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Effect of High-Pressure Pulsation of Some of the Physical–Chemical Properties of Ovalbumin

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The effect of pulsating pressure (2500–5000 atm) on some of the physical–chemical properties of ovalbumin was examined. Conditions reducing electrostatic repulsion promoted extensive aggregation of the pressure-treated protein. Ovalbumin subjected to pressure treatment at pH 4 or 5 in salt-free solution exhibited a rapid equilibrium between a monomer and *n*-mer. Electrophoretic studies and phosphorus analysis revealed a pressure-induced hydrolysis of a phosphate ester at pH $\leq$ 4.0. Chemical analysis did not show any change in the accessibility of sulfhydryl groups resulting from treatment at any pH. However, small conformational changes resulting from pressure treatment were reflected by changes in the reactivity of tyrosyl residues with *N*-acetylimidazole and the accessibility of tyrosyl and tryptophanyl residues to various perturbants.

Numerous studies have shown that proteins are denatured by high-pressure treatments above 1000 atm (Brandts *et al.*, 1970; Bridgeman, 1914; Hawley, 1971; Ikkai and Ooi, 1966; Miyagawa, 1965; Suzuki, 1958, 1963; Suzuki *et al.*, 1962). However, the effect of rapid pulsating high pressures, *i.e.*, high intensity shock waves, on protein structure has not been investigated. The present investigation was undertaken to examine the changes in a protein's structure resulting from electrohydraulic (EH) shock waves. Ovalbumin, which is known to be affected by hydrostatic pressures, was selected for this study.

EXPERIMENTAL PROCEDURES

Materials. Crystalline ovalbumin (Grade III) obtained from Sigma Chemical Co. was used without further purification. For studies of unbuffered solutions, the protein was exhaustively dialyzed against deionized–distilled water followed by adjustment of the pH to the desired value with 0.1 *N* NaOH or 0.1 *N* HCl. Both the untreated (native) and the EH-treated ovalbumin solutions were centrifuged at 13,500 $\times$ *g* and 2° for 15 min; the supernatants were filtered through sterile 0.45-μ Millipore filters and stored in sterile screw-capped vials at 3°.

The A_1 component of ovalbumin was prepared from fresh chicken egg white by the procedure of Rhodes *et al.* (1958) using CM–cellulose chromatography. Analysis showed that the preparation contained 0.13% phosphorus and 5.1 SH groups per 45,000 g. Disk electrophoresis revealed two bands, with the faster migrating component corresponding to ovalbumin-A_1 and representing 90% of the total material.

Electrohydraulic Equipment. The electrohydraulic apparatus was supplied by General Electric Co., Chemical Systems Branch of Research and Development Center, Schenectady, N. Y. Alternating current at line voltage (220 V) was transformed and rectified to a high voltage direct current which was used to charge the capacitors. The capacitor bank consisted of four 6-microfared (μF) capacitors connected in parallel to yield a total of 24 μF. A charging voltage of 13 kV was used throughout this study. The charged capacitors were made to discharge rapidly by means of a secondary switching circuit and an ignition, which is essentially a quick electronic switch. The high-intensity electric field developed across the electrode gap causes ionization of the water molecules and the formation of a gaseous plasma. Since the inertia of the surrounding water exerts an almost rigid opposition to the expansion of the plasma, intense high-pressure shock waves are generated. Martin (1960) has estimated the shock wave pressures to be in the range of 2500–5000 atm and the pressures rise and fall within microseconds. The rate of discharge was held at one discharge per second and a total of 200 discharges was delivered for each treatment in this study. Using the relationship $E = {}^1/_2\, CV^2$, where E is the energy in joules, C the capacitance in μF, and V the voltage in kV, an energy output of 2028 J per discharge or a total of 405,600 J per treatment was calculated.

