STUDIES ON OVOMUCOID*

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Recent correlation by Lineweaver and Murray (1) of the antitryptic factor of chicken egg white with ovomucoid, together with further studies by Lineweaver *et al.* (2, 3) on its interaction with trypsin, have stimulated interest in this protein. From its resistance to heat and to the precipitating action of various reagents, ovomucoid has been considered to be a very stable protein, although no evidence has been presented that such treatments do not alter its structure. The methods used to prepare it (4) involve conditions sufficiently drastic to denature many other proteins. The purpose of this investigation was to develop a method for the separation of ovomucoid under somewhat less drastic conditions and, furthermore, to examine the physical and chemical properties. The relatively high solubility of ovomucoid in trichloroacetate systems, previously recognized (1, 5), has in part formed the basis of the fractionation methods.

EXPERIMENTAL

The ovomucoid was prepared from fresh egg white by using a combination of sodium trichloroacetate precipitation with ethanol fractionation. The various ovomucoid preparations used in the studies on chemical characterization were thoroughly dialyzed against distilled water at the isoelectric point, lyophilized, and then further dried in a vacuum oven at 60° for 24 hours.

Electrophoretic analyses of ovomucoid fractions were carried out in a sodium diethyl barbiturate buffer of ionic strength 0.1 at pH 8.6. Mobility experiments were carried out in buffers of 0.1 ionic strength, sodium chloride constituting 80 per cent of the salt. In the experiments at ionic strength 0.01, buffer salt alone was used.

Velocity sedimentation analyses were performed in the Svedberg oil turbine ultracentrifuge, a schlieren optical system being used to record the boundaries.[1]

Diffusion experiments were carried out in an electrophoretic apparatus

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[1] The sedimentation experiments were carried out by Mr. E. M. Hanson.

at 1.2° with the conventional schlieren lens system. The boundary sharpening technique of Kahn and Polson (6) was utilized. The value for the diffusion constant was corrected to 20° in the usual manner (7).

Heterogeneity constants were determined by the method of Alberty (8). The electrical spreading was determined at a potential gradient of 1.7 volts per cm.

Viscosity determinations were performed in an Ostwald viscometer at 25°, in a solvent 0.1 M in sodium chloride and 0.05 M in sodium acetate buffer at pH 3.9. In studies of the effects of 8 M urea on ovomucoid the solutions were allowed to stand at least 1 hour before viscosity measurements were made.

The partial specific volume of the protein was determined in water at 25° in a pycnometer of approximately 20 ml. volume.

The well known tryptic inhibitor activity of ovomucoid was determined by the procedure of Lineweaver and Murray (1) and was referred to the activity of dry egg white prepared by lyophilization. Tryptic activity was measured by the hemoglobin method of Anson (9).

Nitrogen determinations were carried out by a micro-Kjeldahl procedure.

The carbohydrate content of the protein was measured by the orcinol method of Sörensen and Haugaard (10) and the carbazole method of Dische (11), with mannose as the reference standard. Glucosamine was determined by Hewitt's (12) method on ovomucoid samples hydrolyzed 8 hours at 100° in 2 N HCl. Extinctions were measured with a Beckman spectrophotometer.

Results

The method utilized for the preparation of ovomucoid depends in part upon its relatively high solubility in sodium trichloroacetate solutions. The most suitable conditions of pH and trichloroacetate concentration for the removal of non-ovomucoid proteins were studied with whole egg white at 1:6 dilutions with distilled water. Picrate ion likewise gives satisfactory results in these studies at even lower concentrations than trichloroacetate ion. The other egg white proteins are relatively insoluble in the presence of the above anions at pH values acid to their isoelectric points. The main contaminant of the ovomucoid in the supernatant solution under such conditions is ovalbumin. The solutions, after the initial precipitation carried out with trichloroacetate ion, were dialyzed at pH 3.9 to remove the salt; the protein was then recovered by lyophilization. In Table I are shown the data for yield and purity of ovomucoid as a function of pH and trichloroacetate ion concentration.

The ovomucoid so obtained consists of two closely related electrophoretic components and will be referred to as "crude" ovomucoid (Precipitate II,

Diagram 1). The minor component made up approximately 5 to 8 per cent of the total. In buffers at pH 8.6 and ionic strength 0.1, it migrates

TABLE I

Conditions for Precipitation of Non-Ovomucoid Proteins of Whole Egg White by Trichloroacetate

Precipitating conditions		Composition of supernatant solution		
pH	Trichloroacetate concentration	Protein per liter egg white	Ovomucoid	Ovalbumin
	per cent	*gm.*	*per cent*	*per cent*
4.9	3	72	17.5	75.5
4.0	3		38.0	55
4.0	5	22	72	24
3.5	5	14	99–100	0–1
2.8	3	17	95	4

