes of 0.05 M Tris-HCl buffer, pH 8.6, containing 0.85% sodium chloride. The void volume of the column was determined by measuring the elution volume of Blue Dextran 2,000, 2 mg/ml (Pharmacia Fine Chemicals, Sweden). The column was eluted with the same buffer at a flow rate of 13 ml per h and 3.5 ml fractions were collected using a Gilson Medical Electronics drum-type fraction collector. Fractions were analyzed for protein and sialic acid and those fractions containing the ovomucin (fractions 24 and 25) were combined and exhaustively dialyzed at 4°C against distilled water or centrifugation buffer, containing 0.02% sodium azide. Aliquots for hexose, hexosamines, sialic acid and amino acid analyses were stored at -20°C before analyses. Aliquots for sedimentation equilibrium analysis were stored at 4°C for up to 3 days.

D. Preparation of Ovomucin from Stored Egg White

Egg white from eggs collected within 24 h after laying was stored for 166 h at 30 ± 2°C in a sterilized flask sealed with a cotton plug. All procedures were aseptically carried out. At the end of the given time (166 h) the egg white (pH 9.0) was removed from the flask, blended at the slowest speed in a Sorvall Omni-mixer, diluted with column buffer and prepared for fractionation on Sepharose 4B as before. 10 ml were applied to a Sepharose 4B column and fractionated as previously described. Fractions containing the ovomucin were combined and dialyzed and aliquots were removed for chemical analysis and sedimentation equilibrium as described before. Hereafter this fraction will be referred to as stored ovomucin.

E. Preparation and Fractionation of Reduced Fresh Egg White

2-Mercaptoethanol (0.02%) was added to fresh egg white (30 ml) and then blended for 1 min using a Sorvall Omni-mixer at the slowest speed. Following dilution to 100 ml with column buffer and blending as before, 10 ml were applied to a Sepharose 4B column (2.5 x 40 cm) and fractionated as before. Aliquots from each fraction were removed for sialic acid determination and those containing sialic acid (fractions 17 to 43) were subsequently analyzed for hexose. On the basis of the ratio of sialic acid to hexose, fractions 24 and 37 were identified as containing ovomucin. These fractions, hereafter referred to as reduced ovomucin, were exhaustively dialyzed at 4°C against centrifuge buffer containing 0.02% sodium azide, as before, in preparation for sedimentation equilibrium analysis.

F. Modification of Lysozyme

Acetylation of lysozyme was carried out according to the general method of Yamasaki et al. (1968) as modified by Kato et al. (1975). 5 ml saturated sodium acetate were slowly added with stirring to 5 ml of 4% lysozyme in distilled water and the mixture kept on ice. To this mixture, kept on ice, 0.2 g acetic anhydride was gradually added over a 1 h period (0.01 ml acetic anhydride every 3 min). The pH was maintained above 7.0 over the 1 h period by addition of a small volume of 1 N sodium hydroxide following each addition of acetic anhydride. After addition of the acetic anhydride was complete, the acetylated lysozyme was exhaustively dialyzed at 4°C, against distilled water containing 0.02% sodium azide. The extent of modification of free amino groups in the acetylated lysozyme was determined spectrophotometrically using trinitrobenzene sulfonate, a reagent specific

for amino groups, according to the method of Haynes et al. (1967). To 1 ml of acetylated lysozyme solution (1 mg/ml) were added 1 ml of 4% sodium bicarbonate, pH 8.5, and 1 ml of 0.1% trinitrobenzene sulfonate in water. The mixture was incubated at 40°C for 2 h and then 1 ml of 10% sodium dodecyl sulfate and 0.5 ml of 1 N hydrochloric acid were added. Absorbance was read at 344 nm against a blank treated as above but containing 1 ml of distilled water instead of lysozyme solution and was compared to the absorbance of unmodified lysozyme (1 mg/ml) treated as above.

G. Modification of Ovomucins

Neuraminidase (1.85 μg), E.C. 3.2.1.18 (4.2 units/mg) was added to 2 ml of a 0.3% solution of RA ovomucin complex or native ovomucin in 0.03 M sodium phosphate buffer, pH 6.9, containing 0.02% sodium azide and then incubated at 30°C for 22 h. An aliquot (0.2 ml) was removed for sialic acid determination and the remainder exhaustively dialyzed at 4°C against centrifugation buffer. The extent of enzymatic removal of sialic acid was determined by comparison to the total sialic acid released by acid hydrolysis of unmodified ovomucin with 0.1 N sulfuric acid at 80°C for 1 h.

H. Determination of Native, Stored and Reduced Ovomucin Concentrations

Ovomucin concentrations were estimated qualitatively by absorbance at 280 nm. Quantitative estimation of ovomucin concentrations was made by the method of Lowry et al. (1951) using bovine serum albumin as a standard or by spectrophotometric measurements using the extinction coefficient, $E^{1\%}_{280\ nm,\ 1\ cm} = 7.0$ which was determined experimentally.

I. Amino Acid Analysis of Ovomucin

Amino acid analyses were conducted on 2 mg samples of native and stored ovomucins hydrolyzed with 6 N hydrochloric acid at 105 - 110°C for 22 h in sealed, evacuated ampules previously flushed with nitrogen. After hydrolysis the samples were evaporated to dryness under nitrogen, dissolved in 0.2 M sodium citrate buffer (pH 2.2) and analyzed by the method of Moore and Stein (1954) on a single-column system (Durrum Chem. Corp., Palo Alto, CA) attached to a Phoenix Model M 6800 Amino Acid Analyzer (Phoenix Precision Instrument Co.). Values for amino acids are averages of duplicate analyses with no corrections made for possible destruction of certain amino acids which may have occurred due to the presence of carbohydrates or for possible destruction of serine and threonine during hydrolysis.

J. Carbohydrate Composition of Ovomucins

1. Hexose

Hexose content was determined by the orcinol-sulfuric acid method (Winzler, 1955) using an equimolar mixture of D-galactose and D-mannose as a standard. To 0.2 ml ovomucin solution (99 µg ovomucin) were added 0.5 ml of 1.6% orcinol in 30% sulfuric acid, and 3.0 ml 60% sulfuric acid and the mixture was heated to 80°C for 20 min in the dark. Following this incubation, the mixture was cooled in an ice bath in the dark. Absorbance at 520 nm, or 540 nm for high absorbance samples, was determined spectrophotometrically.

2. Amino Sugars

Hexosamine was determined by the method of Neuhaus and Letzring (1957) in which an equimolar mixture of N-acetylgalactosamine and N-acetylglucosamine was used as a standard. Ovomucin samples (99 μg in 0.2 ml) were evaporated to dryness in glass-stoppered centrifuge tubes by heating in a boiling water bath. One millilitre of 3 N hydrochloric acid was added and the tubes were stoppered and heated in a boiling water bath for 4 h. The remainder of the procedure was carried out exactly as described by Neuhaus and Letzring (1957) except that absorbances at 530 nm were measured in quartz cuvettes instead of Klett colorimeter tubes.

3. Sialic Acid

Sialic acid was measured after acid hydrolysis (total sialic acid) or after enzymatic release, by the thiobarbituric acid method of Warren (1959) with N-acetylneuraminic acid as a standard. Native or stored ovomucin samples (99 μg ovomucin in 0.2 ml) were hydrolyzed with 0.2 ml of 0.2 N sulfuric acid at 80°C for 1 h and free sialic acid was determined exactly as described by Warren (1959) for assay of sialic acid in tissue homogenates.

K. Sedimentation Equilibrium Ultracentrifugation

A Beckman L2-65B Ultracentrifuge equipped with a Prep UV Scanner and integrated with a data acquisition system was employed for sedimentation equilibrium studies. An evaluation of this system for sedimentation equilibrium analysis has been published (Van de Voort and Nakai, 1978). Centrifugal runs were performed at 20 ± 0.5°C, unless otherwise specified, using a black anodized

four place rotor (An-F) holding three, 12 mm filled Epon double sector centerpieces, allowing three samples to be analyzed during the same run. Separate exhaustive dialysis of proteins at 4°C against phosphate buffers (NaH_2PO_4, Na_2HPO_4), pH 6.9 containing 0.02% sodium azide (centrifugation buffer) preceded all analytical centrifugal runs. The outer dialysates were checked spectrophotometrically to ensure that no protein had leaked from the dialysis sac during dialysis and these were used as the reference solutions in sedimentation equilibrium experiments in accord with the requirements specified by Casassa and Eisenberg (1964).

In general, the solvent channel of each centerpiece was filled with 0.19 ml of the appropriate outer dialysate and the sample channel with 0.03 ml fluorocarbon oil (to provide a curved bottom), followed by 0.12 ml protein solution. Sedimentation equilibrium experiments were started by running the rotor at low speed (5,000 to 10,000 rpm) for about 10 min and making a scan to obtain the initial solute concentration. An overspeed run of 3 h at 1½ times the selected equilibrium speed was used to reduce the transient time to obtain the equilibrium condition (except for molecular weight determinations of native and stored ovomucins) and then the rotor was decelerated to the selected equilibrium running speed. The time to reach equilibrium was estimated using the Van Holde-Baldwin equation (Van Holde and Baldwin, 1958) and the system was considered at equilibrium when hourly scans taken after the calculated equilibrium time showed no observable change. After the equilibrium scan was taken the rotor speed was increased to approximately 46,000 rpm for 3 h to deplete the meniscus and then

decelerated to the equilibrium speed where an additional scan was immediately taken to obtain the baseline absorbance, assuming that the absorbance in the depleted region of the cell indicates the baseline for the entire cell.

L. Ovomucin-Lysozyme Interactions

Sedimentation equilibrium was applied to the study of the heterogeneous associations of ovomucin with lysozyme. Dialyzed lysozyme and ovomucin (RA ovomucin complex and native ovomucin) solutions were diluted such that the absorbances at 280 nm were approximately 0.30 and mixed in an ovomucin to lysozyme volume ratio of 1:4, prior to analytical ultracentrifugation. Correcting for UV absorptivity difference between lysozyme and ovomucin, i.e. $E^{1\%}_{280\ nm,\ 1\ cm} = 26.5$ (Canfield, 1963 a) and $E^{1\%}_{280\ nm,\ 1\ cm} = 7.0$ for lysozyme and ovomucin, respectively, gives a calculated ovomucin to lysozyme concentration ratio of 1:1 (w/w) for these mixtures. This ratio was chosen because an insoluble lysozyme-ovomucin aggregate forms in mixtures of lysozyme and ovomucin under conditions where there is an excess of lysozyme (Robinson, 1972; Kato et al., 1975). Robinson (1972) has calculated that 1.97 g of lysozyme are required to cross-link 2 g of ovomucin at pH 7.4.

The effect of ionic strength on the ovomucin-lysozyme interaction was investigated at ionic strengths 0.13 (0.033 M NaH_2PO_4, 0.033 M Na_2HPO_4) and 0.07 (0.017 M NaH_2PO_4, 0.017 M Na_2HPO_4), pH 6.9. The effect of temperature on the interaction was investigated by conducting the sedimentation equilibrium runs at 20 ± 0.5°C and 3 ± 0.5°C for ionic strengths of 0.13 and 0.07. For the equilibrium runs at 3°C, the protein solutions were loaded into the centrifuge

cells and the assembled rotor was pre-cooled to 3 ± 0.5°C prior to centrifugation.

M. Molecular Weight Distributions

Absorbance values were monitored at 278 nm as a function of radial distance and were corrected using a standard curve drawn for ovalbumin. Molecular weight distributions (MWD) were calculated by multiple regression analysis of sedimentation equilibrium data according to the method of Van de Voort et al. (1979) and Nakai and Van de Voort (1979). Rotor speeds were determined by averaging at least 5 odometer readings taken during the course of the run. The partial specific volume of lysozyme was taken as 0.726 ml/g (Deonier and Williams, 1970) and a partial specific volume of 0.662 ml/g was calculated for the RA ovomucin complex according to the method of Howlett and Nichol (1973) using values of 0.701 ml/g and 0.649 ml/g for the partial specific volumes of α- and β- ovomucins (Robinson, 1972) assuming that RA ovomucin complex consists of $(\alpha\text{- ovomicin})_n$: $(\beta\text{- ovomucin})_n$ where n is a positive whole number.

The area under the peaks in the MWD is approximately proportional to the concentration of that molecular weight species and protein-protein interactions between ovomucin and lysozyme were detected as changes in the MWD such as the disappearance of peaks, the appearance of new peaks and/or the change in area under a particular peak. The extent of interaction between ovomucin and lysozyme was determined by measuring the area under the lysozyme peak.

N. Molecular Weight Determinations

Molecular weights of ovomucins (native, stored and reduced) were calculated from sedimentation equilibrium data using the relationship

$$M_{w\ app} = \frac{2RT}{(1-\nu\rho)\omega^2} \frac{(d\ \ln c)}{d(r^2)} \qquad \text{(Schachman, 1959)}$$

where: $M_{w\ app}$ = the apparent weight average molecular weight of the sample

R = universal gas constant
= 8.315×10^7 ergs/degree/mole

T = absolute temperature in degrees Kelvin

ν = partial specific volume, ml/g

ω = angular velocity of rotor in radians per second
= 0.10472 x rpm

c = protein concentration, g/l

r = radial distance from center of rotation, cm

ρ = density of the solution, g/ml

Absorbance values were monitored at 278 nm as a function of radial distance as before and plots of ln absorbance against (radial distance)^2 were constructed for ovomucin samples in 0.07 M sodium phosphate buffer ($\frac{\Gamma}{2} = 0.13$), pH 6.95, containing 0.02% sodium azide at protein concentrations between 0.15 mg/ml and 0.70 mg/ml. The slopes of these plots (d ln A/d(r^2)) were determined and the apparent molecular weight of ovomucin calculated as above. The partial specific volume of native, stored, and reduced ovomucin was assumed to be the same (0.662 ml/g) as that calculated for RA ovomucin complex.

The subscript "app" in this equation denotes that for a nonideal system of charged molecules (i.e. a solution of ovomucin at pH 6.95) the molecular weight is apparent, containing unknown contributions from charge and nonideality effects (Jeffrey and Coates, 1966).

RESULTS

A. Interaction Between Egg White Lysozyme and Ovomucin

1. Effect of Ionic Strength

(a) RA Ovomucin Complex - Lysozyme

Equilibrium experiments were carried out at 20 ± 0.5°C on solutions of lysozyme, RA ovomucin complex, and RA ovomucin complex-lysozyme mixtures (1:4) in 0.07 M ($\frac{\Gamma}{2}$ = 0.13) and 0.03 M ($\frac{\Gamma}{2}$ = 0.07) sodium phosphate buffer, pH 6.9, containing 0.02% sodium azide. For each run three samples were analyzed and because of the large difference in molecular weight between lysozyme and ovomucin it was necessary to use different rotor speeds - a low speed of approximately 10,000 rpm and a higher speed of approximately 20,000 rpm, to obtain good equilibrium patterns for RA ovomucin complex and lysozyme, respectively.

