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PHYSICO-CHEMICAL PROPERTIES OF α-CRYSTALLIN FROM OX LENS*,**

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SUMMARY

α-Crystallin was isolated from ox lenses and purified by repeated precipitation at its isoelectric point. Electrophoretic analyses in acid and alkaline solutions at various ionic strengths indicate α-crystallin to be a single component. Sedimentation studies above pH 3.0 at various ionic strengths also indicate that α-crystallin is a single component. At pH 3.0 and below, depending upon the ionic strength, two or three components appear during sedimentation. Sedimentation and viscosity studies suggest that this dissociation is reversible. Sedimentation, viscosity, and light-scattering data at pH 2.0 at various ionic strengths indicate an unfolding of the protein molecule ultimately leading to dissociation. Sedimentation and light-scattering data at pH 9.1 at six ionic strengths (0.1–0.6) indicate nearly a two-fold aggregation of the protein at the higher ionic strengths, possibly accompanied by a change in the shape of the molecule. Molecular weights and dimensions have been calculated from sedimentation, viscosity, and partial-specific-volume data obtained at pH 9.1 and at six ionic strengths.

* The data presented in this paper are taken from a dissertation submitted by S. K. NIYOGI to the Faculty of Northwestern University in partial fulfillment for the degree, Doctor of Philosophy, in 1961.

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INTRODUCTION

Although a considerable amount of work has been done on the lens proteins in general, comparatively few studies have been made on the individual lens proteins. Recent studies[1-11] on α-crystallin indicate disagreement among the investigators especially with regard to its physico-chemical properties. The present investigation was, therefore, undertaken to elucidate some of the physico-chemical properties of α-crystallin. A thorough knowledge of the physico-chemical characteristics of α-crystallin could conceivably contribute to the understanding of its physiological function. Preliminary reports on these studies have appeared[12, 13].

METHODS

Preparation of pure α-crystallin

Fresh ox eyes were obtained immediately after slaughter from local abattoirs. The eyes arrived packed in dry ice and were immediately transferred to a freezer maintained at −15°. The lenses were removed in a cold room at 5° while the eyes were frozen. α-Crystallin was isolated as needed from the lenses by a method similar to that used by FRANCOIS *et al.*[14]. Extraction of the soluble proteins was accomplished by slow (to prevent foaming) stirring of the lenses in cold distilled water. Four successive 2-h extractions were employed. About 125 ml distilled water were used with each 20 g lenses for each extraction. Only the periphery of the lenses dissolved while the nuclei remained intact, especially if the extracts were gentle. After each extraction, the supernatant was decanted as completely as possible and centrifuged for 0.5 h in a Servall centrifuge at 10000 rev./min to remove the insoluble material. The extracts thus obtained were pooled to serve as the starting material. The insoluble residue and unbroken nuclei were discarded. The pH of the pooled extract was carefully adjusted from 7.5 to 5.2 by slow addition of 0.1 M HCl until the α-crystallin precipitated at its isoelectric point. The precipitate was recovered in a Servall centrifuge at 10000 rev./min for 20 min. The supernatant was discarded. The precipitate was washed with cold distilled water adjusted to pH 5.2. The washings were discarded. The precipitate was re-suspended in distilled water and brought into solution by adding small amounts of cold 0.1 M NaOH, taking care that the pH did not go above 9.0. From this alkaline solution, the α-crystallin was again precipitated as described above. The process was repeated until the protein on precipitation left a clear supernatant. The homogeneity of the preparation was tested both ultracentrifugally and electrophoretically. The appearance of a single boundary in the proper solvent indicated homogeneity.

Determination of protein concentration

The protein concentration of the initial solution was determined by evaporating aliquots of the dialyzed solution and the dialysate at 110° until constant weight was obtained. From the weights of the dried residues from the two solutions, the concentrations were calculated. The protein concentrations of diluted solutions were determined refractometrically using a Brice-Phoenix differential refractometer with light of wavelength of 436 mμ.

Moving-boundary electrophoresis

A Spinco Model H Electrophoresis diffusion apparatus was used. Mobilities were determined according to the method of KOENIG AND HOGNESS[15].

Sedimentation

All sedimentation experiments were performed at 59780 rev./min and at 20°. The Spinco Model E analytical ultracentrifuge provided with automatic temperature control within 0.1° was used. A comparator microscope was used to measure the distances moved by the boundaries in the Schlieren pictures. The formula of SVEDBERG AND PEDERSEN[16] was used to correct the sedimentation coefficients to water as solvent at 20°. The equations of the lines of regression for $s_{20,w}$ on c were calculated by the method of least squares.