The electrode consisted of a stainless steel inner core separated from an aluminum outer tube by epoxy-glass insulation

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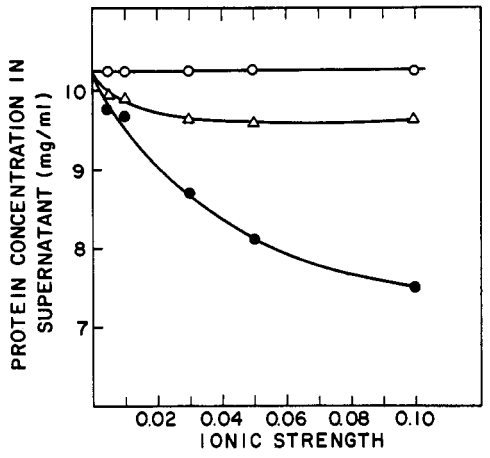

Figure 1. Ionic strength dependence of ovalbumin coagulation by pulsating high pressures at pH 4.0. ○, native ovalbumin; ●, treated ovalbumin; △, ovalbumin dialyzed against water after treatment at the specified ionic strength

(the center rod was the high voltage electrode and the outer sheath the ground). An aqueous solution of 0.3% NaCl was used to fill the tank submerging the electrode. A neoprene diaphragm ($^1/_8$-in. thickness) separated the compartment, thus eliminating the effect of electrical discharge *per se* on the protein. Measured temperature of the protein solution (22–25°) was the same before and after treatment.

Methods. Protein concentration of various samples was determined from their 290-nm absorbances in 0.1 *M* NaOH using an experimentally determined absorptivity of 0.72 ml mg^{-1} cm^{-1} from the refractive index difference at 436 nm using an experimentally determined specific refractive increment of 0.178 ml/g, and from total nitrogen determined by a microKjeldahl procedure assuming a nitrogen content of 15.76% (Chibnall *et al.*, 1943; Lee and Montgomery, 1961). Reactive sulfhydryl groups were determined by Ellman's (1959) procedure.

Determination of phosphorus content was performed according to Berenblum and Chain (1938). Native or treated ovalbumin solutions were passed over a column of Bio-Rad AG 502-X8 mixed bed resin in order to remove all ions prior to analysis.

Viscosity measurements were made at 20.0 ± 0.05° in Cannon–Ubberlohde semimicro dilution viscometers. Dust and fibers were removed from the solutions by filtration through 0.45-μ Gelman Alpha Metricell filters.

Beds of Sephadex G-150 (1.5 × 23-cm) were prepared in Pharmacia columns according to the method of Flodin (1962). The eluate was monitored continuously by absorption measurement at 280 nm and fractions were collected in a Gilson Medical Electronics Linear Fractionator.

Acetylation of "free" tyrosine residues of native and shock-treated ovalbumin was performed according to the procedure of Riordan *et al.* (1965). A 1. 1 × 10^{-4} *M* ovalbumin solution was treated with a 60-fold excess of the reagent.

Free-boundary electrophoresis of native and EH-treated ovalbumin was performed in a Perkin-Elmer Model 38A electrophoresis apparatus using a 2-ml Tiselius cell at 1° and the mobilities were calculated from the descending arm using the conductivity of the protein solution.

Disk electrophoresis in polyacrylamide gels was performed with a Canalco Model 6 apparatus. Sample and stacking gels of 2.5% gel concentration were prepared by photopolymerization (riboflavin–TEMED System) in Tris–HCl buffer, pH 6.7. A persulfate-*n,n,n,n*-tetramethylethylene-

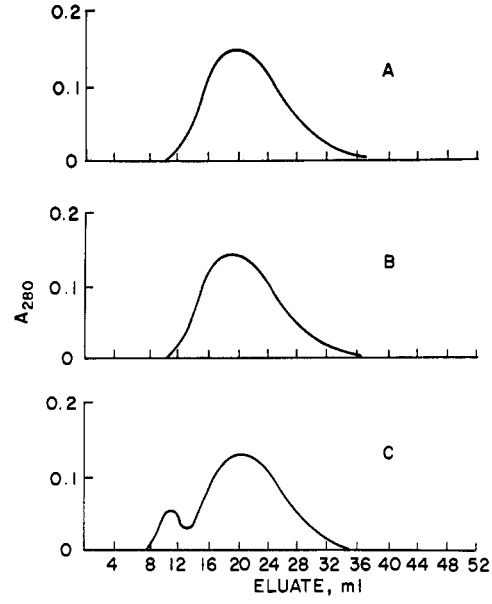

Figure 2. Elution profiles from Sephadex G-150. The eluting buffer was 0.025 *M* sodium phosphate, pH 8.0, and the initial protein concentration was 10 mg/ml. A, native ovalbumin dissolved at pH 4, 5, 6, 7, or 8. B, ovalbumin treated at pH 6, 7, or 8. C, ovalbumin treated at pH 4 or 5

diamine (TEMED) catalyst system was used for preparation of the 7% separation gels in Tris–HCl buffer, pH 8.9. Gels were stained with Amido Schwarz in 7% acetic acid solution for 1.5 hr and electrophoretically destained in the apparatus. A plot of the peak area, obtained from a gel scan of the stained gels using a Photovolt Model 542 densitometer, *vs.* the total protein concentration showed that Beer's law was obeyed up to 60 μg of protein sample. Hence, disk electrophoresis experiments with native and EH-treated ovalbumin were performed using 40 μg of sample.