The molecular weight distribution (MWD) of proteins computed from sedimentation equilibrium data are presented in Figures 1, 2 and 3. The result from a typical experiment is shown in Figure 1. Panel A shows the MWD from the cell containing lysozyme only and panels B and C show the MWD patterns from the cells containing RA ovomucin complex-lysozyme mixtures (1:4) at ionic strengths of 0.13 and 0.07, respectively. It can be seen that the area under the lysozyme peak in the MWD of RA ovomucin complex-lysozyme mixtures decreases with decreasing ionic strength of solution. In other words, an ionic strength - dependent interaction between RA ovomucin complex and lysozyme is indicated. Although the interaction is much stronger at ionic strength 0.07, a slight interaction can be detected at ionic strength 0.13 when the area under the lysozyme

peak in the MWD in panel A (corrected for sampling volume) is compared to that in panel B. Only a lysozyme peak is seen in these MWD patterns because the much larger molecules of RA ovomucin complex and RA ovomucin complex-lysozyme interaction product sediment to the cell bottom at the equilibrium speed that is needed to obtain a good lysozyme pattern.

The extent of interaction of lysozyme with ovomucin can be calculated as the per cent lysozyme that forms a complex with ovomucin and sediments to the cell bottom. In other words, the percentage decrease in the area of the lysozyme peak in the MWD of ovomucin-lysozyme mixtures relative to the area of the lysozyme peak from the cell containing lysozyme only, can be used to express the extent of interaction. Thus at ionic strength 0.13, the extent of interaction is 5.6% while at ionic strength 0.07 it is 70%.

To obtain further information on the interaction, especially on changes in ovomucin, the ionic strength experiments were repeated using a much slower equilibrium speed. Figure 2 shows the interaction of RA ovomucin complex with lysozyme at ionic strength 0.13. Panel A shows the MWD of the cell containing lysozyme only. Panel B shows the MWD of the cell containing RA ovomucin complex, yielding a trimodal distribution consisting of α-ovomucin peaks (component molecular weights 115,700 and 493,600) and a β-ovomucin peak (component molecular weight 1,113,300). Panel C shows the MWD pattern of the cell containing an RA ovomucin complex-lysozyme mixture (1:4). When RA ovomucin complex and lysozyme were mixed, a decrease in the area under the lysozyme and α-ovomucin peaks and a complete disappearance of the β-ovomucin peak are observed. Thus a measurable interaction

(extent 6.9%) exists at ionic strength 0.13 and a preferential interaction of lysozyme with β-ovomucin is indicated.

The interaction of RA ovomucin complex with lysozyme at ionic strength 0.07 is shown in Figure 3. Panel A shows the MWD of the cell containing lysozyme only and panel B the MWD pattern of the cell containing RA ovomucin complex only. Self association of α-ovomucins is seen in panel B as evidenced by a bimodal distribution consisting of a single α-ovomucin peak (component molecular weight 221,100) and a β-ovomucin peak (component molecular weight 1,149,200). Panel C shows the effect of mixing RA ovomucin complex and lysozyme. Complete disappearance of the β-ovomucin component can be seen as well as the appearance of a new peak (component molecular weight 460,500) which may represent a complex of associated α-ovomucin with lysozyme. A greater decrease in the area under the lysozyme peak at ionic strength 0.07 (extent of interaction, 73%) as compared to ionic strength 0.13 supports the previous finding that the RA ovomucin-lysozyme interaction is ionic strength dependent, being stronger at the lower ionic strength.

(b) Native Ovomucin-Lysozyme

To investigate the effect of ionic strength on the interaction of native ovomucin with lysozyme, equilibrium experiments at 20 ± 0.5°C were carried out on solutions of lysozyme and native ovomucin-lysozyme mixtures (1:4) in 0.07 M and 0.03 M sodium phosphate buffers, pH 6.9, containing 0.02% sodium azide. The MWD patterns of these solutions are shown in Figure 4. An equilibrium speed of about 31,000 rpm was chosen for this experiment in order

to look at the lysozyme component only as a low speed run to look at ovomucin only was not possible because even at rotor speeds as low as 4,000 rpm the large native ovomucin molecules and molecules of native ovomucin-lysozyme complex immediately sedimented to the cell bottom. Panel A shows the MWD of the cell containing lysozyme only while panels B and C show the MWD patterns of the cells containing native ovomucin-lysozyme mixtures (1:4) at ionic strengths 0.13 and 0.07, respectively. It can be seen that the area under the lysozyme peak of native ovomucin-lysozyme mixtures decreases with decreasing ionic strengths (extents of interaction being 5.7% and 27% at ionic strengths 0.13 and 0.07, respectively), indicating an ionic strength dependent interaction between native ovomucin and lysozyme. This result is similar to that obtained using RA ovomucin complex-lysozyme mixtures. However, the extent of interactions of native ovomucin with lysozyme at ionic strength 0.07 is only about 0.4 times as strong as that between RA ovomucin and lysozyme at the same ionic strength.

2. Effect of Chemical Modification

(a) Modification of Lysozyme

The extent of acetylation of amino groups in lysozyme was 100 per cent as determined spectrophotometrically. Thus all seven amino groups of lysozyme were modified. To investigate the effect of modification of amino groups in lysozyme on the ovomucin-lysozyme interaction, solutions of acetylated lysozyme, acetylated lysozyme-RA ovomucin complex (4:1) and acetylated lysozyme-native ovomucin (4:1) in 0.03 M sodium phosphate buffer, pH 6.9, containing 0.02%

sodium azide were prepared and equilibrium experiments were carried out at 20 ± 0.5°C using rotor speeds of about 34,000 rpm. The MWD patterns of these solutions are shown in Figure 5. By this modification of lysozyme the extent of interaction with RA ovomucin complex at ionic strength 0.07 is 10.6% and that with native ovomucin under similar conditions is 6.8%. In other words, under conditions (0.03 M sodium phosphate buffer, pH 6.9) where the extent of lysozyme-RA ovomucin interaction is 70%, acetylation of lysozyme decreased this interaction to 10.6% and where the extent of lysozyme-native ovomucin interaction is 27%, acetylation of lysozyme decreased this to 6.8%.

(b) Modification of Ovomucin

The extent of removal of sialic acid by treatment of RA ovomucin complex and native ovomucin with neuraminidase was 100% and the MWD of neuraminidase treated RA ovomucin complex was similar to that of untreated RA ovomucin complex indicating little, if any proteolysis by contaminating enzymes. To investigate the effect of modification of ovomucin on the ovomucin-lysozyme interaction, solutions of lysozyme, asialo RA ovomucin complex, asialo RA ovomucin complex-lysozyme mixture (1:4), asialo native ovomucin, and asialo native ovomucin-lysozyme mixture (1:4) in 0.03 M sodium phosphate buffer, pH 6.91, containing 0.02% sodium azide were prepared and equilibrium experiments were carried out at 20 ± 0.5°C. The effect of modification of RA ovomucin complex on the ovomucin-lysozyme interaction is shown in Figure 6. Panel B shows the MWD pattern of the cell containing an RA ovomucin complex-lysozyme mixture yielding a bimodal distribution consisting of a lysozyme peak and an associated α-ovomucin component

of molecular weight 252,500 with the β-ovomucin-lysozyme complex having sedimented to the cell bottom. Here the extent of interaction of lysozyme with RA ovomucin is 72% as calculated from the area under the lysozyme peak in panel A. Panel C shows the bimodal MWD pattern of the asialo, RA ovomucin complex-lysozyme mixture consisting of a lysozyme component and an α-ovomucin component of molecular weight 262,700. Probably the asialo, β-ovomucin-lysozyme interaction products have sedimented to the cell bottom, thus not being shown in the pattern. By complete removal of sialic acid residues from RA ovomucin complex, the extent of the ovomucin-lysozyme interaction is 76% at ionic strength 0.07 and no effect on the preferential interaction of lysozyme with β-ovomucin can be seen. In other words, under conditions (0.03 M sodium phosphate, pH 6.91) where the extent of the interaction of lysozyme with RA ovomucin is 72%, removal of sialic acid residues has only a slight effect.

Figure 7 shows the effect of modification of native ovomucin on the ovomucin-lysozyme interaction. Panel A shows the MWD of the cell containing lysozyme only. Panel B shows the MWD of the cell containing a native ovomucin-lysozyme mixture (1:4) and panel C shows the MWD of the cell containing an asialo, native ovomucin-lysozyme mixture (1:4). The extent of interaction of lysozyme with native ovomucin is 19.5%, the same as that for the interaction with modified native ovomucin. In other words, complete removal of sialic acid residues from native ovomucin has no effect on the extent of the ovomucin-lysozyme interaction at pH 6.9, ionic strength 0.07.

3. Effect of Temperature

The effect of temperature on the ionic strength-dependent ovomucin-lysozyme interaction was studied by conducting equilibrium experiments at 20 ± 0.5 and 3 ± 0.5°C using solutions of lysozyme and RA ovomucin complex-lysozyme mixtures (1:4) in 0.07 M and 0.03 M sodium phosphate buffers, pH 6.92, containing 0.02% sodium azide. The MWD patterns are shown in Figure 8. At ionic strength 0.13 the extent of interaction is virtually the same, 6.4% and 6.8% at temperatures of 3 and 20°C, respectively. However, at ionic strength 0.07, the extent of interaction is much stronger at 3°C (100%) than at 20°C (77%).

B. Molecular Weight of Ovomucin During Egg White Thinning

1. Preparation of Native, Stored and Reduced Ovomucins

Figure 9 shows the elution profiles of native, stored and reduced egg white on Sepharose 4B. Young and Gardner (1972) and Hayakawa and Sato (1976) reported that during gel filtration of blended egg white, ovomucin emerged in the void volume from columns of Sepharose 4B in Tris-HCl buffer, pH 8.6, containing 0.85% NaCl. As shown in panels A and B, there is little difference between the elution profiles of native and stored white, except for a little broader void volume peak in the profile of stored white. The extinction coefficient, $E^{1\%}_{280\ nm,\ 1\ cm}$ of the ovomucin fraction was 7.0 for a pH 8.6 solution and the content of the ovomucin fraction calculated from the absorbance in Figure 9 was about 3 per cent of the total native egg white solids. Robinson (1972) reported that ovomucins comprise 1.5 to 3.0 per cent of total egg

white solids, hence it appears that most of the ovomucin in egg white can be isolated in a soluble state by gel filtration on Sepharose 4B after blending and dilution at pH 8.6. Figure 9C shows the elution pattern of egg white reduced with 0.02% 2-mercaptoethanol. The void volume fraction decreased while the lower molecular weight fractions (fractions 35 to 40) increased, indicating a completely different profile from that of fractionation of stored white.

2. Amino Acid Composition of Ovomucin

Table 1 shows the amino acid composition of the ovomucin fraction from native egg white obtained in this experiment and the compositions of ovomucin and ovomucin complex reported earlier are also given for comparison. There is generally good agreement between our results and the composition of native ovomucin as reported by Young and Gardner (1972) except for the sulfur containing amino acids, methionine and cysteine. With few exceptions there is reasonably good agreement between our results and the other seven sets of data. The variability can be attributed to the different methods of preparation of ovomucin.

Table 2 shows the amino acid compositions of ovomucin from native and stored egg white. It can be seen that the amino acid composition of the ovomucin fraction did not change during storage at 30°C for 166 h under conditions where the pH of the egg white increased from 7.8 to 9.0 and natural thinning occurred.

3. Carbohydrate Composition of Ovomucins

Table 3 gives the carbohydrate content of the same ovomucin preparations listed in Table 2. The data for ovomucin from fresh

white is in general agreement with earlier reports (Adachi et al., 1973; Kato et al., 1973), however considerable variability in carbohydrate content of ovomucin has been reported (Smith et al., 1974) due to the fact that almost all glycoproteins contain their carbohydrate units in varying stages of completion (Robinson, 1972). Hence it may not be meaningful to compare carbohydrate compositions amongst different ovomucin preparations. It can be seen that the carbohydrate composition of the ovomucin fraction did not change during storage for 166 h at 30°C under conditions in which natural thinning occurred. In contrast, previous studies reported a decrease in sialic acid, hexose and hexosamine content of ovomucin accompanying storage of thick white (Kato et al., 1972; Robinson and Monsey, 1972 a, b).

4. Molecular Weight of Ovomucins

To get information about the molecular weight of ovomucin during natural thinning, sedimentation equilibrium analysis was carried out. The running speed was set at a low speed, 4,000 rpm, to get stable equilibrium patterns. At rotor speeds much higher than 4,000 rpm the ovomucin molecules sedimented to the cell bottom quite rapidly. Figure 10 shows representative results of equilibrium ultracentrifugal analysis of native and stored ovomucins, at initial protein concentrations between 0.15 to 0.67 mg/ml and 0.14 to 0.60 mg/ml, respectively. The plots of ln A versus r^2 for native and stored ovomucin fractions showed linear relationships with higher slopes observed for lower protein concentrations. The slopes of the plots of ln A versus r^2, which are proportional to molecular weight, were used to calculate the molecular weights as a function of ovomucin concentration according to the relationship

$$M_{w\ app} = \frac{2\ RT}{(1-\nu\rho)\omega^2} \quad \frac{(d\ \ln c)}{d\ (r^2)}$$

as previously described.

Tables 4 and 5 list the calculated molecular weights of native and stored ovomucins as a function of protein concentration and these data are plotted in Figure 11. As shown in this figure, the molecular weight of both native and stored ovomucins are markedly dependent on the protein concentration - $M_{w\ app}$ of 5.6 - 7.5 x 10^6 for ovomucin concentrations of 0.15 to 0.67 mg/ml. By using linear regression analysis, the following relationships between apparent molecular weight ($M_{w\ app}$) and protein concentration (C) have been calculated: $M_{w\ app} \times 10^{-6} = -4.95\ C + 5.64$ for native ovomucin and $M_{w\ app} \times 10^{-6} = -4.89\ C + 5.25$ for stored ovomucin, suggesting that there is little difference between the molecular weight of ovomucin from fresh versus stored egg white.

Sedimentation equilibrium patterns (ln A vs r^2) of reduced ovomucin fractions 24 and 37 from gel filtration of egg white reduced with 0.02% 2-mercaptoethanol (Figure 9C) are shown in Figure 12. The molecular weights calculated from the slopes of these plots were 726,200 and 309,500, respectively.