Viscosity

The flow times were measured in standard Ostwald viscometers at 25° in a bath thermostated to within 0.01°. The densities of the solutions were determined by pycnometry. The data were fitted by the method of least squares to the equation,

$$\eta/\eta_r = 1 - Kc,$$

where η_r is relative viscosity, K is weight intrinsic viscosity, and c is concentration in g/100 ml. Volume intrinsic viscosity is K(100)/partial specific volume.

Partial specific volume

The method of DRUCKER[17] was used to determine the apparent partial specific volume. Ostwald-type pycnometers were used. The methods of procedure and calculation were similar to those reported by KOENIG[18].

Light-scattering

The Aminco Microphotometer[19] was used for the light-scattering measurements. The procedure followed was that described by SOWINSKI, OHARENKO AND KOENIG[20]. The dissymmetry method described by STACEY[21] was used for the determination of the molecular weight. Dust-free solutions were obtained by filtration under air pressure through coarse membrane filters of pore size, 0.4 μ. The refractive index increment was determined in a Brice-Phoenix differential refractometer at 436 mμ. The light-scattering measurements were also made at 436 mμ. The data were fitted to the following equation by the method of least squares:

$$Hc/\tau = 1/M + 2Bc,$$

where c is protein concentration in g/l, τ is turbidity, M is weight-average molecular weight,

$$H = 32\pi^3 n^2 (\Delta n/c)^2 / N\lambda^4$$

n is refractive index of solvent, $\Delta n/c$ is the refractive index increment for the protein, N is Avogadro's number, and λ is wavelength in cm (436 mμ). The slope of the above line is the interaction constant, $2B$, which is a measure of the departure of the solution from ideal behavior. The intercept of the line is the reciprocal of the weight-average molecular weight.

Estimates of the molecular weights and dimensions were made from the sedimentation, viscosity, and partial specific volume data. The method of calculation was that

used by PERRY AND KOENIG[11]. In the present study, calculations were made assuming the molecules to be both unhydrated prolate and oblate ellipsoids of revolution. Molecular weights were also calculated according to the equation of SCHERAGA AND MANDELKERN[23] which has been recommended by SCHACHMAN[24]. A value of $2.16 \cdot 10^6$ was assumed for an average β in the SCHERAGA AND MANDELKERN equation.

Phosphorus determination

The phosphorus content of α-crystallin was determined according to the method of GOMORI[22].

RESULTS

When α-crystallin obtained by a single precipitation at pH 5.2 was dissolved in 0.1 M Na_2HPO_4 (pH 9.1) a slowly sedimenting impurity appeared upon examination in the ultracentrifuge. Reprecipitation of this α-crystallin at pH 5.2 removed the impurity so that a single boundary was observed upon re-examination in the ultracentrifuge. α-Crystallin, homogeneous on the ultracentrifuge when dissolved in 0.1 M Na_2HPO_4 (pH 9.1), produced two or three boundaries on the ultracentrifuge when dissolved in NaCl–HCl (pH 3.0) and below. The dissociation of the α-crystallin begins between

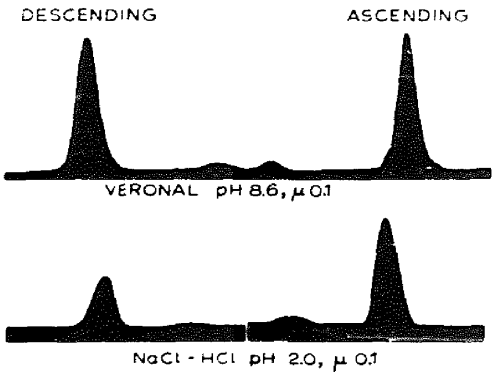

Fig. 1. Electrophoretic patterns of ox-lens α-crystallin in basic and acidic buffers.