Sedimentation velocity experiments were performed with a Beckman Model E Analytical Ultracentrifuge equipped with a RTIC temperature control unit and a phase plate for schlieren optics. The effect of temperature on the sedimentation rate of native and shocked ovalbumin was studied by equilibrating the solution at 3, 20, or 35° for 2.5 hr. The rotor was equilibrated to the desired temperature prior to the experiment.

Solvent perturbation difference spectra were obtained for native ovalbumin and that shocked at pH 4 or 8 in distilled water. The procedures followed were essentially those developed by Herskovits and Laskowski (1962) and Herskovits (1967). All of the perturbant chemicals were reagent or spectroscopic grades. The ovalbumin model mixture was prepared by mixing the *N*-acetyl–ethyl esters of L-tyrosine, tryptophan, and phenylalanine (Mann Research Laboratories) in the proper molar proportions, Phe–Tyr–Trp, 8:3:1 (Lewis *et al.*, 1950). The spectral measurements were performed with a Cary 15 spectrophotometer using a 0–0.1 slide-wire and a matched pair of cylindrical-tandem cells.

RESULTS

Preliminary studies showed that EH treatment had an adverse effect on protein stability. For example, there was an 8% decrease in soluble ovalbumin when preparations were shocked in 0.1 *M* sodium phosphate buffer (pH 8.0) and a 25% decrease when shocked at pH 4.8 in sodium acetate buffer (0.1 *M*).

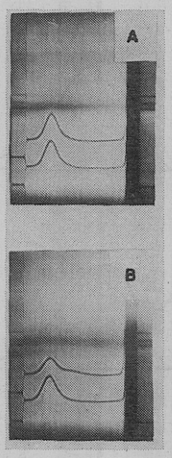

Figure 3. Sedimentation velocity patterns of native and treated ovalbumin in 0.1 *M* NaCl. A, upper boundary, ovalbumin shocked at pH 7; lower boundary native ovalbumin at pH 7. B, upper boundary, ovalbumin shocked at pH 4; lower boundary, native ovalbumin at pH 4. Conditions: rotor speed, 59,780 rpm; temperature, 20°; time, 64 min; diaphragm angle, 60°

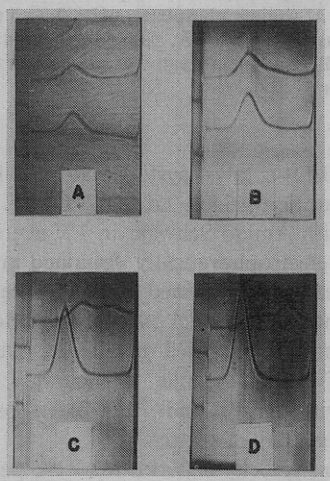

Figure 4. Effect of varying protein concentration on sedimentation of native ovalbumin and ovalbumin treated at pH 4 in salt-free solution. For each case the pattern for native ovalbumin appears in the lower curve and the pattern for treated ovalbumin appears in the upper curve. Protein concentrations are: A, 4.1 mg/ml; B, 8.2 mg/ml; C, 15.3 mg/ml; D, 20.4 mg/ml. Conditions: rotor speed, 59,780 rpm; temperature, 20°; time, 64 min; diaphragm angle, 60°; salt-free solution adjusted to pH 4

Ovalbumin was subjected to EH treatment while dispersed in NaCl solutions of varying ionic strengths at pH 4.0 (Figure 1). The extent of coagulation was small at ionic strengths less than 0.01, but increased sharply at higher ionic strengths. Shocked ovalbumin solutions, when dialyzed against distilled water at 4° for 4 days, showed that some of the coagulated protein was resolubilized.

In view of the above results, subsequent treatments were performed in salt-free media at different pH values.