DISCUSSION

A. Interaction Between Lysozyme and Ovomucin

1. Effect of Ionic Strength

(a) RA Ovomucin Complex-Lysozyme

It has been shown by direct measurement of protein concentrations that the interaction between RA ovomucin complex and lysozyme, at pH 6.9, is electrostatic, judging by the dependence of the interaction on ionic strength (Kauzmann, 1959), as proposed in earlier reports (Klotz and Walker, 1948; Hawthorne, 1950; Cotterill and Winter, 1955; Robinson and Monsey, 1969b; Kato and Sato, 1972; Kato et al., 1975; Kato et al., 1976; Garibaldi et al., 1968). However previous measurements of this interaction (Cotterill and Winter, 1955; Dam and Bennett, 1963; Robinson and Monsey, 1969 b; Dam, 1971; Kato et al., 1971; Kato and Sato, 1972; Robinson, 1972; Kato et al., 1975; Kato et al., 1976) were based on the assumption that complexes between ovomucin and lysozyme are insoluble, thus possible soluble interaction complexes were ignored in determining the extent of interaction. Moreover, none of these methods measured protein concentration directly, hence they may not be reliable. For example, Cotterill and Winter (1955) studied the effect of pH on the ovomucin-lysozyme interaction by measuring the volume of precipitate formed upon addition of solutions of lysozyme to ovomucin. Dam and Bennett (1963) and Dam (1971) studied the ovomucin-lysozyme interaction by measuring the lysozyme binding ability of ovomucin preparations following centrifugation of turbid solutions formed upon mixing lysozyme and

ovomucin solutions. Turbidity determinations at 450 nm (Robinson and Monsey, 1969 b; Robinson, 1972), 550 nm (Kato et al., 1975; Kato et al., 1976) and 600 nm (Kato et al., 1971; Kato and Sato, 1972) of mixtures of ovomucin and lysozyme were used as a measure of the amount of ovomucin-lysozyme complex, hence the extent of interaction between lysozyme and ovomucin, assuming that all interaction products produce turbidity.

The usefulness of sedimentation equilibrium ultracentrifugation for studying heterogeneous protein-protein associations in interacting mixtures of RA ovomucin complex and lysozyme, under conditions (0.07 M phosphate buffer, pH 6.9) where an interaction could not be detected using turbidimetric measurements (Kato et al., 1975; Kato et al., 1976) can be seen in Figure 2. Here information on both the extent of interaction and the participating components in the interaction can be obtained. For example, by comparing the area under the lysozyme peak in the MWD pattern of the cell containing lysozyme only to that in the cell containing a lysozyme-ovomucin mixture, an extent of interaction of 6.9% was calculated. Furthermore, by comparison of the MWD pattern of the cell containing RA ovomucin complex only to that of the cell containing lysozyme-ovomucin mixture, a preferential interaction between the β-ovomucin component and lysozyme is seen. Kato et al. (1975) have reported that the interaction of β-ovomucin (F-ovomucin) with lysozyme was much stronger than that with α-ovomucin (S-ovomucin) in 0.07 M phosphate, pH 5.4. However, this pH is very far removed from the in vivo egg white pH of approximately 7.4 to 7.8.

In Figure 3 it can be seen that the interaction between RA ovomucin complex and lysozyme is stronger at ionic strength 0.07 and that self-association of α-ovomucin occurs. Robinson and Monsey (1971) have reported self-association of reduced α-ovomucins at pH 7.5 in the absence of the dissociating agent, guanidine hydrochloride. The ionic strength-dependent interaction between α-ovomucins at pH 6.9 may be electrostatic as indicated by the present results, although Van der Waals forces and hydrogen bonds may also be involved (Waugh, 1954). The importance of the self-association of α-ovomucins to the structure of thick egg white and mechanism of egg white thinning needs investigation.

(b) Native Ovomucin-Lysozyme

Although the interaction of RA ovomucin complex with lysozyme has been studied, there have been no previous reports on the interaction of native ovomucin, that is ovomucin solubilized at neutral pH without chemical modification, with lysozyme. The importance of such a study to the understanding of the *in vivo* interaction of lysozyme with ovomucin is obvious. It was found in the present study that the interaction between native ovomucin and lysozyme was also dependent upon ionic strength and hence electrostatic in nature. However, while there was little difference in the extents of interaction of lysozyme with native ovomucin compared to that with RA ovocumin complex at ionic strength 0.13, the interaction of lysozyme with native ovomucin at ionic strength 0.07 was only 0.4 times as strong as that with RA ovomucin complex at the same ionic strength. It is known that native ovomucin consists of aggregated and polymerized molecules of interacting α- and β-ovomucins

cross-linked by specific bonds (Adachi et al., 1973; Hayakawa and Sato, 1976). Similarly the structure of ovomucin complex is known to consist of polymerized chains of α- and β-ovomucins, the individual subunits being held together by disulfide bonds (Kato et al., 1971; Robinson and Monsey, 1971, 1975; Tomimatsu and Donovan, 1972). Reduction and alkylation of the thiol groups in this complex results in depolymerization of the α- and β-ovomucins to give a heterogeneous mixture of α- and β-subunits. Since it has been reported that optical rotatory dispersion experiments of mercaptoethanol-reduced ovomucin indicate that the conformation of the polypeptide chains is not altered by reduction (Donovan et al., 1970) it is assumed that the greater interaction of lysozyme with RA ovomucin complex is due to more lysozyme binding sites being exposed in the depolymerized or partly associated α- and β-subunits of the RA ovomucin complex than in the much larger native ovomucin molecule which migrates as a single component during sedimentation. This assumption is supported by observations that pronase or trypsin digested, reduced ovomucins interact more strongly with lysozyme than do untreated reduced ovomucins (Kato et al., 1975; Kato et al., 1976) and that the carbohydrate side chains of ovomucin which contain the lysozyme binding sites seem to be concentrated along portions of the protein core (Kato et al., 1973). In other words, it is proposed that dissociation of subunits caused by reduction of sulfhydryl bonds exposes the carbohydrate side chains of ovomucin which contain the lysozyme binding sites. Zeta potential or surface charge measurements of RA ovomucin complex versus native ovomucin may confirm this proposal.

2. Effect of Chemical Modification

(a) Lysozyme

The isoionic pH of lysozyme is 10.7 (Osuga and Feeney, 1977) while that of ovomucin is 4.5 to 5.0 (Donovan et al., 1970), hence at pH 6.90 lysozyme will carry a net positive charge and ovomucin a net negative charge. Lysozyme has six lysyl ε-amino groups and an α-amino group (Canfield, 1963 b) carrying positive charges at pH 6.9 and these groups can be chemically modified by acetylation resulting in a decrease in the net positive charge. It was shown in the present study that the extent of interaction of lysozyme with both RA ovomucin complex and native ovomucin decreased but was not completely abolished following 100% modification of the free amino groups in lysozyme. Hence the lysyl ε-amino groups in lysozyme are essential for the electrostatic ovomucin-lysozyme interaction. Kato et al. (1975, 1976) also showed that the lysozyme -RA ovomucin interaction was markedly decreased by acetylation of lysyl amino groups in lysozyme and that the interaction was completely abolished when 6 amino groups were modified. It has been shown that no conformational change occurs in the polypeptide chain as a result of acetylation of lysozyme (Yamasaki et al., 1968), hence it is possible that the residual interaction of acetylated lysozyme with RA ovomucin complex and native ovomucin represent electrostatic bonding between other positively charged groups such as histidine or arginine in lysozyme (Steiner, 1953) or hydrogen bonding (Robinson and Monsey, 1969 a). That this residual interaction was not detected using turbidimetric measurements (Kato et al., 1975) is a further indication of the decreased sensitivity of that technique for detecting protein-protein interactions.

(b) Ovomucin

It was shown in the present study that complete removal of sialic acid residues (which are negatively charged at pH 6.9) from RA ovomucin complex and native ovomucin by treatment with neuraminidase had little effect on the ovomucin-lysozyme interaction at ionic strength 0.07 and that a preferential interaction of lysozyme with β-ovomucin occurred even though its sialic acid residues had been removed. Dam and Bennett (1963) and Dam (1971) also reported that enzymatic removal of sialic acid residues from ovomucin had little effect on lysozyme binding. Thus, if the interaction between ovomucin and lysozyme is electrostatic in nature, there must be sufficient negatively charged polar groups still present to account for the interaction of lysozyme with asialo ovomucin. It is known that ovomucin also contains ester sulfate which would be negatively charged at pH 6.91 and that the ratio of sialic acid to ester sulfate is approximately 1:1 (Donovan et al., 1970; Robinson and Monsey, 1971; Kato and Sato, 1971; Kato et al., 1978 c). Since β-ovomucin contains sialic acid and ester sulfate while α-ovomucin does not (Kato et al., 1972; Kato et al., 1973) and since there is a preferential interaction of lysozyme with β-ovomucin even after complete removal of negatively charged sialic acid residues, it seems likely that it is the net negative charge of the ovomucin molecule made up of contributions from sialic acid and ester sulfate, that is important for the electrostatic ovomucin-lysozyme interaction. In other words, there is still sufficient negative charge carried by ester sulfate after removal of sialic acid residues for a lysozyme-ovomucin interaction. Other acidic

groups such as the carboxyl groups of glutamic and aspartic acids, present in ovomucin, may be the active groups which react with lysozyme (Robinson, 1972). This seems unlikely though, as α-ovomucin contains more of these groups than does β-ovomucin. It is possible, however, that in addition to electrostatic interactions, a more specific type of interaction between the N-acetylglucosamine groups of β-ovomucin and the active site of lysozyme is involved (Howlett and Nichol, 1973) as β-ovomucin contains more than 1.6 times as much N-acetylglucosamine as does α-ovomucin (Smith et al., 1974).

The results of the present study are surprising in view of the previous reports of Kato et al. (1975) who found that the ovomucin-lysozyme interaction decreased correspondingly at a rate depending on the extent of modification of ovomucin with neuraminidase and of Kato et al. (1976) who reported that sialic acid in ovomucin is commonly essential for its interaction with ovalbumin and conalbumin and for its inhibition of the aggregation of κ-casein by rennin (Kato et al., 1974). In fact a detailed study by Kato et al. (1976) showed that the binding site for the ovomucin-lysozyme interaction was the carboxyl group of the sialic acid residue. It should be noted that these studies were done at pH 5.4 and ionic strength 0.13 using turbidimetric measurements to detect insoluble ovomucin-protein interactions. Loss of negatively charged sialic acid groups may result in a change in shape or folding of ovomucin due to a reduction in electrostatic repulsion (Gottschalk and Thomas, 1961; Sachdev et al., 1979) and such a change in conformation might affect the ability of the asialo ovomucin to form an insoluble or turbid interaction

complex with lysozyme at pH 5.4 thus affecting its detection by turbidimetric measurements. It seems possible to resolve the question of whether the ester sulfate is the binding group in the lysozyme -β- ovomucin interaction or whether it is the net negative charge of β-ovomucin contributed by both sialic acid residues and ester sulfate that is important, by enzymatic removal of ester sulfate.

3. Effect of Temperature

That the interaction between lysozyme and RA ovomucin complex was stronger at 3° than 20°C at ionic strength 0.07 is suggestive of an electrostatic interaction and/or hydrogen bonding between lysozyme and ovomucin (Douzou and Balny, 1978). That the extent of interaction at ionic strength 0.13 was apparently unaffected by temperature may be due to the "strong" salt solution (high ionic strength) which can reduce electrostatic protein-protein interactions (Dixon and Webb, 1961) and suggests that hydrogen bonding may not be important. Dam (1971) reported little difference in the effect of temperature on the amount of lysozyme bound by chicken ovomucin at pH 7.2 yet found that much more lysozyme was bound to duck ovomucin at 2° than at 40° at the same pH. As stated previously, these binding measurements were based on the assumption that all complexes between lysozyme and ovomucin are insoluble, hence they may not be reliable. The difference in binding observed between chicken and duck ovomucins may be a reflection of the different properties (e.g., solubility of ovomucins) between these particular species.

B. Molecular Weight of Ovomucin During Egg White Thinning

Ovomucin, a polydisperse glycoprotein fraction, precipitates from egg white when the salt concentration is reduced by dilution of the albumen with water and becomes increasingly insoluble when washed with 2% KCl during further purification. Since ovomucin prepared in this manner is not sufficiently soluble at acidic or neutral pH in non-denaturing solvents and is only partially soluble in alkaline solvents, characterization has been difficult. Most studies therefore have been done on chemically modified ovomucin, that is ovomucin solubilized by reduction with mercaptoethanol followed by alkylation of thiol groups with iodoacetate (Robinson and Monsey, 1964, 1971, 1975; Kato et al., 1971; Donovan et al., 1970). However, physical measurements on ovomucin solubilized without chemical modification (so called "native" ovomucin) are necessary to determine the relation of ovomucin to the gel-like properties of thick egg white.

As calculated from the absorbance values in Figure 9A most of the ovomucin in egg white was solubilized by blending egg white which had been previously diluted with 0.05 M Tris-HCl buffer, pH 8.6, containing 0.85% NaCl. Ovomucin thus obtained (native ovomucin) has an amino acid composition that is in general agreement with that previously reported (Table 1) and a carbohydrate composition (Table 2) which although different from previous reports, reflects on the variability in carbohydrate content of ovomucins (Sleigh et al., 1973; Smith et al., 1974).

Native ovomucin showed an apparent molecular weight (extrapolated to infinite dilution) of 5.64×10^6 and a remarkable

dependence of molecular weight on the concentration of ovomucin which may reflect a strong association of ovomucin molecules at dilute concentrations. A similar dependence of sedimentation coefficients on the concentration of ovomucin and of mucoproteins in general has been reported (Adachi et al., 1973; Robinson and Monsey, 1975; Morawiecki, 1964).

Table 6 shows the molecular weights of soluble ovomucins that have been reported and it is apparent that the molecular weight varies according to the method of solubilization. It should be noted that these published molecular weight determinations were done at single concentrations of ovomucin so it is necessary to compare them to the range of apparent molecular weights ($2.43 - 5.15 \times 10^6$) determined in the present study. Thus native ovomucin has a molecular weight in general agreement to the values of 7.6×10^6 and 8.3×10^6 reported by Lanni et al. (1949) and Hayakawa and Sato (1976), respectively.

The molecular weight of the ovomucin fraction decreased only slightly during the natural thinning of thick white induced by storage of albumen at $30^{\circ}C$ for one week and no change in the amino acid and carbohydrate compositions between the native and stored ovomucins was observed. Moreover, the molecular weight of stored ovomucin (5.25×10^6) was much higher than that determined for reduced ovomucins (mol wt. 726,000 and 309,500). Since ovomucin gel in thick white is solubilized by low speed blending, it seems possible that the thick white gel structure consists of aggregated high molecular weight ovomucins (mol wt. 5.6×10^6) linked inter-molecularly by non-covalent forces such as hydrogen bonding or

hydrophobic interactions (Robson et al., 1975) and that disaggregation of these molecules might occur during natural thinning. Self-aggregation of mucin type glycoproteins of epithelial secretions has been observed (Hill et al., 1977).