TABLE I

ELECTROPHORETIC MOBILITIES OF α-CRYSTALLIN IN VARIOUS SOLVENTS

Solvent		*Ionic strength* (μ)	*Mobility* ($cm^2/V \cdot sec$) 10^5
Composition	*pH*		
Na_2HPO_4	9.1	0.10	−5.23
Na_2HPO_4	9.1	0.20	−5.79
Na_2HPO_4	9.1	0.30	−4.74
Na_2HPO_4	9.1	0.40	−4.70
Veronal buffer	8.6	0.10	−5.60
Phosphate buffer	7.7	0.20	−4.86
NaCl–HCl	2.0	0.05	7.39
NaCl–HCl	2.0	0.10	6.62
NaCl–HCl	2.0	0.15	5.34
NaCl–HCl	2.0	0.20	6.03

pH 3.0–3.5 and continues below pH 3.0. This dissociation is apparently reversible, because α-crystallin appearing as two components in NaCl–HCl (pH 2.0, $\mu = 0.05$) sedimented as a single boundary when the pH was increased to 4.0 and above. Readjustment of the solution to pH 2.0 caused the α-crystallin to sediment as two boundaries.

α-Crystallin existed as single boundaries electrophoretically when examined either at acid pH or at alkaline pH. Electrophoretic patterns in veronal buffer (pH 8.6, $\mu = 0.10$) and NaCl–HCl (pH 2.0, $\mu = 0.10$) are shown in Fig. 1. The mobilities are listed in Table I.

Studies in solutions of Na_2HPO_4 (pH 9.1) at various ionic strenghts

A single boundary was obtained when α-crystallin was analyzed electrophoretically at $\mu = 0.1$, 0.2, 0.3 and 0.4. The mobilities are given in Table I.

Single boundaries were obtained when α-crystallin was examined on the ultra-

TABLE II

PHYSICAL DATA FOR α-CRYSTALLIN IN SOLUTIONS OF Na_2HPO_4 (pH 9.1)

Sedimentation

Ionic strength	*Line of regression*	*Correlaion coefficient*	*Number of determinations*	*Concentration range (g/l)*
0.10	$s_{20,w} = 11.08 + 0.022c$	0.125	9	0.42–2.15
0.20	$s_{20,w} = 12.12 + 0.519c$	0.874	8	0.93–3.05
0.30	$s_{20,w} = 18.82 - 0.506c$	0.917	10	0.84–8.35
0.40	$s_{20,w} = 17.61 - 0.306c$	0.909	10	2.34–11.73
0.50	$s_{20,w} = 22.16 - 0.295c$	0.975	8	3.02–13.57
0.60	$s_{20,w} = 18.91 - 0.380c$	0.822	10	4.16–8.30

Viscosity

Ionic strength	*Partial specific volume*	*Line of regression*	*Correlation coefficient*	*Number of determinations*	*Concentration range (g/100 ml)*
0.10	0.742	$1/\eta_r = 1 - 0.083c$	0.971	8	0.066–0.656
0.20	0.757	$1/\eta_r = 1 - 0.085c$	0.920	8	0.026–0.262
0.30	0.742	$1/\eta_r = 1 - 0.084c$	0.986	8	0.048–0.477
0.40	0.742	$1/\eta_r = 1 - 0.084c$	0.971	8	0.035–0.317
0.50	0.728	$1/\eta_r = 1 - 0.084c$	0.978	8	0.059–0.527
0.60	0.748	$1/\eta_r = 1 - 0.081c$	0.976	8	0.090–0.627

Light-Scattering

Ionic strength	*Line of regression*	*Correlation coefficient*	*Molecular weight*	*Dissymmetry*	*Δn/c*
0.10	$*Hc/\tau = 0.399 - 0.026c$	0.875	2 505 000	1.726	0.00182
0.20	$Hc/\tau = 0.400 - 0.010c$	0.398	2 499 000	1.931	0.00188
0.30	$Hc/\tau = 0.419 - 0.067c$	0.954	2 384 000	1.678	0.00185
0.40	$Hc/\tau = 0.197 - 0.163c$	0.970	5 082 000	1.789	0.00196
0.50	$Hc/\tau = 0.164 - 0.185c$	0.927	6 089 000	1.865	0.00182
0.60	$Hc/\tau = 0.196 - 0.212c$	0.951	5 114 000	1.763	0.00209

* Hc/τ is multiplied by 10^6.

centrifuge at $\mu = 0.1$, 0.2, 0.3, 0.4, 0.5 and 0.6. The boundaries were symmetrical at $\mu = 0.1$ and 0.2. At $\mu = 0.3$ and above, the boundaries were less symmetrical with a curved base-line which is characteristic of phosphate buffers. When $s_{20,w}$ was plotted *versus* concentration, a straight line was obtained. The regression lines for $s_{20,w}$ on concentration are given in Table II. In addition to the equations for the lines, the correlation coefficients, number of determinations, and the range of concentrations are listed. The intercepts of the lines are the sedimentation constants at infinite dilution.