Gel Filtration Studies. Gel filtration on Sephadex G-150 did not show any differences between the ovalbumin shocked at pH 6.0, 7.0, or 8.0 and the corresponding native proteins (Figure 2A, B). In each case, the shocked ovalbumin emerged from the column with an elution profile identical to that of the native ovalbumin. Elution profiles of ovalbumin shocked at pH 4.0 or 5.0 showed two fractions (Figure 2C). The faster component was eluted from the column immediately following the void volume, while the slower component was eluted at a volume comparable to that of the native albumin. The relative concentrations of the fast and slow components were 10 and 90%, respectively. The presence of the faster component indicated that soluble aggregates of ovalbumin were formed by EH treatment at the lower pH values.

Table I. Effect of Protein Concentration on the Sedimentation Coefficients and Composition of Treated Ovalbumin[a]

Protein concentration, mg/ml	Slow component		Fast component	
	Relative area	$S_{20,w}$	Relative area	$S_{20,w}$
4.1	1.00	3.34	0	
8.2	1.00	3.30	0	
10.3	0.80	3.29	0.20	6.81
15.3	0.53	3.29	0.47	6.86
20.4	0.40	3.32	0.60	6.95

[a] Ovalbumin was subjected to pressure pulsation and sedimentation in salt-free solution adjusted to pH 4.0. (See Figure 4 for the conditions of sedimentation.)

Different eluants altered the relative amount of aggregate eluted from the gel column. Elution with 0.01 *M* sodium acetate buffer (pH 4.0) resulted in amounts of 20 and 80% for the fast and slow components, respectively. Elution with 0.2 *M* NaCl (pH 4.0) recovered only the slow components, indicating the aggregate fraction had precipitated on the column. Native ovalbumin was completely recovered when the latter eluant was used.

Sedimentation Velocity Studies. Prior to sedimentation analysis, salt-free ovalbumin solutions shocked at the different pH values were dialyzed against 0.1 *M* NaCl (at 3°). Ovalbumin solutions treated at pH 6.0, 7.0, or 8.0, as well as the native protein, sedimented as a single component with a symmetrical boundary shape as illustrated for pH 7.0 in Figure 3A. The sedimenting boundary of shocked ovalbumin at pH 4.0 or 5.0 was skewed markedly on the leading edge (Figure 3B).

Regardless of the pH, sedimentation coefficients of the major component were essentially identical with those of the native protein (*ca.* 3.2 S at a protein concentration of 10 mg/ml).

Additional sedimentation studies were performed on ovalbumin shocked at pH 4.0 without prior dialysis against 0.1 *M* NaCl. A bimodal sedimenting boundary was observed. The slow component sedimented at essentially the same rate as native ovalbumin (3.3 S), while sedimentation of the fast component was much greater (6.8 S). Relative amounts of the fast and slow components were estimated to be 20 and 80%, respectively.

Since gel chromatography and coagulation studies had suggested a reversible reaction, the concentration-dependence of the sedimentation velocity data was examined for ovalbumin which had been shocked in salt-free media at pH 4.0. These data showed that the sedimentation rate of the fast component increased as the total protein concentration increased, whereas that of the slow component remained relatively constant (Figure 4, Table I). Furthermore, the relative concentration of the slow component remained constant, while that of the fast component increased with increasing protein concentration (Table I).

Variation of the temperature (20° → 5° → 35° → 20°) demonstrated the temperature dependence of the association equilibrium. The relative area corresponding to the fast sedimenting species increased greatly as the temperature was increased (Figure 5).

Viscosity Measurements. Intrinsic kinematic viscosities

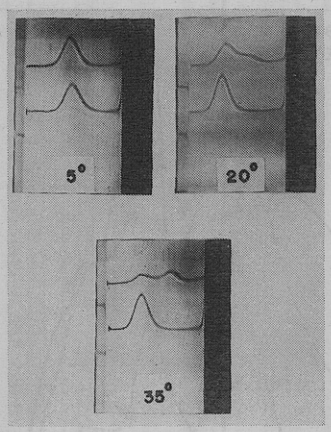

Figure 5. Effect of temperature on the association of ovalbumin treated at pH 4 in salt-free solution. Protein concentration was 10 mg/ml. Other conditions were the same as those listed in Figure 4. Identical patterns were obtained at 20°, regardless of the temperature history