Thick egg white is also solubilized by 2-mercaptoethanol producing low molecular weight ovomucins. Previous studies (Robinson and Monsey, 1971, 1975) showed that reduction of the disulfide bonds of ovomucin splits ovomucin into α- and β-subunits (mol wt. 210,000 and 720,000 respectively). The production of similar glycoprotein subunits by reduction of thick egg white shows that cleavage of intramolecular disulfide bonds in ovomucin results in the breakdown of the intermolecular non-covalent interactions responsible for gel formation. However, as there were no low molecular weight ovomucins produced during natural thinning that were produced in the presence of reducing agents, it seems that intermolecular disulfide cleavage of ovomucin does not occur during natural thinning.

It should be noted that gel filtration of blended egg white isolates all soluble ovomucins, i.e., those which may be disaggregated from the thick white gel and are thus present in the thin white and those that were present in the gel but were solubilized by blending. Thus it is difficult, if not impossible, to distinguish between these two classes of ovomucins in the present experiment. To show that disaggregation of ovomucin molecules does occur during natural thinning, the following experiments are suggested. After separation of fresh thick and thin whites by ultracentrifugation (59,000 x g for 60 min) according to the method of Kato et al. (1971), determine the molecular weight and chemical composition of the ovomucin fractions

obtained upon gel filtration of blended thick white versus unblended thin white. Repeat the experiment using naturally thinned egg white (i.e., albumen stored at 30°C for one week). Comparison of the results from these experiments may suggest that there is an equilibrium between aggregation and disaggregation of ovomucin molecules which is in favour of the aggregated state at the pH of fresh albumen and is shifted in favour of the disaggregated state as the pH of the white increases during storage (Powrie, 1980).

GENERAL DISCUSSION

Although many mechanisms have been proposed for the role of ovomucin in the thinning of thick white (Feeney, 1955; Osuga and Feeney, 1977) those that have attracted the most study can be classified into three general groups. One of these is based on the formation of a complex between lysozyme and ovomucin. Another is based upon a physical or chemical change in ovomucin such as disaggregation or chemical hydrolysis and the third is essentially a combination of the first two. The purpose of this general discussion is to relate the results obtained in the present study of ovomucin to these three mechanisms of egg white thinning.

1. Lysozyme-Ovomucin Complex

According to this mechanism the rigidity or structure of thick white is due to the formation of a complex between lysozyme and ovomucin. The non-covalent dissociation of this complex accompanying the increase in pH, (from 7.8 to 9.5), during storage of shell eggs at room temperature, results in thinning. It has been reported (Brooks and Hale, 1959, 1961) that a simple network of ovomucin chains will not account for the mechanical properties of thick white and that the gelatinous structure is better explained by assuming that chains of an ovomucin-lysozyme complex are cross-linked into a network, which is in accord with accepted hypotheses concerning the nature of gel structure (Ferry, 1948). Moreover, mucin-albumin interactions have been shown to affect the properties of mucin solutions notably enhancement of viscosity (List *et al.*, 1978). Thus a requirement of this mechanism for application to egg white thinning is that lysozyme and ovomucin interact under the conditions (ionic strength and pH) found in fresh thick white. A further requirement of this

mechanism is that the extent of the protein-protein interaction decrease with increasing pH.

In this study, direct evidence has been obtained that the interaction between ovomucin and lysozyme *in vitro* is electrostatic, involving the positively charged lysyl ε-amino groups of lysozyme and the net negative charge (i.e., not that carried exclusively by the sialic acid residues) of ovomucin. Moreover, it was shown that there is an interaction between lysozyme and native ovomucin at ionic strength 0.10, which is the approximate ionic strength of egg white (Donovan *et al.*, 1972; Sato *et al.*, 1976). These results suggest but do not prove the existence of an interaction between lysozyme and ovomucin, *in vivo*. Although the extent of the interaction as a function of pH was not measured in the present study, because of the tendency of ovomucin and lysozyme to precipitate out of dilute salt solutions above pH 7.4, it can be concluded that if the interaction between lysozyme and ovomucin is electrostatic, an increase in pH would cause a decrease in the extent of interaction (assuming no conformational changes) due to a decrease in the positive charge of lysozyme as its isoionic pH of 10.7 is approached.

However, there has been little information on whether the lysozyme-ovomucin interaction exists *in vivo* in thick white. Kato *et al.* (1978a) studied the changes in lysozyme during thinning and found that the relative concentration of lysozyme in the gel fraction was 1.4 times that of the liquid fraction in thick white. After storage of thick white for 5 days, some differences in lysozyme content between the gel and liquid fractions were found. These results suggest that lysozyme has a strong affinity for the thick white gel (which is

mainly composed of ovomucin) and that lysozyme dissociates from the gel during storage.

In addition to interacting with ovomucin, basic lysozyme has been shown to bind with some of the other proteins in egg white - ovalbumin (Howlett and Nichol, 1973; Nakai and Kason, 1974) and conalbumin (Ehrenpreis and Warner, 1956) both of which are present in much larger amounts than ovomucin in egg white. As well, ovomucin has been shown to interact non-specifically with ovalbumin and conalbumin (Kato et al., 1976). The relations of these interactions to the rigidity of thick white and their possible role in thinning is unknown and needs investigation.

2. Chemical or Physical Change in Ovomucin During Thinning

A consistent observation on the differences between thick and thin white in fresh eggs (aside from the obvious structural viscosity) has been that thick white contains approximately four times as much ovomucin as thin white (McNally, 1933; Forsythe and Foster, 1949; Lanni et al., 1949; Feeney et al., 1952; Feeney et al., 1955; Brooks and Hale, 1961; Baliga et al., 1971). This, in addition to the gel like property of purified ovomucin, led many to believe that the glycoprotein ovomucin plays an important role in determining the physical properties of thick egg white and that thinning is caused by a chemical change in ovomucin.

That the viscosity of thick white or isolated ovomucin gel was readily decreased upon addition of a small amount of reducing agent such as 2-mercaptoethanol, dithiothreitol, thioglycollic acid etc. (MacDonnell et al., 1950, 1951; Robinson and Monsey, 1964; Dam, 1971; Kato et al., 1971) suggested that thinning was caused by a reduction

of disulfide bonds (disulfide cleavage) of ovomucin and attempts were made to determine the presence and possible identity of a natural reducing agent in egg white. Since attempts to find significant amounts of reducing agents failed (Ducay et al., 1960) and since the time scale for the natural thinning is of a completely different order than the simulation of natural thinning by reducing agents, it was suggested and later shown that alkaline hydrolysis of disulfide bonds caused a decrease in viscosity of egg white and isolated ovomucin gel (Donovan et al., 1972). These experiments were carried out at pH 11.5 in order to reduce the time necessary to observe a significant amount of thinning. As the rate of the thinning reaction was nearly equal to the rate of alkaline hydrolysis of protein disulfide bonds (Donovan, 1967; Donovan and White, 1971) it seemed possible that the thinning of egg white accompanying storage of shell eggs was due to alkaline hydrolysis of the disulfide bonds of ovomucin.

Determination of the molecular weight of ovomucin was therefore needed to substantiate these theories. In the presence of 6 M guanidine-HCl and 0.2 M 2-mercaptoethanol an average molecular weight of 1.5×10^5 was reported (Donovan et al., 1970) while Robinson and Monsey (1971, 1975) reported molecular weights of 2.1×10^5 and 7.2×10^5 for ovomucin reduced with 0.3 M 2-mercaptoethanol in 6 M guanidine-HCl. Tomimatsu and Donovan (1972) determined the weight average molecular weights of ovomucin under various conditions of pH and ionic strength and noted a large difference in molecular weight of ovomucin as a function of pH, being $210 - 270 \times 10^6$ for a pH 6.2 solution of ovomucin compared to $27 - 56 \times 10^6$ for a pH 7.9

solution. Since the molecular weight at neutral pH in 6.5 M guanidine-HCl was found to be 23 x 10^6 it was concluded that ovomucin is highly aggregated at pH 6.2 and only partially aggregated at pH 7.9. Moreover, time-dependent changes in the molecular weight of aggregated ovomucin at pH 11.5 that could be accounted for by alkaline hydrolysis of disulfide bonds, yielded a low molecular weight ovomucin (2.2 x 10^5). Thus two processes -- disaggregation and degradation of ovomucin occur at alkaline pH. Since natural thinning occurs between pH 7.8 and 9.7 and since the alkaline degradation process is very slow at 9.7, these authors concluded that much of the thinning accompanying storage of shell eggs might be produced by disaggregation of gel-like aggregates of high molecular weight ovomucin molecules rather than by alkaline degradation of disulfide bonds.

To determine whether a chemical change (degradation) or a physical change (disaggregation) occurs during natural thinning it is necessary to study the change in molecular weight of unmodified or native ovomucin accompanying the natural thinning of egg white. A requirement of the disulfide cleavage mechanism be it by reducing agents present in the white or by alkaline hydrolysis, is that low molecular weight ovomucins be formed. That these were not detected during natural thinning in the present study argues against this mechanism. As little change in the molecular weight and chemical composition of ovomucin from fresh versus naturally thinned white could be detected and since ovomucin gel in fresh thick white could be solubilized by low speed blending, non-covalent disaggregation of high

molecular weight ovomucin molecules might occur during natural thinning. This disaggregation may be caused by a change in conformation of the ovomucin molecule as the pH of the white increases (Jonathan et al., 1977) or may be due to charge conditioned repulsive forces (Blundell et al., 1972) and needs further investigation.

Ovomucin has an asymmetric distribution of carbohydrate side chains (Kato et al., 1973) and it is possible that ovomucin gel may be partially held by hydrophobic interactions as those carbohydrate moieties may induce a specific folding of the polypeptide chains allowing maximum exposôre of their hydrophobic regions (Mizrahi et al., 1978). The presence of hydrophobic binding regions in glycoproteins of the mucin type has been reported (Sachdev et al., 1979). This idea is supported by the results of Adachi et al. (1973) who reported that the sedimentation coefficient of ovomucin which was isolated by gel filtration of egg white solubilized with 0.5 - 1.0% sodium dodecyl sulfate, remained unchanged before and after liquefaction. Fractionation of a soluble ovomucin (without prior blending of egg white, if possible) and subsequent molecular weight determination (if sedimentation equilibrium ultracentrifugation at much lower running speed is possible) would be a further approach to the study of this mechanism.

3. Dissociation of an Ovomucin Complex Stabilized by Lysozyme

Detailed chemical and physical studies of ovomucin revealed that ovomucin has a subunit structure consisting of carbohydrate rich (β-ovomucin) and carbohydrate poor (α-ovomucin) glycoproteins (Kato et al., 1970 a; Kato and Sato, 1971; Robinson and Monsey, 1971;

Hayakawa and Sato, 1976; Kato et al., 1977). Thus to elucidate the mechanism of egg white thinning the chemical or physiochemical changes which ovomucin undergoes during thinning were studied. It was found (Kato et al., 1970 b; Kato et al., 1971; Kato and Sato, 1972; Kato et al., 1972; Robinson and Monsey, 1972 a) that the carbohydrate contents of ovomucin gel remarkably decreased during storage and that the carbohydrate rich component also diminished. Accompanying the decrease in concentration of carbohydrate rich component from ovomucin gel was a decrease in lysozyme content. Thus a model of ovomucin gel structure and mechanism of thinning were proposed (Kato et al., 1971). According to this model the swollen, rigid gelatinous structure of thick white is due to association of polymerized α-ovomucin subunits and polymerized β-ovomucin subunits supported by lysozyme and thinning results from the dissociation of β-ovomucin and lysozyme. It was assumed that α-ovomucin and/or β-ovomucin subunits were polymerized through disulfide bonds because ovomucin gel is solubilized by treatment with 0.01 M 2-mercaptoethanol yielding α- and β-subunits. Subsequently it was shown that the interaction between lysozyme and ovomucin was electrostatic involving the negatively charged terminal sialic acid residues of ovomucin and the positively charged lysyl ε-amino groups of lysozyme, and that the interaction of lysozyme with β-ovomucin was much stronger than that with α-ovomucin (Kato et al., 1975). As it was later observed that the O-glycosidically linked carbohydrate units of β-ovomucin (containing the negatively charged terminal sialic acid residues) were liberated from serine or threonine residues by alkali treatment (Kato et al., 1978 b, c) it was suggested that the specific solubilization

of β-ovomucin which occurs during thinning might be due to the natural degradation of β-elimination of the O-glycosidically linked carbohydrate units. β-elimination of O-glycosidically linked carbohydrate units of egg white proteins was observed during thinning and seemed to be caused by β-elimination of the O-glycosidically linked carbohydrate units of ovomucin because most of the O-glycosidic linkages in egg white are found in ovomucin (Kato et al., 1979), supporting this model.

The data in the present study lend support to this mechanism in that a preferential interaction between β-ovomucin and lysozyme was shown, as required by this model, although the importance of the negatively charged sialic acid residues for the interaction is doubtful. However, the carbohydrate side chains also contain a negatively charged ester sulfate which was shown in the present study to be important for the lysozyme-β-ovomucin interaction. If β-elimination of O-glycosidic linkages does occur during natural thinning, it would decrease this interaction.

Whether or not the β-elimination reaction is significant has yet to be proven. For example, a difference in carbohydrate content (notably sialic acid) between ovomucin from fresh egg white versus naturally thinned white stored for 7 days could not be detected in the present study. This may not be unreasonable as it was reported that only 10% of the total β-elimination was shown to occur in egg white after 30 days of storage (Kato et al., 1979). That only 10% β-elimination was shown to occur may indicate that this reaction is not important in terms of thinning. If this is so, a dissociation of

β-ovomucin could still occur as the extent of the electrostatic interaction of lysozyme with ovomucin would decrease with the increasing pH of egg white during storage.

Whether such changes in the interaction of ovomucin with lysozyme (in view of the fact that ovomucin comprises only a very small portion of total egg white protein) can result in the drastic change in the physical properties of thick white observed during thinning remains in doubt.

The role of lysozyme in the possible stabilization of a gel formed by non-covalent association of polymerized α-ovomucins and polymerized β-ovomucins needs more attention but may not be important. To this end an interesting explanation for the possible disaggregation of ovomucin during thinning was recently proposed (Kato, 1980). According to this mechanism the ovomucin of fresh thick white has a swollen structure because of its large water binding capacity (one gram of ovomucin binds over 200 grams of water), the water being held by the carbohydrate side chains. The large amount of water bound to ovomucin is unusual (being about 1.5 g per g protein for most proteins) and can account for its gel like properties at low concentration (Ferry, 1948). Disaggregation of ovomucin molecules could thus be caused under conditions in which the ordered structure of bound water is destroyed, such as blending, sonication, or partial degradation of the O-glycosidically linked carbohydrate units of ovomucin.