The regression lines for $1/\eta_r$ on concentration at each ionic strength studied by sedimentation are given in Table II. The correlation coefficients, number of determinations, range of concentrations, and the partial specific volumes ($\bar{v}$) are also listed.

The results of the light-scattering studies at various ionic strengths are tabulated in Table II. The equations for the regression lines of Hc/τ on protein concentration, weight-average molecular weights, the dissymmetry values, correlation coefficients, and the values for refractive index increment are also listed. Each line represents turbidity measurements on at least eight different concentrations and in some cases ten concentrations.

TABLE III

MOLECULAR WEIGHTS AND DIMENSIONS OF α-CRYSTALLIN IN SOLUTIONS OF Na_2HPO_4 (pH 9.1) CALCULATED FROM SEDIMENTATION AND VISCOSITY DATA

η, Volume intrinsic viscosity; f/f_0, frictional ratio; a and b, minor and major axes respectively; mol. wt. (S.M.), molecular weight from SCHERAGA–MANDELKERN equation.

Ionic strength	η	Prolate ellipsoid				Oblate ellipsoid				Mol. wt. (S.M.)
		f/f_0	Mol. wt.	a(Å)	b(Å)	f/f_0	Mol. wt.	a(Å)	b(Å)	
0.10	11.09	1.466	338 100	22.68	194.37	1.622	393 600	8.21	119.13	391 300
0.20	11.20	1.469	417 400	24.38	210.64	1.625	485 700	8.78	128.71	495 100
0.30	11.31	1.473	729 800	29.10	253.17	1.629	848 400	10.44	154.51	881 000
0.40	11.51	1.478	631 000	27.51	242.18	1.640	737 500	9.80	147.98	770 400
0.50	11.54	1.478	862 900	30.47	268.14	1.640	1 008 000	10.82	163.88	1 073 700
0.60	10.83	1.456	756 000	29.88	250.99	1.606	875 400	10.93	154.11	953 200

The molecular weights and dimensions calculated from sedimentation, viscosity, and partial specific volume data are listed in Table III.

Studies in solutions of NaCl–HCl (pH 2.0) at various ionic strengths

Single boundaries were obtained when α-crystallin was analyzed electrophoretically at $\mu = 0.05$, 0.10, 0.15 and 0.20 (Fig. 1). The mobilities are listed in Table I.

Three components appeared when sedimentation was performed at $\mu = 0.05$ while two components were evident at $\mu = 0.1$. Values of $s_{20,w}$ plotted against values of concentration gave straight lines. The equations of the regression lines for $s_{20,w}$ on concentration for each component at each ionic strength are listed in Table IV. The correlation coefficients, number of determinations and the concentration ranges are also given.

The equations for the lines of regression of $1/\eta_r$ on concentration at $\mu = 0.05$, 0.10, 0.15 and 0.20 are given in Table IV. The correlation coefficients, number of determinations, and concentration ranges, and the partial specific volumes ($\bar{v}$) are also listed.

The light-scattering data at $\mu = 0.05$ and 0.10 are listed in Table IV. The equations for the regression lines of Hc/τ on concentration, correlation coefficients, weight-average molecular weights, values of dissymmetry, and the values for refractive index increment are listed. The determinations at $\mu = 0.05$ were made at nine different concentrations of protein, while at $\mu = 0.10$, ten different concentrations of protein were studied.

TABLE IV

PHYSICAL DATA FOR α-CRYSTALLIN IN NaCl–HCl (pH 2.0)

Sedimentation

Component	*Line of regression*	*Correlation coefficient*	*Number of determinations*	*Concentration range (g/l)*
	$\mu = 0.05$			
I	$s_{20,w} = 7.10 - 0.155c$	0.976	10	1.67–19.73
II	$s_{20,w} = 3.85 - 0.020c$	0.983	5	7.05–19.73
III	$s_{20,w} = 1.22 - 0.004c$	0.227	10	1.67–19.73
	$\mu = 0.10$			
I	$s_{20,w} = 8.28 - 0.043c$	0.174	10	0.42–3.95
II	$s_{20,w} = 1.45 - 0.018c$	0.296	7	1.50–3.95