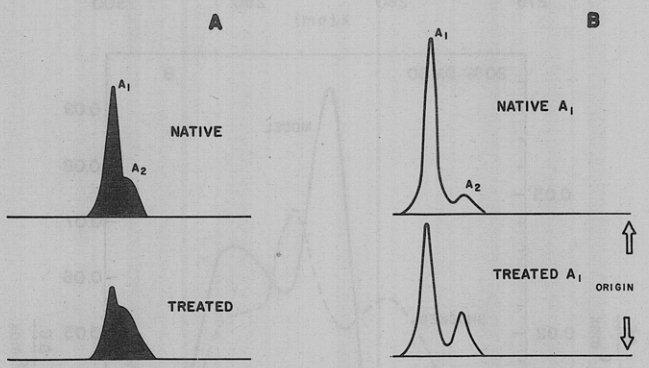

Figure 6. Electrophoresis studies of native and pH 4-treated ovalbumin. A, free boundary experiment for unfractionated ovalbumin performed in 0.05 *M* sodium acetate buffer, pH 4.9. Schlieren pattern of the ascending boundary photographed 7200 sec after current application. B, densitometer traces from disk gel electrophoresis of fractionated ovalbumin A_1 in Tris–HCl buffer, pH 8.9

were determined for native ovalbumin and solutions EH-treated at different pH values in distilled water. Essentially the same value was obtained for each case: 3.9 cm^3/g for native protein at pH 4 or 8 and an average of 3.5 cm^3/g for protein shocked at pH 4, 5, 6, 7, or 8.

Electrophoretic Studies. Moving boundary electrophoresis in 0.05 *M* sodium acetate, pH 4.9, of native and pH 4-shocked ovalbumin indicated a change in the relative amounts of A_1 and A_2 components as a result of treatment (Figure 6A). The mobilities and the presence of only two boundaries in a 50:50 mixture of native and pH 4-shocked ovalbumin suggested the identity of the components in treated ovalbumin as A_1 and A_2 (Table II). EH treatment in distilled water at pH 8 did not cause a shift in the relative amounts of the components.

These observations were substantiated by disk gel electrophoresis of the preparation of ovalbumin A_1 which had been subjected to EH treatment at pH 4.0 (Figure 6B). Measurement of intensities of the protein bands by densitometry showed that the A_1 component decreased 20% and the A_2 component increased accordingly as a result of treatment.

Phosphorus Analysis. Ovalbumin shocked at pH 3.5 and 4.0 had 15% less phosphorus than the native protein and shocked A_1 had 20% less phosphorus than the untreated (Table III). However, solutions of protein treated at pH 4.5 or 5.0 did not exhibit a reduction in phosphorus content.

Sulfhydryl Group Analysis. EH treatment had no measurable effect on reactivity of sulfhydryl groups. No reaction

Table II. Electrophoretic Mobilities[a] of Native Ovalbumin, pH 4-Treated Ovalbumin, and a 50:50 Mixture of Each

Ovalbumin components	Mobility × 10^5 ($cm^2/V/sec$) Native	Treated	50:50 mixture
Fast component (A_1)	−1.81 (64%)[b]	−1.82 (46%)	−1.85 (56%)
Slow component (A_2)	−1.52 (36%)	−1.63 (54%)	−1.69 (44%)

[a] Solutions were dialyzed against 0.05 *M* acetate buffer, pH 4.90, prior to electrophoresis. [b] The values in parentheses are the percent areas of the fast and slow components of the respective solutions.

Table III. Percent Phosphorus in Native Ovalbumin and in Ovalbumin Treated at Different pH Values

	Average phosphorus, %	Standard deviation, %
Native	0.099[a]	0.001
Treated		
pH 5.0	0.100[b]	0.001
4.5	0.103[b]	0.003
4.0	0.084[c]	0.001
3.5	0.085[b]	0.001
Ovalbumin-A_1		
Native	0.130[b]	0.003
Treated	0.101[b]	0.003

[a] The average of ten determinations. [b] The average of four determinations. [c] The average of six determinations.