CONCLUSION

The interaction between lysozyme and ovomucin at neutral pH is electrostatic involving positively charged lysyl-amino groups of lysozyme and the net negative charge of ovomucin, and a preferential interaction between lysozyme and β-ovomucin was observed. A large difference in the extent of interaction of lysozyme with reduced and alkylated ovomucin complex as compared to that with native ovomucin at low ionic strength was observed, hence it is recommended that future studies on the properties of ovomucin be done using native or unmodified ovomucin. The existence of such a lysozyme-ovomucin interaction *in vivo* needs to be demonstrated and until then its importance in contributing to the gelatinous property of thick white remains doubtful.

The gelatinous structure of thick white appears to be due to association of high molecular weight ovomucin molecules held together by non-covalent interactions (hydrogen bonding, hydrophobic interactions, etc.). It seems unlikely that chemical degradation of ovomucin occurs during thinning although a small amount of alkaline hydrolysis of glycosidic linkages may occur. Instead, it is probable that disaggregation of associated high molecular weight ovomucin molecules causes thinning of thick white. The cause of this disaggregation which accompanies the pH increase observed upon the storage of shell eggs needs further investigation as does the quaternary structure of native ovomucin.

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TABLE 1

Amino Acid Composition of Whole Ovomucin. Values expressed as g amino acid per 100 g of protein.

Amino Acids	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Aspartic Acid	9.77	9.31	12.29	9.18	10.11	9.76	11.66	10.98	12.39
Threonine	7.02	8.50	7.68	7.51	6.53	5.76	5.93	6.48	7.40
Serine	7.11	7.76	5.97	7.11	6.54	6.79	7.22	6.36	7.00
Glutamic Acid	11.94	11.98	11.94	11.49	12.83	12.76	10.81	11.87	13.63
Proline	5.62	5.82	4.44	5.51	4.89	6.79	1.99	4.15	5.65
Glycine	3.85	3.67	4.27	2.96	3.58	3.07	4.40	3.71	3.59
Alanine	4.32	4.00	3.58	2.97	3.78	4.11	3.96	4.62	5.66
Valine	5.88	5.92	5.63	5.38	5.93	6.68	6.89	5.03	5.38
Methionine	2.49	0.78	2.39	2.36	2.27	3.07	1.95	1.98	2.31
Isoleucine	4.78	4.71	4.95	4.33	4.76	5.22	5.78	5.84	4.50
Leucine	7.28	7.45	7.34	6.63	7.15	7.83	6.55	8.46	6.49
Tyrosine	4.30	4.12	5.46	4.19	6.12	4.64	5.00	5.09	4.95
Phenylalanine	5.17	4.86	5.63	5.07	5.28	5.83	6.74	5.37	5.82
Lysine	6.18	6.38	7.34	7.13	6.94	6.20	7.66	7.59	7.88
Histidine	2.70	2.43	2.73	2.81	2.56	2.40	2.40	2.20	2.79
Tryptophan	ND*	ND*	ND*	1.36	ND*	2.72	ND*	ND*	ND*
Arginine	4.21	4.54	5.12	4.35	4.94	4.76	4.84	4.76	4.55
Cysteine	4.04	7.75	3.24	6.56	5.80	3.93	6.37	5.51	ND*

*Not determined.

(1) This study.
(2) Young and Gardner, 1972.
(3) Kato et al., 1973.
(4) Robinson and Monsey, 1971.
(5) Osuga and Feeney, 1968.
(6) Donovan et al., 1970.
(7) Adachi et al., 1973.
(8) Sato and Hayakawa, 1977.
(9) Smith et al., 1974.

TABLE 2

Amino Acid Composition of Ovomucins from Native and Stored Egg White

Amino Acid	Ovomucin from fresh thick egg white	Ovomucin from thick egg white held at 30 ± 2°C for 166 h
Aspartic acid	9.77	9.96
Threonine	7.02	7.14
Serine	7.11	7.67
Glutamic acid	11.94	11.72
Proline	5.62	5.51
Glycine	3.85	3.74
Alanine	4.32	4.19
Valine	5.88	5.95
Methionine	2.49	2.26
Isoleucine	4.78	4.60
Leucine	7.28	7.42
Tyrosine	4.30	4.45
Phenylalanine	5.17	5.38
Lysine	6.18	6.06
Histidine	2.70	2.74
Tryptophan	ND*	ND*
Arginine	4.21	4.13
Cysteine	4.04	4.22

[a]Values for amino acids are averages of duplicate analyses on 22 h acid hydrolysates.

*Not determined.

TABLE 3

Carbohydrate Composition of Ovomucins from Fresh and Stored Egg White, g per 100 g dry weight.

Component	fresh	stored
Sialic acid	4.65	4.63
Hexose	9.77	9.74
Hexosamine	10.14	10.23

TABLE 4

$M_{w\ app}$ of Native Ovomucin as a Function of Protein Concentration

Protein concentration, mg/ml*	$M_{w\ app}$ X 10^{-6}
0.150	5.153
0.169	4.865
0.199	4.611
0.226	3.987
0.336	4.334
0.369	3.944
0.453	3.014
0.671	2.426

*Calculated from molar extinction coefficient, $E^{1\%}_{280\ nm,\ 1cm} = 7.0$

TABLE 5

$M_{w\ app}$ of Stored Ovomucin as a Function of Protein Concentration

Protein concentration, mg/ml*	$M_{w\ app}$ X 10^{-6}
0.144	4.433
0.149	4.579
0.179	4.758
0.364	3.234
0.387	3.046
0.446	2.852
0.457	3.045
0.521	2.830
0.600	2.574

*Calculated from molar extinction coefficient, $E^{1\%}_{280\ nm,\ 1cm}=7.0$

TABLE 6

Molecular Weight of Soluble Ovomucins

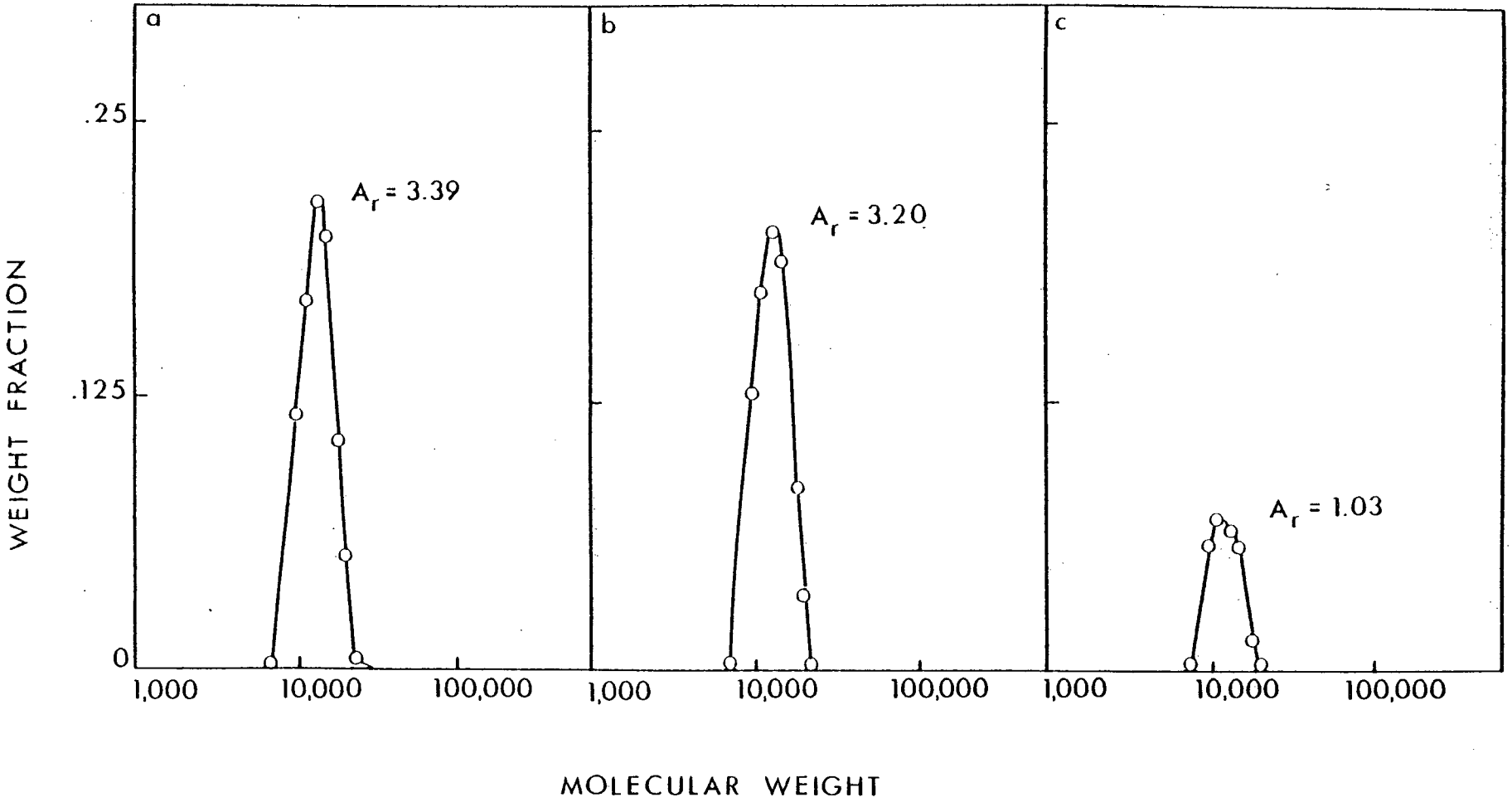
Solubilization method	Molecular weight (million)	Determination	Reference
0.1 N NaOH	0.22	Light scattering	(1)
0.3 M 2-mercaptoethanol	0.21	Sedimentation equilibrium	(2)
	0.72		
Sonication	1.1	Light scattering	(3)
Blending in mild alkaline buffer, pH 8.6	3.0	Molecular exclusion on Sepharose 4B	(4)
Blending in mild alkaline buffer, pH 8.6	5.5-7.5	Sedimentation equilibrium	(5)
0.06 M phosphate buffer, pH 7.2	7.6	Sedimentation velocity	(6)
Blending in mild alkaline buffer, pH 9.0	8.3	Light scattering	(3)
6.5 M guanidine-HCl	11.5	Sedimentation velocity	(7)
1 M KCl, pH 7.9	40.0	Light scattering	(1)
1 M KCl, pH 6.2	240.0	Light scattering	(1)

(1) Tomimatsu and Donovan, 1972.
(2) Robinson and Monsey, 1971.
(3) Hayakawa and Sato, 1976.
(4) Young and Gardner, 1972.
(5) This study.
(6) Lanni et al., 1949.
(7) Robinson and Monsey, 1975.

FIGURE 1

Molecular weight distribution of an RA ovomucin complex-lysozyme mixture at ionic strengths 0.13 and 0.07. (A) Lysozyme at ionic strength 0.13. Initial absorbance, 0.298; rotor speed, 19,329 rpm. (B) RA ovomucin complex-lysozyme (1:4) at ionic strength 0.13. Initial absorbance, 0.298; rotor speed, 19,329 rpm. (C) RA ovomucin complex-lysozyme mixture (1:4) at ionic strength 0.07. Initial absorbance, 0.300; rotor speed, 19,944 rpm.

For all figures, true value for A_r can be obtained by dividing by 4.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b
c
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.125
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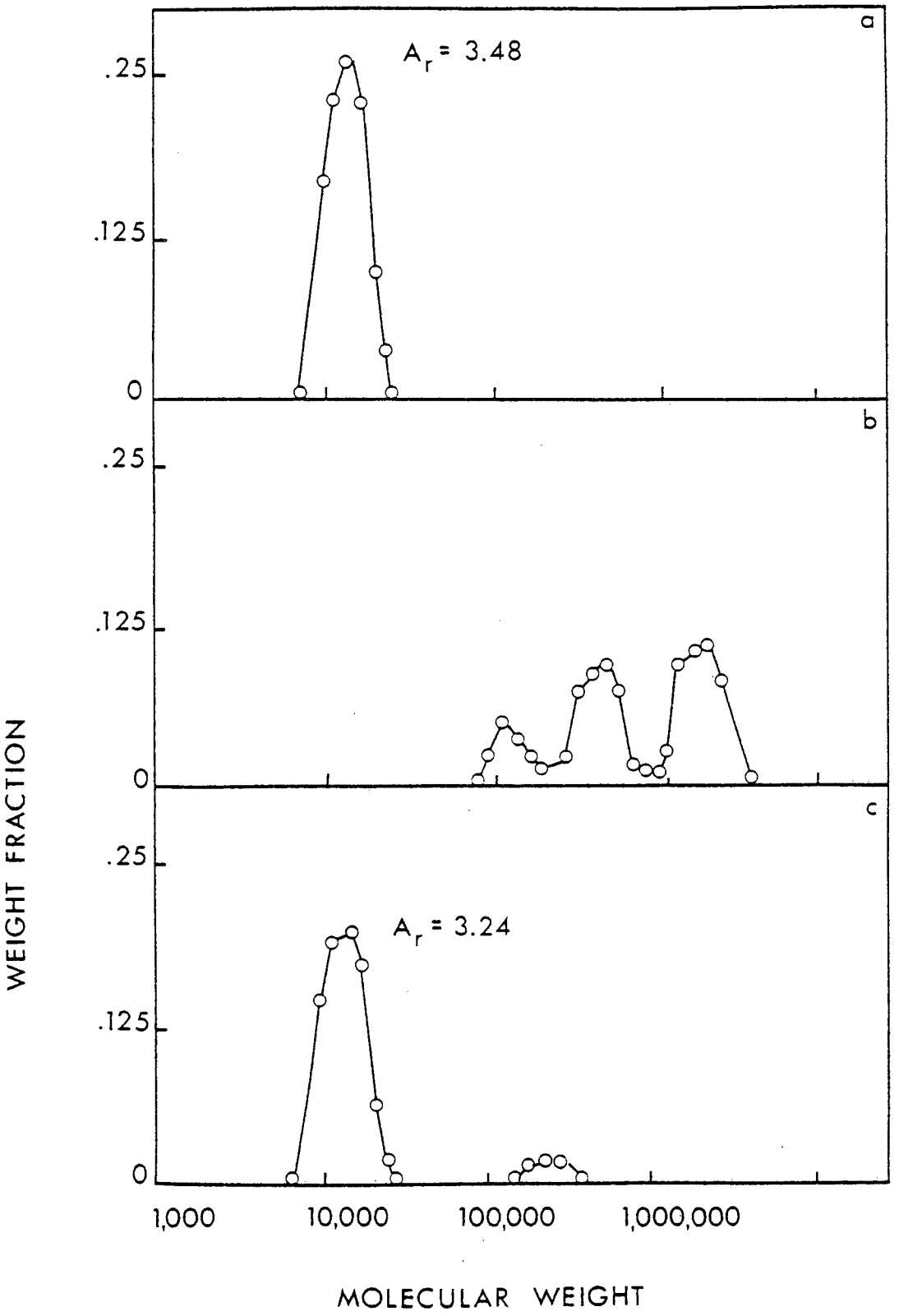
A_r = 3.39
A_r = 3.20
A_r = 1.03
1,000
10,000
100,000
WEIGHT FRACTION
MOLECULAR WEIGHT