Viscosity

Ionic strength	*Partial specific volume*	*Line of regression*	*Correlation coefficient*	*Number of determinations*	*Concentration range (g/100 ml)*
0.05	0.740	$1/\eta_r = 1 - 0.144c$	0.988	8	0.089–0.794
0.10	0.754	$1/\eta_r = 1 - 0.133c$	0.998	8	0.072–0.504
0.15	0.756	$1/\eta_r = 1 - 0.154c$	0.994	8	0.049–0.443
0.20	0.748	$1/\eta_r = 1 - 0.219c$	0.994	7	0.041–0.277

Light-Scattering

Ionic strength	*Line of regression*	*Correlation coefficient*	*Mol. wt.*	*Dissymmetry*	*Δn/c*
0.05	$^{*}Hc/\tau = 1.246 + 0.010c$	0.746	802 900	2.860	0.00188
0.10	$Hc/\tau = 0.505 - 0.006c$	0.876	1 980 500	2.226	0.00195

* Hc/τ is multiplied by 10^6.

Studies in phosphate buffer (pH 7.7)

One boundary was observed when α-crystallin was analyzed electrophoretically at $\mu = 0.2$. The mobility value is given in Table I.

The equation of the regression line for $1/\eta_r$ on concentration at $\mu = 0.2$ is given in Table V. The correlation coefficient, number of determinations, concentration range, and the partial specific volume are also listed.

The light-scattering data at $\mu = 0.2$ and $\mu = 0.3$ are listed in Table V. The equations for the regressior lines of Hc/τ on concentration, correlation coefficients, molecular weights, values of dissymmetry, and the values for refractive index

increment are included. Light-scattering determinations at $\mu = 0.2$ were made on six concentrations ranging from 1.5 g/l to 5.4 g/l, while at $\mu = 0.3$, on eight concentrations ranging from 0.25 to 2.1 g/l.

Quantitative evidence for the reversibility of the dissociation of α-crystallin at pH 2.0

Ox-lens α-crystallin manifested two boundaries in the ultracentrifuge in NaCl–HCl (pH 2.0, $\mu = 0.10$). The same solution when adjusted to pH 9.1, and dialyzed against Na_2HPO_4 (pH 9.1, $\mu = 0.30$) formed a single boundary in the ultracentrifuge.

TABLE V

PHYSICAL DATA FOR α-CRYSTALLIN IN PHOSPHATE BUFFER (pH 7.7)

Viscosity

Ionic strength	Partial specific volume	Line of regression	Correlation coefficient	Number of determinations	Concentration range (g/100 ml)
0.20	0.722	$1/\eta_{sp} = 1 - 0.056c$	0.910	7	0.063–0.564

Light-Scattering

Ionic strength	Line of regression	Correlation coefficient	Molecular weight	Dissymmetry	$1/\tau_0$
0.20	$*Hc/\tau = 0.3303 - 0.004c$	0.762	3 306 100	1.433	0.00194
0.30	$Hc/\tau = 0.2811 - 0.020c$	0.395	3 553 500	1.658	0.00184

* Hc/τ is multiplied by 10^6.

TABLE VI

PHYSICAL DATA DEMONSTRATING QUANTITATIVELY THE REVERSIBLE DISSOCIATION OF α-CRYSTALLIN

Sedimentation

Component	Line of regression	Correlation coefficient	Number of determinations	Concentration range (g/l)
	NaCl - HCl (pH 2.0) μ = 0.10			
I	$s_{20,w} = 6.58 - 0.050c$	0.861	10	2.82–14.13
II	$s_{20,w} = 1.38 - 0.003c$	0.260	10	2.82–14.13
	Na_2HPO_4 (pH 9.1) μ = 0.30			
I	$s_{20,w} = 17.83 - 0.183c$	0.955	8	2.54–11.43

Viscosity

Line of regression	Correlation coefficient	Number of determinations	Concentration range (g/100 ml)
NaCl - HCl (pH 2.0) μ = 0.10			
$1/\eta_{sp} = 1 - 0.133c$	0.998	8	0.072–0.504
Na_2HPO_4 (pH 9.1) μ = 0.30			
$1/\eta_{sp} = 1 - 0.094c$	0.977	8	0.083–0.583

The sedimentation patterns are shown in Fig. 2. The equations of the lines of regression for $s_{20,w}$ on concentration are shown in Table VI. At pH 2.0, the components have $s_{20,w}$ values of 8.58 S and 1.38 S, whereas at pH 9.1, the single component has a $s^{\circ}_{20,w}$ value of 17.83 S. The reversibility of the dissociation is indicated.