Table IV. Relative Exposure[a] of Tyrosyl and Tryptophanyl Residues in Native and Treated Ovalbumin as Measured by Solvent Perturbation Difference Spectra

	Native (pH 4.0)		pH 4.0-treated		pH 8.0-treated	
Perturbant	Tyr	Trp	Tyr	Trp	Tyr	Trp
Sucrose	0.32	0.16	0.30	0.30	0.29	0.32
Ethylene glycol	0.43	0.34	0.45	0.47	0.45	0.46
DMSO	0.17	0.18	0.30	0.34	0.30	0.34
D_2O	0.18	0.20	0.33	0.34	0.33	0.34

[a] R_m values calculated relative to the model compound mixture.

was observed for either native or treated ovalbumin in the absence of sodium dodecylsulfate (SDS), whereas five groups reacted in either case in the presence of SDS. Furthermore, EH treatment in the presence of iodoacetamide or mercaptoethanol did not alter these results.

Reaction with *N*-Acetylimidazole. The effect of EH treatment on exposure of tyrosyl residues was examined by reaction with excess *N*-acetylimidazole. The number of reactive residues, calculated from spectral measurements at 278 nm, was 1.6 for native ovalbumin and 3.1 for the treated protein, indicating that additional residues became exposed during EH treatment.

Solvent Perturbation Difference Spectra. Relative exposure of Trp and Tyr residues in treated ovalbumin was compared with native ovalbumin at pH 4.0 and 8.0 using sucrose, ethylene glycol, dimethylsulfoxide (DMSO), and D_2O as perturbants (Table IV, Figures 7 and 8). The magnitude of the difference spectra was a linear function of perturbant concentration and extrapolated to the origin in each case, indicating that the perturbants did not alter the conformation (Cross and Fisher, 1966). The data show an increase of 0.12–0.15 for relative exposure (R_m) of tyrosyl residues and 0.14–0.16 for R_m of tryptophanyl residues as a result of treatment.

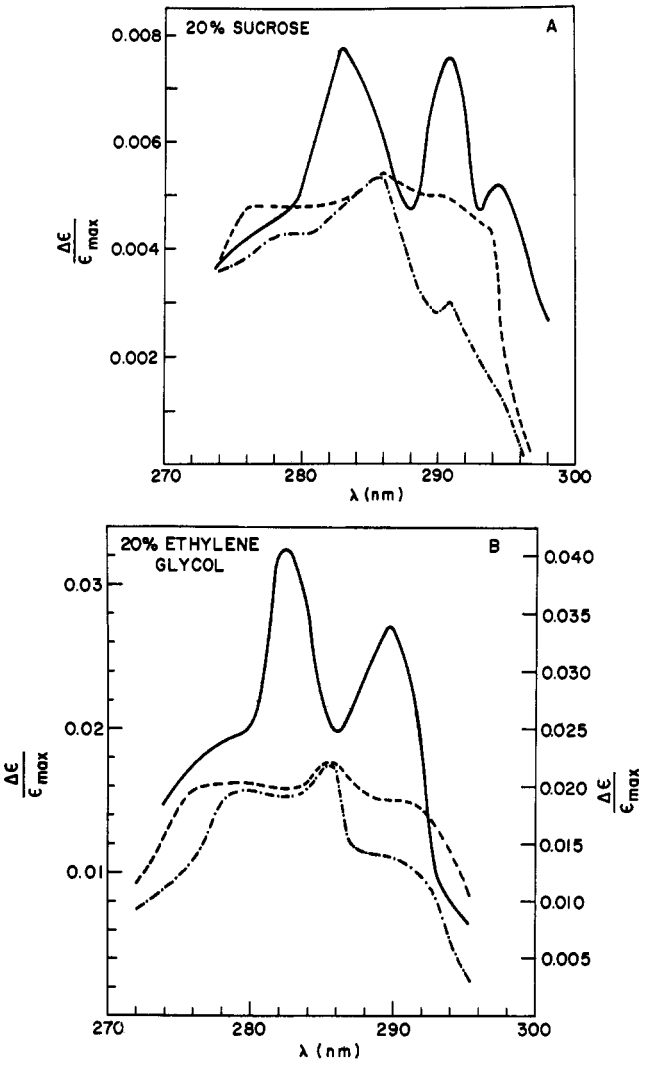

Figure 7. Solvent perturbation difference spectra for native and pH 4.0-treated ovalbumin and the model compound mixture in salt-free solution at pH 4.0. Solid line: model compound solution. Dashed line: treated ovalbumin. Dashed-dot line: native ovalbumin. A, the perturbant was 20% sucrose. B, the perturbant was 20% ethylene glycol. The values for the model compound solution are plotted according to the right ordinate