FIGURE 2

Molecular weight distribution of an RA ovomucin complex-lysozyme mixture at ionic strength 0.13. (A) Lysozyme. Initial absorbance, 0.300; rotor speed, 19,939 rpm. (B) RA ovomucin complex. Initial absorbance, 0.300; rotor speed, 9,866. (C) RA ovomucin complex-lysozyme mixture (1:4). Initial absorbance 0.298; rotor speed, 9,866 rpm.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c
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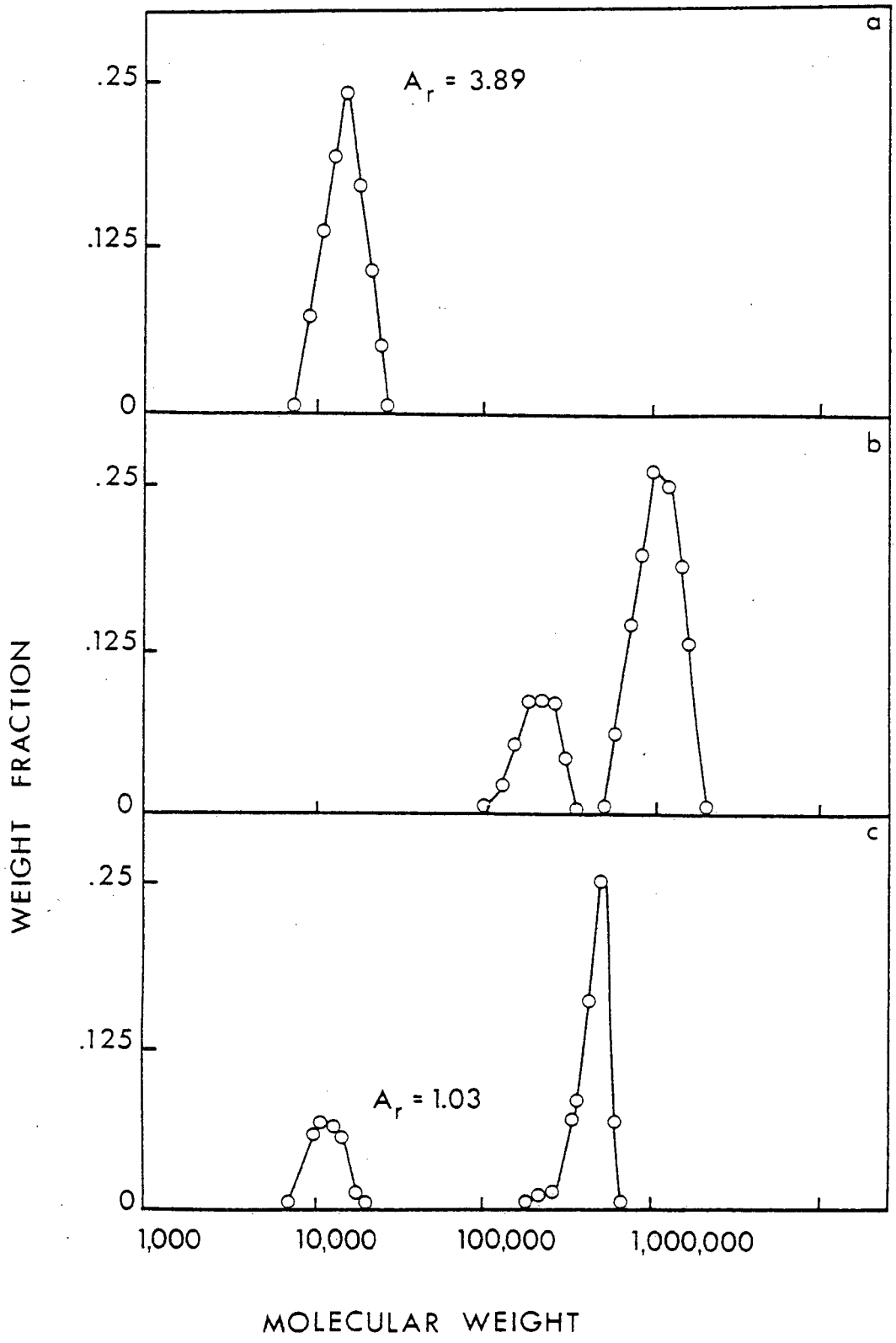
.125
0
1,000
10,000
100,000
1,000,000
WEIGHT FRACTION
MOLECULAR WEIGHT

FIGURE 3

Molecular weight distribution of an RA ovomucin complex-lysozyme mixture at ionic strength 0.07. (A) Lysozyme. Initial absorbance, 0.300; rotor speed, 19,944 rpm. (B) RA ovomucin complex. Initial absorbance, 0.303; rotor speed, 9,974 rpm. (C) RA ovomucin complex-lysozyme mixture (1:4). Initial absorbance, 0.305; rotor speed, 9,974 rpm.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b
c
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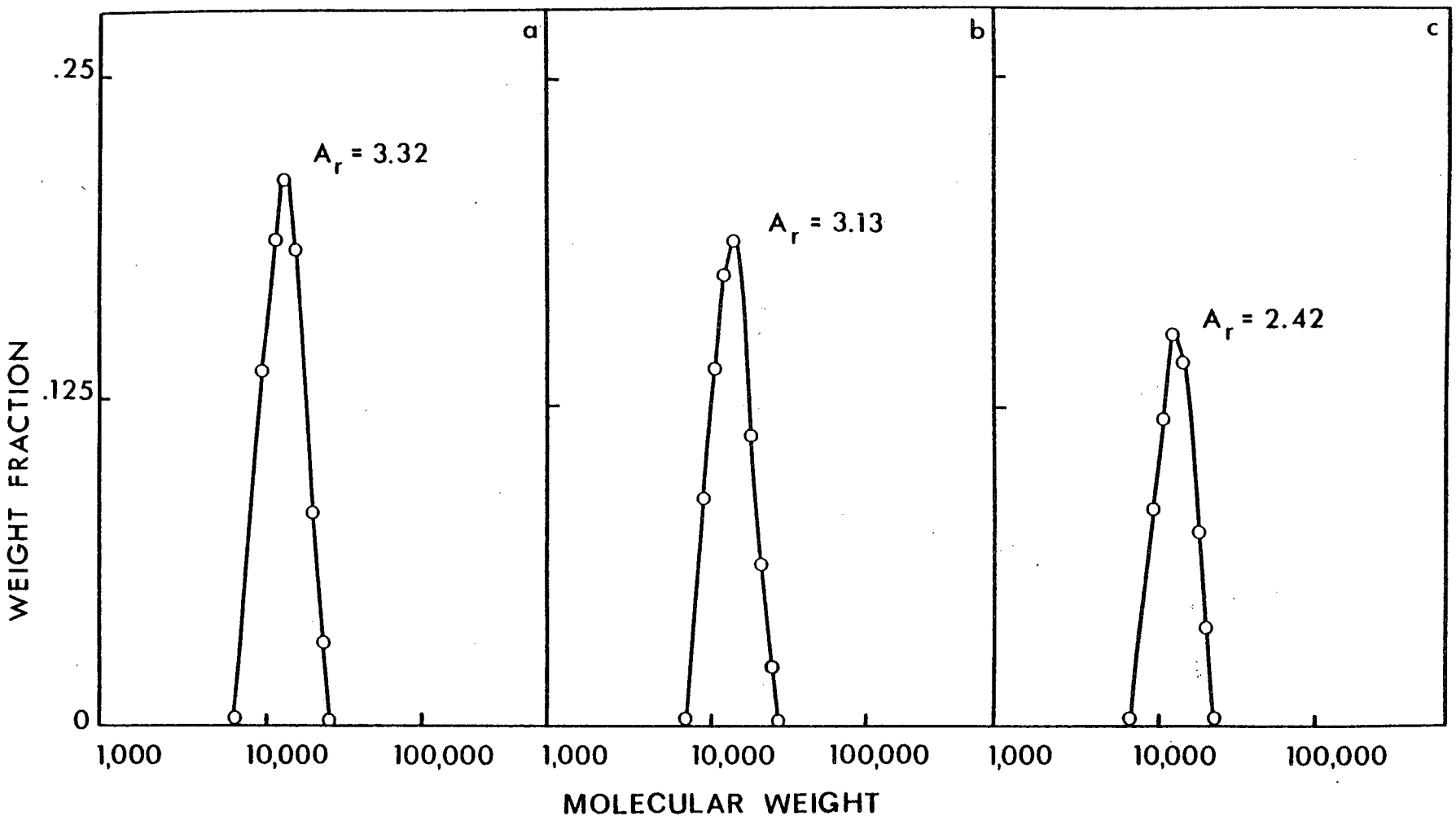
A_r = 1.03
WEIGHT FRACTION
1,000
10,000
100,000
1,000,000
MOLECULAR WEIGHT

FIGURE 4

Molecular weight distribution of a native ovomucin-lysozyme mixture at ionic strengths 0.13 and 0.07. (A) Lysozyme at ionic strength 0.13. Initial absorbance, 0.305. (B) Native ovomucin-lysozyme mixture (1:4) at ionic strength 0.13. Initial absorbance, 0.300. (C) Native ovomucin-lysozyme mixture (1:4) at ionic strength 0.07. Initial absorbance, 0.298. Rotor speed, 31,908 rpm.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0
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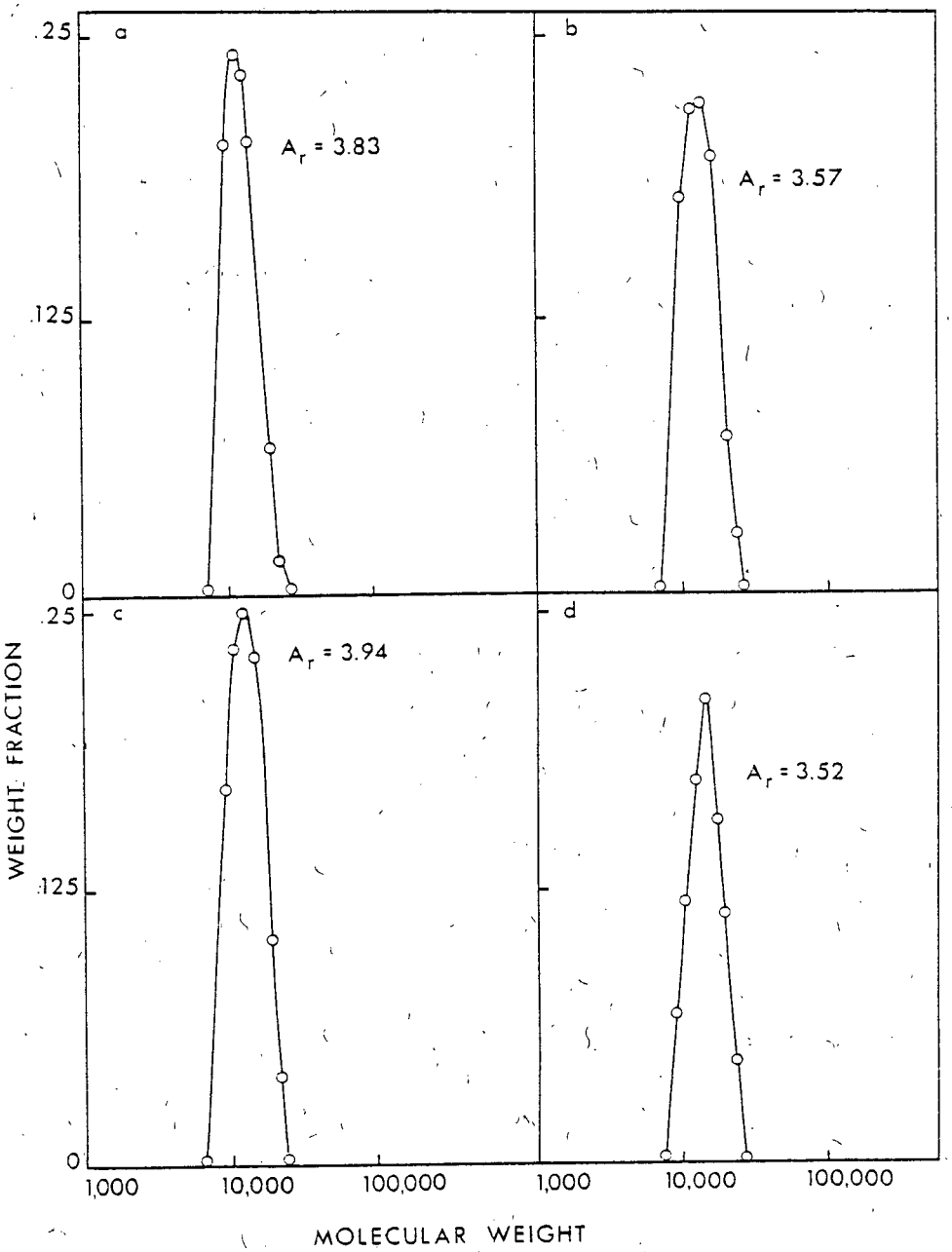
A_r = 3.13
A_r = 2.42
WEIGHT FRACTION
1,000
10,000
100,000
MOLECULAR WEIGHT