The solution of α-crystallin at pH 2.0, $\mu = 0.10$, having a weight intrinsic viscosity of 0.133, was adjusted to pH 9.1, and allowed to dialyze against Na_2HPO_4 (pH 9.1, $\mu = 0.30$) with frequent changes of dialysate. The weight intrinsic viscosity at pH 9.1 was 0.094. The viscosity data further indicate the apparent reversible transformation of α-crystallin with pH. The viscosity data are shown in Table VI.

The phosphorus content of α-crystallin was found to be 0.08 %.

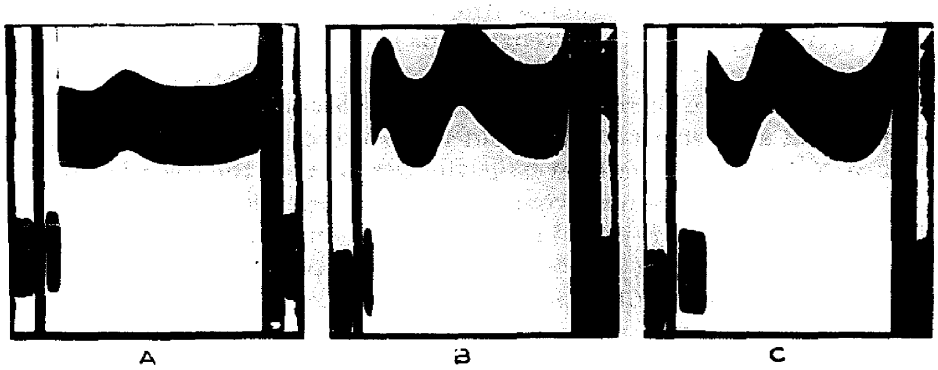

Fig. 2. Sedimentation boundaries of ox-lens α-crystallin showing reversible dissociation. (A) Before acid treatment, in 0.033 M Na_2HPO_4 (pH 8.9) $\mu = 0.1$, 2.15 g protein/l, after 25 min, bar angle 30°. (B) In NaCl–HCl (pH 2.0) $\mu = 0.1$, 9.89 g protein/l, after 46 min, bar angle 25°. (C) After acid treatment, in 0.1 M Na_2HPO_4 (pH 9.0) $\mu = 0.3$, 7.62 g protein/l, after 16 min, bar angle 25°.

DISCUSSION

Sedimentation and electrophoretic studies indicate the efficiency of isoelectric precipitation as a method of purification of α-crystallin. These results are different from those of BLOEMENDAL AND TEN CATE[6], who found α-crystallin gave two boundaries in the ultracentrifuge using 1.0 M sodium acetate (pH 7.4) and during electrophoresis using barbiturate buffer (pH 7.7, $\mu = 0.025$). Two boundaries during sedimentation in sodium acetate–glycine buffer (pH 7.8) were also observed by BON[9] with α-crystallin. For the isoelectric precipitation of α-crystallin, these investigators used dilute acetic acid which gives incomplete precipitation.

The dissociation of ox-lens α-crystallin starts below pH 3.5; the protein remains stable at pH 3.5 and above. Similar stability ranges have been reported for several other proteins, for example, tobacco-seed globulin, exelsin and edestin[25], bovine-serum albumin[26], and β-lactoglobulin[27]. The dissociation of ox-lens α-crystallin at pH 2.0 was also found to be a function of ionic strength. Two components appeared in the ultracentrifuge at $\mu = 0.10$, 0.15 and 0.20, whereas at an ionic strength of 0.05, a third component appeared at the higher protein concentrations. At pH 2.0, $\mu = 0.10$ the sedimentation constants at infinite dilution of the two components are 8.28 S and 1.45 S. At $\mu = 0.05$, the three components have sedimentation constants at infinite dilution of 7.10 S, 3.85 S and 1.22 S. Apparently, the heavier component at $\mu = 0.10$ splits into two components when the ionic strength is lowered to 0.05.