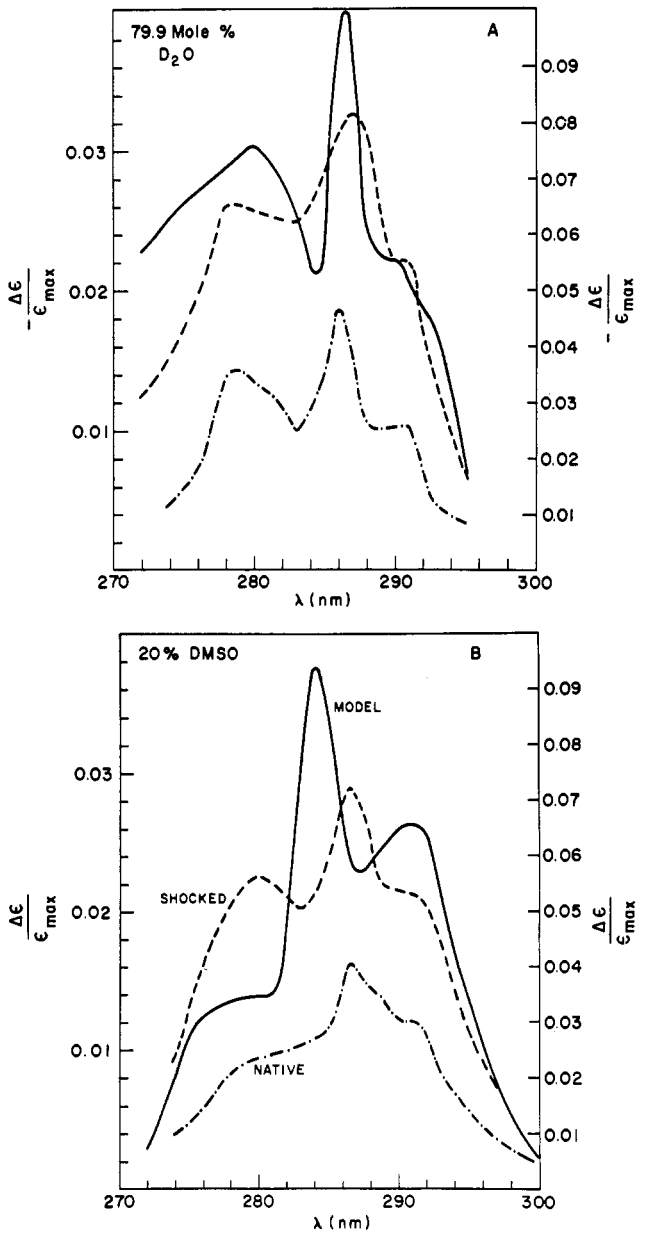

Figure 8. Solvent perturbation difference spectra. Conditions are the same as for Figure 7. Solid line: model compound solution. Dashed line: treated ovalbumin. Dashed-dot line: native ovalbumin. A, the perturbant was 79.9 mol% D_2O. B, the perturbant was 20% DMSO. The values for the model compound solution are plotted according to the right ordinate for both cases

DISCUSSION

Association and Aggregation Induced by Pressure Pulsation. Aggregation and subsequent coagulation of shocked ovalbumin could be prevented by increasing electrostatic repulsion, *i.e.*, by raising the pH above the isoelectric point or lowering the ionic strength. The presence of associated species was observed only after treatment at pH 4.0 or 5.0 in distilled water. Solubility of this species was very sensitive to ionic strength, as shown by the decrease in the area of the leading boundary in sedimentation analysis when the solution was dialyzed against 0.1 *M* NaCl and by the resolubilization of coagulated ovalbumin by dialysis against water. No associated species, and hence no coagulation, was observed after treatment in distilled water at pH 7.0 or 8.0.