FIGURE 5

Molecular weight distributions of ovomucin-acetylated lysozyme mixtures at ionic strength 0.07. (A) Acetylated lysozyme. Initial absorbance, 0.298; rotor speed, 33,570 rpm. (B) Acetylated lysozyme-native ovocumin (4:1). Initial absorbance, 0.300; rotor speed, 33,570 rpm. (C) Acetylated lysozyme. Initial absorbance, 0.302; rotor speed, 34,224 rpm. (D) Acetylated lysozyme-RA ovomucin complex (4:1). Initial absorbance, 0.300; rotor speed, 34,224 rpm.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d
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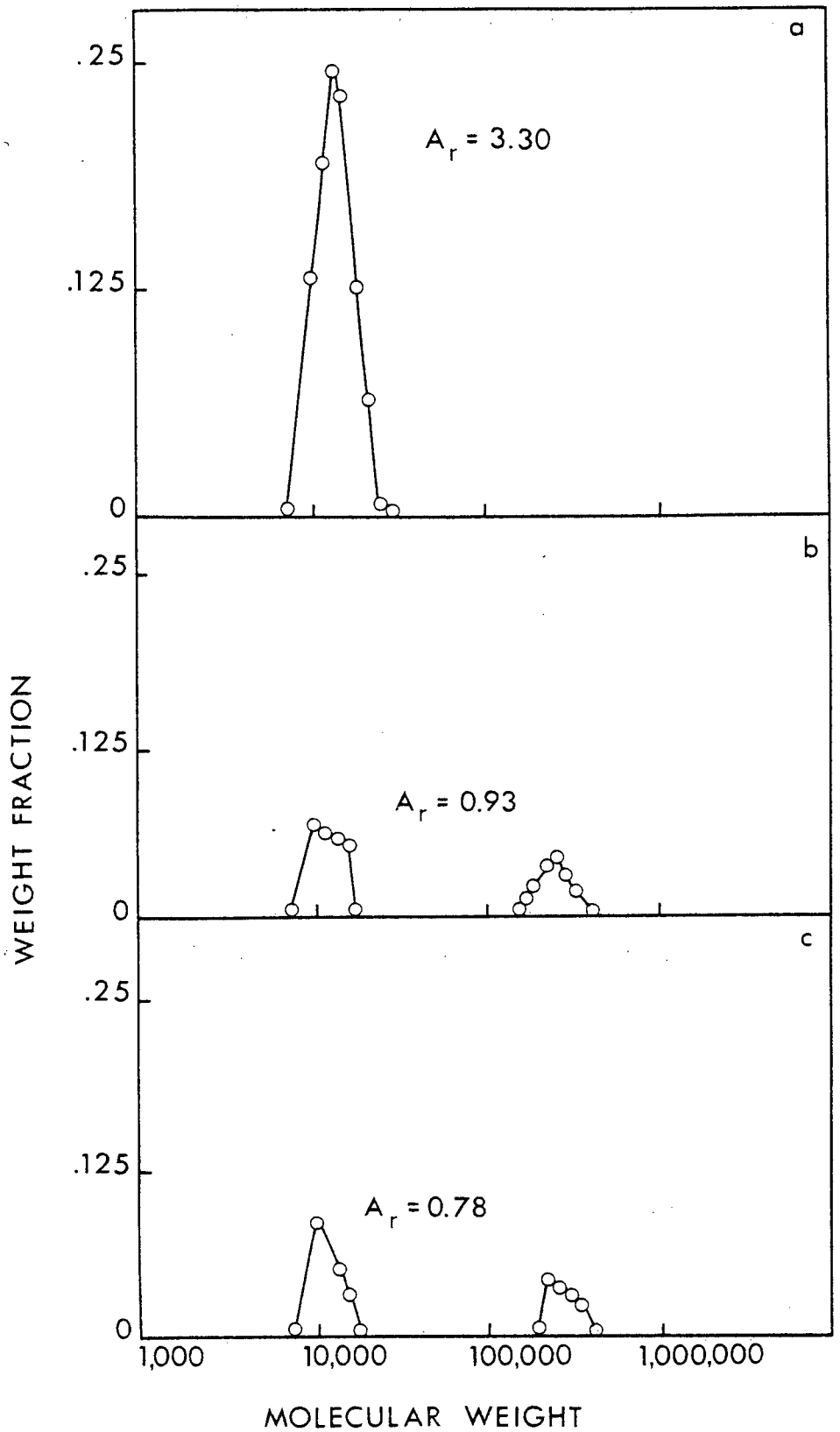
A_r = 3.94
A_r = 3.52
.25
.125
0
WEIGHT FRACTION
1,000
10,000
100,000
MOLECULAR WEIGHT

FIGURE 6

Molecular weight distribution of an asialo, RA ovomucin complex-lysozyme mixture at ionic strength 0.07. (A) Lysozyme. Initial absorbance, 0.297; rotor speed, 35,235 rpm. (B) RA ovomucin complex-lysozyme mixture (1:4). Initial absorbance, 0.298; rotor speed, 9,263 rpm. (C) Asialo, RA ovomucin complex-lysozyme mixture (1:4). Initial absorbance, 0.300; rotor speed, 9,263 rpm.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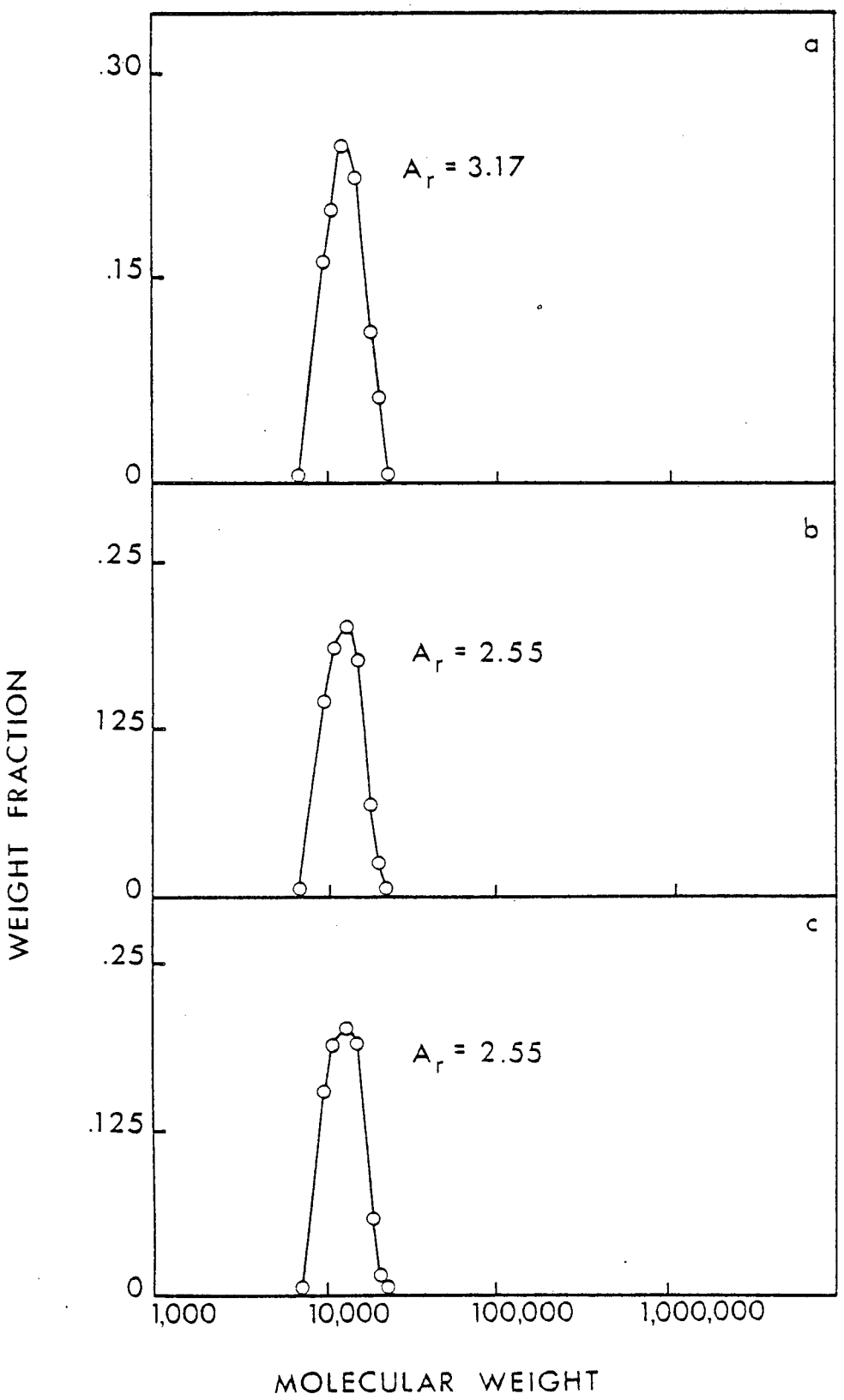
0
A_r = 3.30
b
.25
.125
0
A_r = 0.93
c
.25
.125
0
A_r = 0.78
WEIGHT FRACTION
1,000
10,000
100,000
1,000,000
MOLECULAR WEIGHT

FIGURE 7

Molecular weight distribution of an asialo, native ovomucin-lysozyme mixture at ionic strength 0.07. (A) Lysozyme. Initial absorbance, 0.300. (B) Native ovomucin-lysozyme mixture (1:4). Initial absorbance, 0.300. (C) Asialo, native ovomucin-lysozyme mixture (1:4). Initial absorbance, 0.300. Rotor speed, 35,798 rpm.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A_r = 3.17
.30
.15
0
b
A_r = 2.55
.25
125
0
c
A_r = 2.55
.25
.125
0
1,000
10,000
100,000
1,000,000
WEIGHT FRACTION
MOLECULAR WEIGHT

FIGURE 8

Molecular weight distributions of RA ovomucin complex-lysozyme mixtures (1:4) (⊣O⊢) and lysozyme (-•-) at 20° and 3°C. Initial absorbances of all solutions, 0.250. (A), (B). Temperature, 20 ± 5°C. Rotor speed, 33,696 rpm. (C), (D). Temperature, 3 ± 5°C. Rotor speed, 34,416 rpm. Ionic strengths of solutions: 0.13 (A), (C); 0.07 (B), (D).

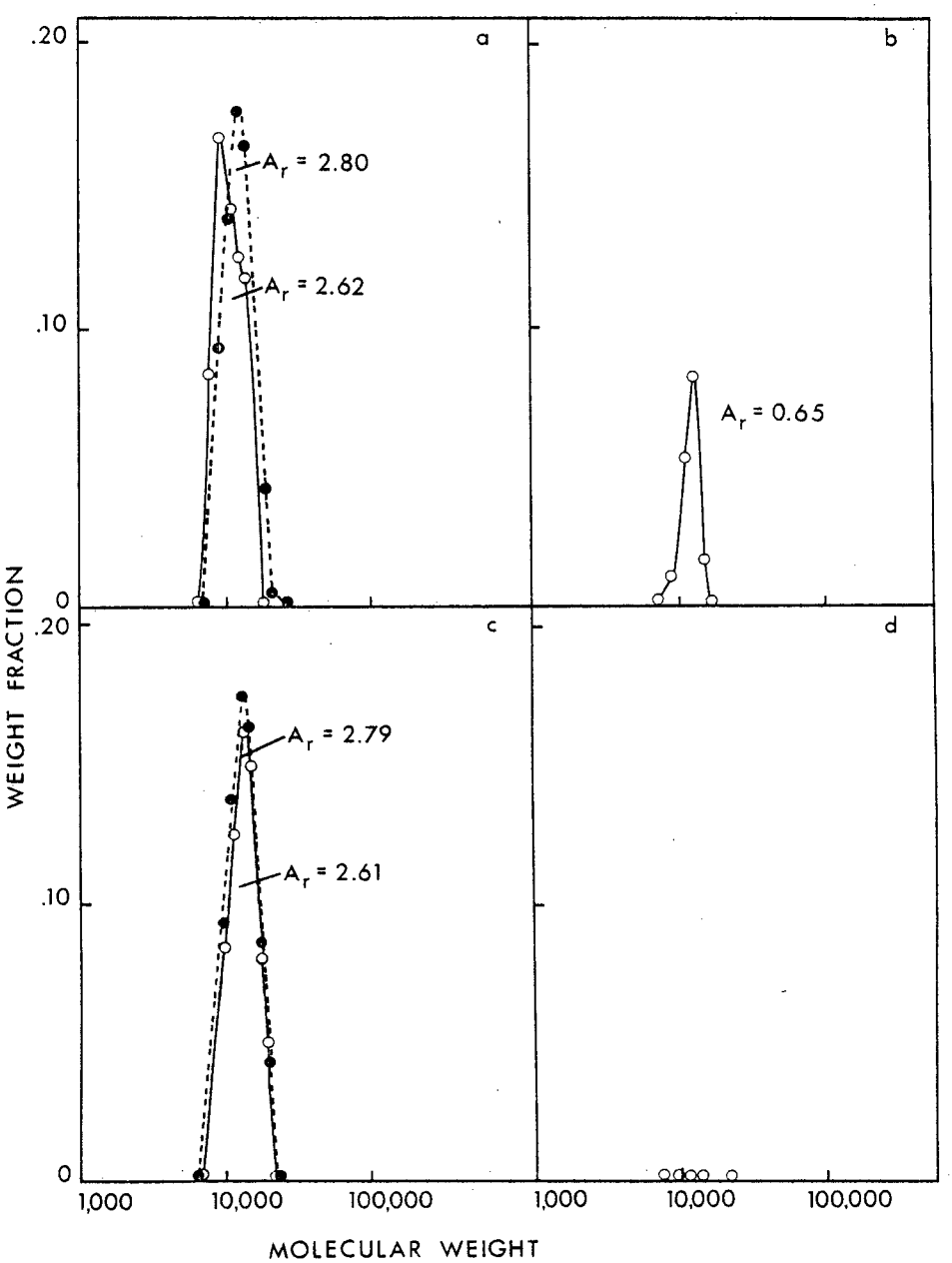

.20
.10
0
a
b
c
d
A_r = 2.80
A_r = 2.62
A_r = 0.65
A_r = 2.79
A_r = 2.61
WEIGHT FRACTION
1,000
10,000
100,000
MOLECULAR WEIGHT

FIGURE 9

Gel filtration of egg white on Sepharose 4B. 10 ml of egg white (prepared as described in "Materials and Methods") was applied to a column (2.5 X 40 cm) of Sepharose 4B equilibrated at 20°C with 0.04 M Tris-HCl buffer, pH 8.6, containing 0.85% NaCl, and eluted with the same buffer. The effluant was collected in 3.5 ml fractions and analyzed for protein (-●-) and sialic acid (-■-).