Electrophoretic analyses at pH 2.0, $\mu = 0.05$, 0.10, 0.15 and 0.20 showed a single boundary. These results indicate that the components at pH 2.0 possess the same charge properties. The appearance of a hypersharp ascending boundary as compared to the broad descending boundary can be partly explained on the basis of Donnan equilibrium which gives the protein solution less conductivity than the buffer solution. Superimposed on the conductivity is also a pH effect which can be appreciable when removed from the isoelectric point. This behavior is especially evident at high protein concentrations and low ionic strength. As the ionic strength increases, the boundaries become more enantiographic. The non-enantiographic character of the electrophoretic patterns may also be explained on the basis of mobility variation due to a continual readjustment of ionic equilibria across the moving boundaries of the components present at a given pH. Similar systems have been discussed by LONGWORTH AND JACOBSEN[28].

RESNIK[4] reported the dissociation of calf lens α-crystallin below pH 3.5 at low ionic strength, and showed that this could be reversed at higher ionic strengths for example 0.1 M NaCl. The present investigations show that the dissociation of ox-lens α-crystallin at pH 2.0 persists even when the ionic strength is raised to 0.20 with NaCl. The dissociation was readily reversed by increasing the pH to 3.5, 4.0, 7.7 and 9.2 and maintaining the ionic strength at 0.05 or 0.10. The reappearance of two boundaries in the ultracentrifuge when the pH was readjusted to 2.0, and the quantitative results from the sedimentation and viscosity experiments provide additional evidence for the reversible nature of the dissociation. It should be emphasized, however, that the sedimentation constant at infinite dilution, the concentration dependence, and the weight intrinsic viscosity of the pH-readjusted solutions were 17.8 S, 0.183 S/g/l, and 0.094 dl/g respectively, as compared to the values of 18.8 S, 0.506 S/g/l and 0.084 dl/g when the protein was dissolved originally in Na_2HPO_4 (pH 9.1, $\mu = 0.3$) without undergoing the pH adjustment. The differences between the two sets of values may indicate a slight irreversible change that has occurred within the molecule.

It was thought that suitably placed phosphate groups might be partly responsible for the reversible dissociation of ox-lens α-crystallin at low pH inasmuch as an appreciable amount of phosphorus was detected in α-crystallin. The phosphorus seems to be tightly bound to the protein and could not be removed even after prolonged dialysis in several solvents. Since carbohydrates and nucleic acids were found to be absent, the phosphorus is probably attached to amino acids in the protein chain, possibly serine. Further work is needed to explain the nature and role of phosphorus in this protein.

At pH 9.1, the weight intrinsic viscosity remains fairly constant in spite of a change in the intensity of the electrostatic interaction. At first sight, this might indicate that the size and shape of the α-crystallin at pH 9.1 remain unchanged with a change in the intensity of the electrostatic interaction. Sedimentation and light-scattering studies at pH 9.1, however, indicate a two-fold aggregation at the higher ionic strengths. The viscosity data, therefore, lead to the possibility that the shape of the protein may also change during the process of aggregation, but in such a way as to compensate for the increase in viscosity due to an increase in size and volume. The change in the hydration of the protein due to a change in ionic strength of the solvent may also be a factor in maintaining the constancy of the viscosity.

The concentration dependence of the sedimentation constants at pH 9.1, at

$\mu = 0.3$, 0.4, 0.5 and 0.6 (Table II) are of the usual type showing negative slopes. The slope at $\mu = 0.1$ is slightly positive, that at $\mu = 0.2$ is more positive, showing that an association–dissociation reaction is apparently taking place between the macromolecules in solution. The standard errors of the slopes ((0.027–0.118)) indicate significant differences between the slopes at $\mu = 0.1$ and 0.2, at $\mu = 0.2$ and 0.3, and at $\mu = 0.3$ and 0.4. The standard errors of the intercepts ((0.09–0.40)) indicate significant differences between the intercepts at $\mu = 0.1$ and 0.2, at $\mu = 0.2$ and 0.3, at $\mu = 0.3$ and 0.4, at $\mu = 0.4$ and 0.5, and at $\mu = 0.5$ and 0.6. On the basis of the formation of aggregates, two or more boundaries could be expected at pH 9.1. A single boundary, though somewhat broad, was obtained at each of these ionic strengths. This behavior suggests that the rate of association is sufficiently rapid so that equilibrium is maintained during sedimentation[16,29,30]. The single boundary apparently moves with the weight-mean velocity of its components. Similar associating–dissociating systems have been described by GILBERT[31–33].