Transport studies, gel filtration, and sedimentation velocity indicate that the associated species and the monomer exist in equilibrium. The observation of a bimodal boundary with the leading component increasing in sedimentation rate and relative area with increasing protein concentration suggests a rapid monomer $\rightleftharpoons$ *n*-mer equilibrium with $n > 2$, since a single boundary is predicted for monomer–dimer or consecutive equilibria (Fujita, 1962). Temperature dependence of the association equilibrium was evidenced by a reversible increase in the relative area of the faster sedimenting boundary which occurred as the temperature was raised. Thus, it appears most likely that association occurs primarily as a result of hydrophobic interactions. Similar observations have been made with numerous other proteins, *e.g.*, tobacco mosaic virus protein (Stevens and Lauffer, 1965), carboxypeptidase A (Bethune, 1965), phycocyanin (Scott and Berns, 1965), and ox liver sulfatase A (Nichol and Roy, 1965).

Hydrolysis of the Phosphate Ester. Electrophoretic analysis of both native and pH 4-shocked ovalbumin and native and pH 4-shocked component A_1 indicated a 15–20% decrease for the relative amount of A_1 and a concomitant increase for the amount of component A_2. Since the only known difference between the two components is the number

of phosphate monoester groups (Perlmann, 1955), two for A_1 and one for A_2, it seems reasonable to propose that one of the groups is pressure labile. This conversion was not observed when the preparations were treated at pH 8. This conclusion is substantiated by a 15–20% decrease in the amount of protein-bound phosphate as a result of treatment at pH 4.0. Furthermore, loss of phosphate was only observed at pH 3.5 and 4.0 and not at pH 4.5 or 5.0, illustrating the pH dependence of the reaction. Seemingly only one of the two phosphate groups is labile to treatment since component A_3, which does not contain phosphate, was not observed.

Changes in Relative Exposure of Side-Chain Residues. Suzuki *et al.* (1962) have reported changes in reactivity of sulfhydryl groups of ovalbumin at high hydrostatic pressures. These side-chain residues have also been implicated in coagulation of ovalbumin by various treatments (Argus *et al.*, 1966; Mirsky and Anson, 1935). However, changes in sulfhydryl reactivity with DTNB as a result of pressure pulsation were not observed in this study. Furthermore, it is doubtful that sulfhydryl groups are involved in such coagulation since association was also observed in solutions which had been shocked in the presence of either iodoacetamide or mercaptoethanol.

Acetylation of an additional tyrosyl residue as a result of pressure pulsation was observed. The number of reactive residues for native protein was in agreement with the value given by Riordan *et al.* (1965). Presumably extensive reversible unfolding does not occur at the "peak" of the pressure pulse, since the degree of tyrosyl group exposure as measured by acetylation during treatment was the same as that indicated by solvent perturbation studies.

Further evidence for subtle conformational changes was obtained from results of solvent perturbation difference spectra. Data for exposure of tyrosyl residues in native ovalbumin can be interpreted in agreement with chemical reactivity studies of Gorbunoff (1969). Two residues appear to be readily accessible to the aqueous environment; one is located in a crevice large enough to allow perturbation by sucrose but too small to allow complete perturbation by "short-range" probes, and a fourth is located in a still smaller crevice allowing only "long-range" perturbation by ethylene glycol. Regardless of the specific interpretation, tyrosyl residues do become more accessible to the aqueous environment following pressure treatment. Presumably, three residues are completely exposed to the aqueous environment, whereas a fourth remains in a crevice "seen" only by ethylene glycol. As with native protein, five residues remain completely unperturbed by any of the agents used.

Similar changes were noted for accessibility of tryptophanyl residues after treatment. Apparently only one of three residues was partially accessible to various perturbants before pressure pulsation, whereas afterward one residue was completely exposed and another was affected by ethylene glycol.

Proposed Model. Observations made in this study are consistent with the following model.

$$\text{native ovalbumin} \xrightarrow{1} \text{"altered" ovalbumin} \overset{2}{\rightleftharpoons} n\text{-mer} \overset{3}{\rightleftharpoons} \text{precipitation}$$

Viscosity data, chemical reactivities, and difference spectra are indicative of only small irreversible conformational changes as a result of a pressure pulsation, step 1. Increase in the exposure of hydrophobic residues (Tyr, Trp) occurs providing potential sites for association by hydrophobic interactions. The conformationally altered monomer exists in rapid equilibrium with an *n*-mer, the temperature dependence being consistent with such interactions, step 2. The *n*-mer undergoes further polymerization, leading to a precipitate when electrostatic repulsive forces are lowered, step 3. The precipitate is slowly resolubilized when the ionic strength is decreased, indicating some reversibility of this step.

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