A. Native egg white. (Fractions 23-28 combined for chemical analysis.)

B. Stored (166 h) egg white. (Fractions 23-28 combined for chemical analysis.)

C. Egg white reduced with 0.02% 2-mercaptoethanol.

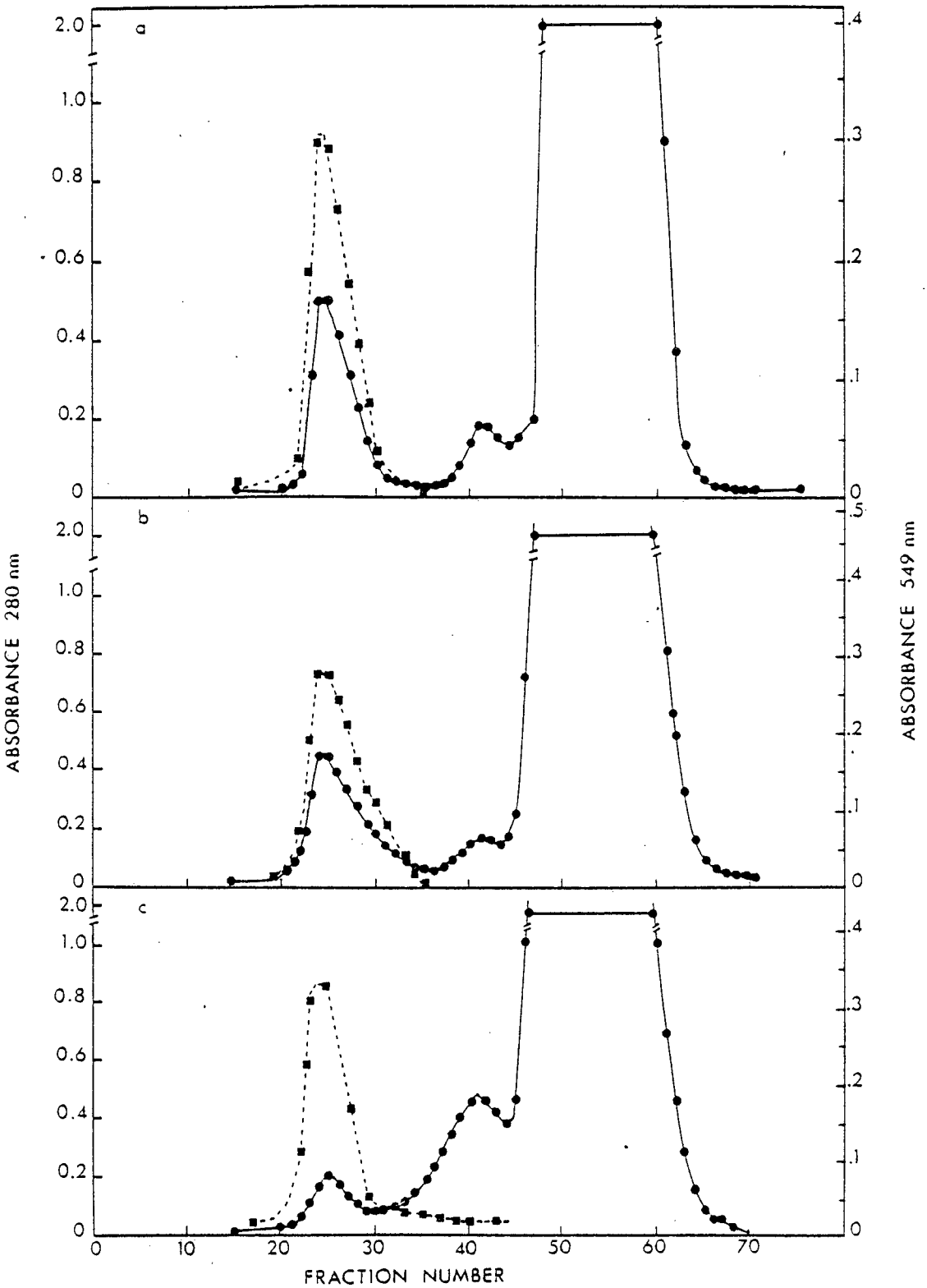

a
b
c
ABSORBANCE 280 nm
ABSORBANCE 549 nm
FRACTION NUMBER
0
10
20
30
40
50
60
70
2.0
1.0
0.8
0.6
0.4
0.2
0
.4
.3
.2
.1
.5

FIGURE 10

Sedimentation equilibrium patterns of ovomucins, ln A vs r^2. All samples were dialyzed against 0.07 M sodium phosphate, pH 6.95, containing 0.02% sodium azide with a column height of 3 mm. The preparations, initial protein concentrations and rotor speeds were as follows: A, native ovomucin, 0.45 mg/ml, 4,085 rpm; B, native ovomucin, 0.23 mg/ml, 4,048 rpm; C, stored ovomucin, 0.52 mg/ml, 4,014 rpm; D, stored ovomucin, 0.36 mg/ml, 4,034 rpm.

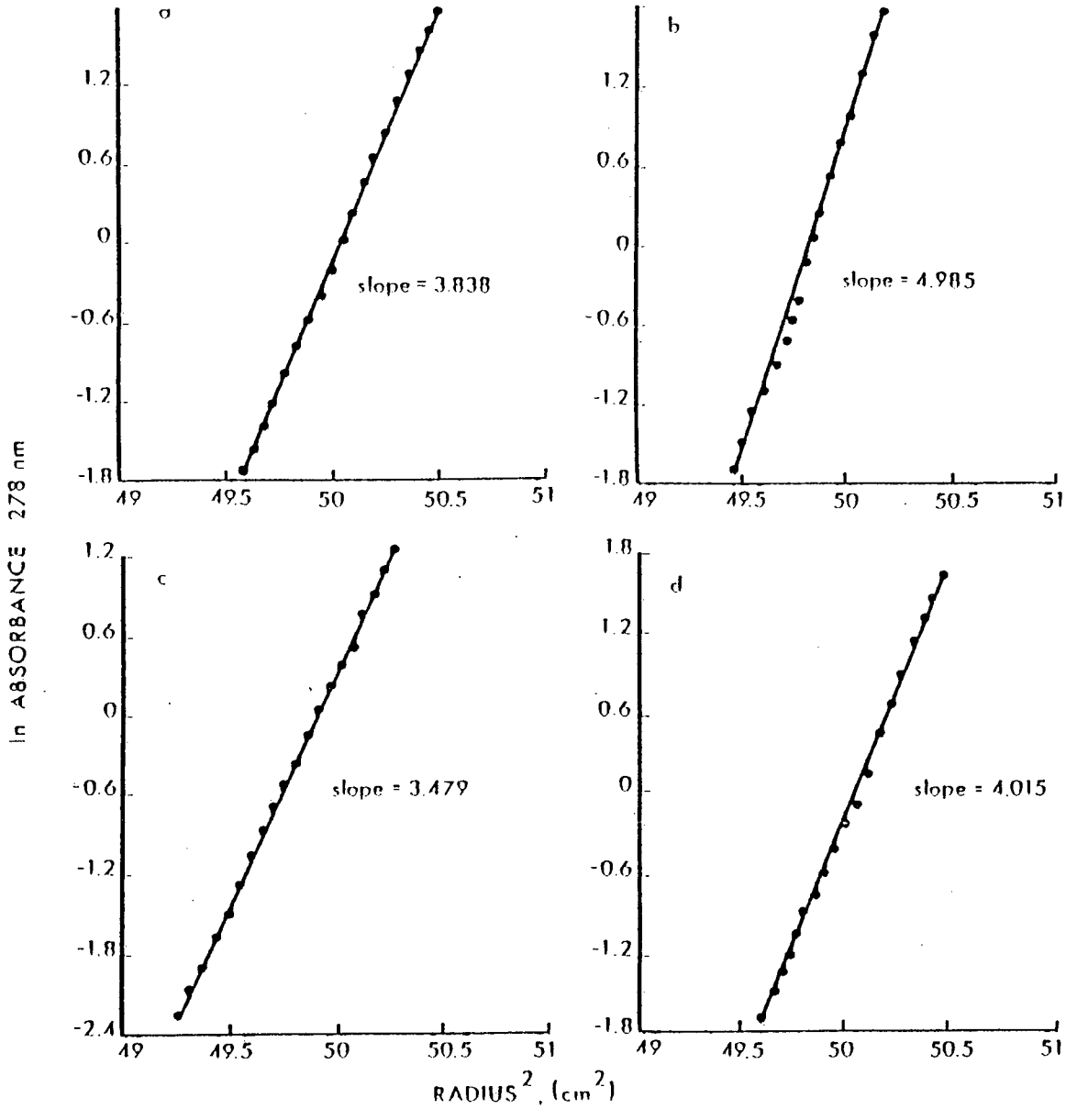

a
b
c
d
slope = 3.838
slope = 4.985
slope = 3.479
slope = 4.015
ln ABSORBANCE 278 nm
RADIUS^2, (cm^2)
1.2
0.6
0
-0.6
-1.2
-1.8
-2.4
49
49.5
50
50.5
51

FIGURE 11

Apparent molecular weight ($M_{w\ app}$) of ovomucins as a function of protein concentration in 0.07 M sodium phosphate containing 0.02% sodium azide, pH 6.95, 20°C.

(a) Native ovomucin.

(b) Stored ovomucin.

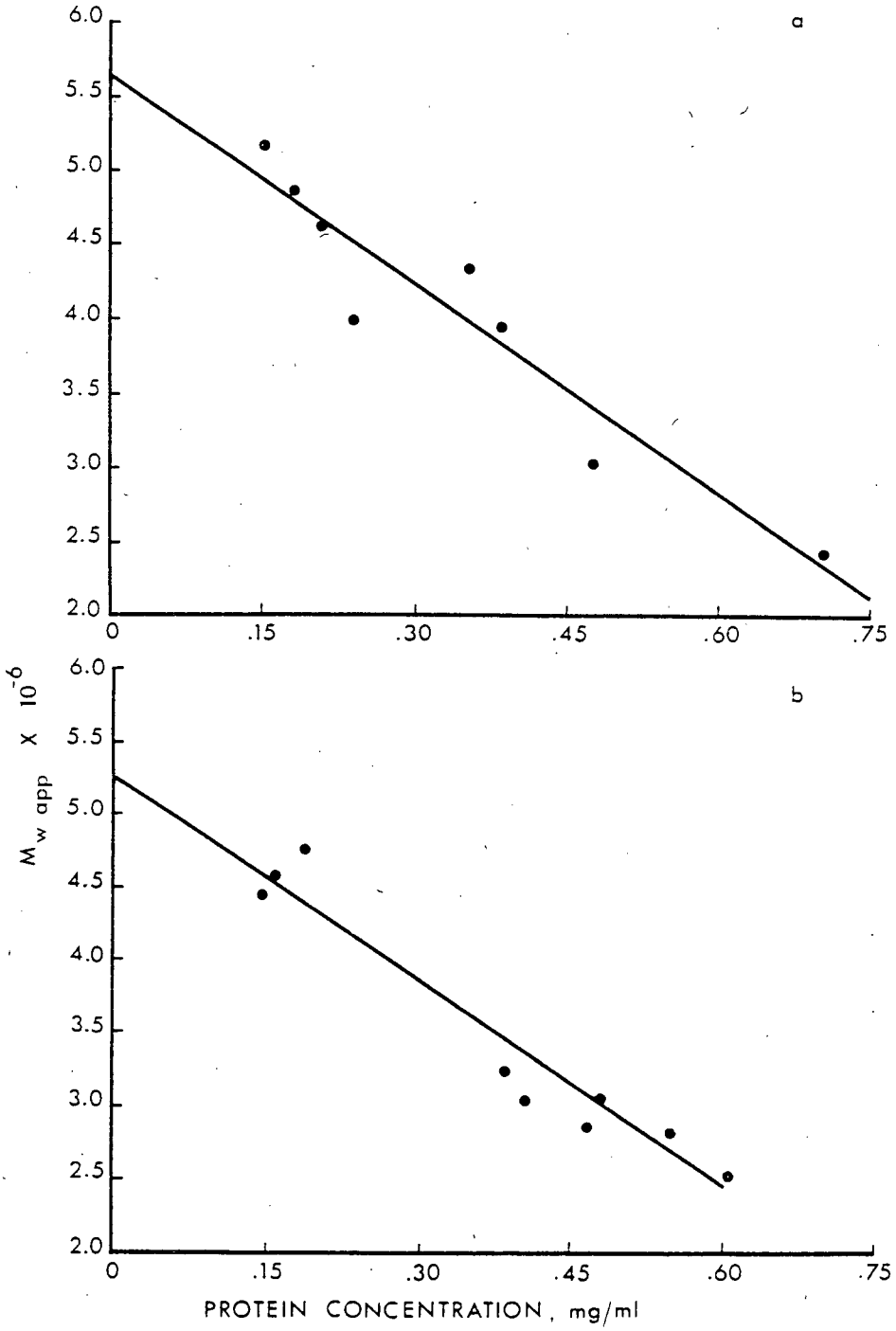
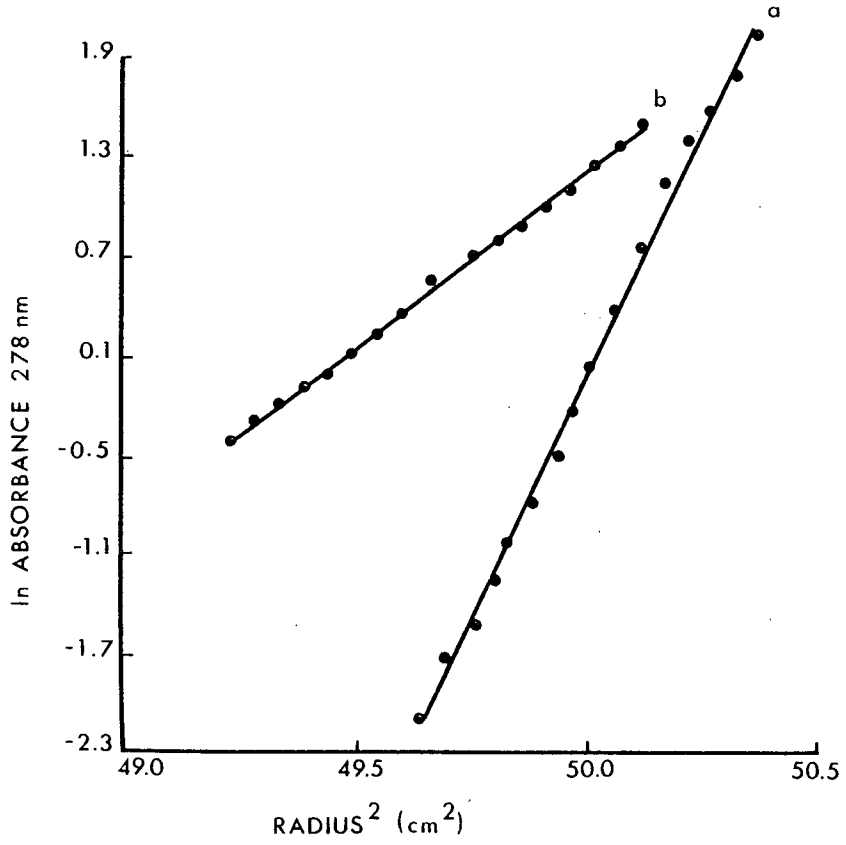

a
b
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
0
.15
.30
.45
.60
.75
M_w app X 10^{-6}
PROTEIN CONCENTRATION, mg/ml

FIGURE 12

Sedimentation equilibrium ultracentrifugation of fractions 24 and 37 from gel filtration of egg white reduced with 0.02% 2-mercaptoethanol. Samples were dialyzed against 0.07 M sodium phosphate, pH 6.95, containing 0.02% sodium azide. Final concentrations were 0.09 and 0.11 mg/ml for fractions 24 and 37, respectively. Rotor speed, 9,891 rpm.

(a) Fraction 24.

(b) Fraction 37.


a
b
1.9
1.3
0.7
0.1
-0.5
-1.1
-1.7
-2.3
ln ABSORBANCE 278 nm
49.0
49.5
50.0
50.5
RADIUS^2 (cm^2)