The viscosity studies at pH 2.0 show a significant increase in weight intrinsic viscosities at pH 2.0 over those at pH 9.1. The weight average molecular weights at pH 2.0, however, are significantly lower than those at pH 9.1. The shape of the molecules at pH 2.0 is probably contributing to the increase in viscosity. Sedimentation studies, as mentioned earlier, actually show that the protein breaks up into two or more components at pH 2.0. Apparently there seems to be an opening-up of the compact structure of the molecule thereby giving it a more asymmetric form. This interpretation is supported by light-scattering studies. The dissymmetry values at pH 2.0 (average value is 2.5) are significantly higher than those at pH 9.1 (average value is 1.7). Increased acid binding at pH 2.0 could lead to more hydration with a consequent swelling of the particle. At pH 2.0, the swelling may account partly for the increase in viscosity. The increase in partial specific volumes at pH 2.0 further suggests a swelling of the molecule. This swelling may be the result of two opposing factors[34]: the increase in volume due to increased acid binding, and the decrease in volume due to the destruction of the native configuration of α-crystallin at pH 2.0. The opening-up of the compact structure of the α-crystallin molecule, ultimately leading to a splitting of the polypeptide chain, is apparently due to electrostatic forces and a manifestation of reversible denaturation that occurs at pH 3.0 and below. SANTAMARIA *et al.*[11] report somewhat higher intrinsic viscosities at pH 2.65 and 18°.

The weight intrinsic viscosity at pH 2.0 increased with the ionic strength of the solvent, seemingly going against the electroviscous effect. The viscosity determinations were, however, carried out presumably at ionic strengths high enough to quench the electroviscous effect. Sedimentation studies at pH 2.0 indicate that at $\mu = 0.10$ there are two components having sedimentation constants at infinite dilution of about 8.3 S and 1.4 S. As μ is increased to 0.15 and 0.20, the relative concentration (from composition analysis of the patterns) of the heavier component gradually increases at the expense of the smaller one, leading to an increase in viscosity. On the other hand, at $\mu = 0.05$, the heavier component breaks up into two smaller components thus leading to a decrease in the viscosity.

Molecular weights and dimensions calculated from sedimentation, viscosity, and partial specific volume data obtained at pH 9.1 indicate nearly a two-fold aggregation of the protein at higher ionic strengths. The molecular weight at $\mu = 0.3$ was about 730000 for a prolate ellipsoid and 848000 for an oblate ellipsoid of revolution. These

values are slightly lower than those reported by PERRY AND KOENIG[10] for ox lens α-crystallin and lower than that reported for calf lens α-crystallin by RESNIK[2]. The closeness of the present values of the molecular weight obtained for the two models indicates that ox lens α-crystallin at pH 9.1 apparently possesses a globular form. The dissymmetry values obtained by light-scattering are also within the range for hydrated globular particles. The molecular weights calculated by the method of SCHERAGA AND MANDELKERN[23] are consistently closer to the corresponding values obtained by assuming an oblate ellipsoid of revolution. These findings tend to suggest that at pH 9.1 ox lens α-crystallin behaves as an oblate ellipsoid of revolution.

The molecular weights obtained by the dissymmetry method from light-scattering data at pH 9.1 are much higher than those calculated from sedimentation, viscosity, and partial specific volume data. This discrepancy can be partly explained by considering the conditions under which these measurements are made. The static conditions during light-scattering are conductive to the formation of aggregates, especially in the case of strongly interacting macromolecules such as α-crystallin. RESNIK[3], for example, reported that at alkaline pH, α-crystallin showed filamentous aggregates under the electron microscope. The dynamic conditions that prevail during sedimentation and viscosity studies may be sufficient to prevent extensive aggregation from occurring. The light-scattering studies were undertaken not so much to determine the absolute value of the molecular weight, but the relative changes in the molecular weight as a function of the pH and ionic strength.

The viscosity at pH 7.7, $\mu = 0.2$ is not much different from that at pH 9.1, $\mu = 0.2$. From light-scattering studies, the molecular weight at pH 7.7, $\mu = 0.2$, is somewhat greater than that at pH 9.1, $\mu = 0.2$. The increase in viscosity that could result from this increase in size is possibly compensated for by a change in the shape of the molecule as indicated by the fact that the dissymmetry value at pH 7.7, $\mu = 0.2$ is lower than that at pH 9.1, $\mu = 0.2$. The static conditions of light-scattering determinations at pH 7.7 which is closer to the isoelectric point may also favor aggregate formation which might not be detected by the viscosity measurements.

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