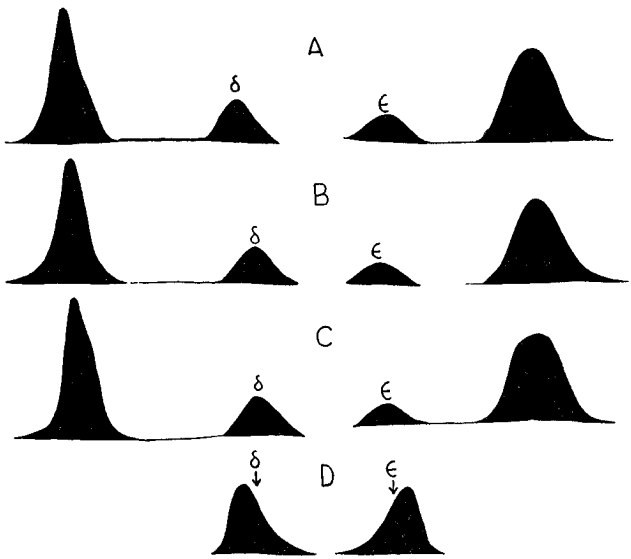


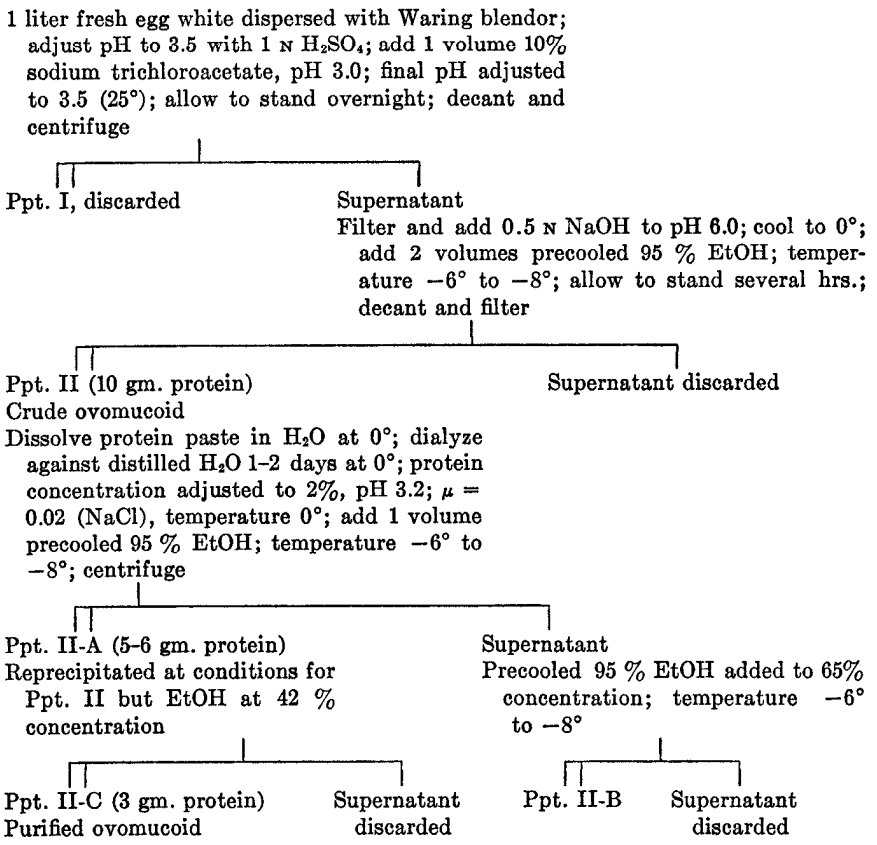
FIG. 1. Electrophoretic patterns of ovomucoid fractions at ionic strength 0.1. Duration of Experiments A, B, and C, 10,800 seconds at a potential gradient of 6 volts per cm. *A*, crude ovomucoid at pH 8.6 (Precipitate II, Diagram 1); *B*, purified ovomucoid at pH 8.6 (Precipitate II-C, Diagram 1); *C*, purified ovomucoid residue at pH 8.6 (Precipitate II-B, Diagram 1); *D*, purified ovomucoid at pH 4.1 (8100 seconds, potential gradient 3.7 volts per cm.).

as a slow and poorly resolved shoulder. An ovomucoid fraction which is free of this minor component was prepared by ethanol precipitation. Conditions for its separation as well as the preliminary fractionation steps are

shown in Diagram 1. To avoid the use of excessive volumes and reagents, all fractionations were carried out on undiluted egg white. The success of the several separations is indicated by the electrophoretic patterns in Fig. 1. The material which has given rise to Fig. 1, *B* will be designated hereafter as "purified" ovomucoid (Precipitate II-C, Diagram 1). This

DIAGRAM 1

Preparation of Ovomucoid



protein was extensively characterized. As indicated by Diagram 1, this substance represents the less soluble portion of the crude ovomucoid. The more soluble portion (Precipitate II-B, Diagram 1, and Fig. 1, *C*) shows an enhanced amount of the more slowly migrating component.

Electrophoretic Studies

The so called purified ovomucoid showed a single boundary upon electrophoretic study in buffers of 0.1 ionic strength, although, as is evident

in Fig. 1, *D*, there is some asymmetry near the isoelectric point. A plot of electrophoretic mobilities *versus* pH (Fig. 2) gives an isoelectric point of 3.9. This value is somewhat lower than that reported by Longsworth, Cannan, and MacInnes (13) and by Hesselvik (14) for ovomucoid prepared by differential heat denaturation of the other egg white proteins.

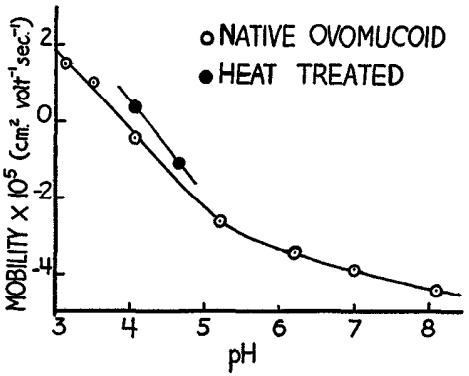


FIG. 2. pH-mobility curve of ovomucoid at ionic strength 0.1

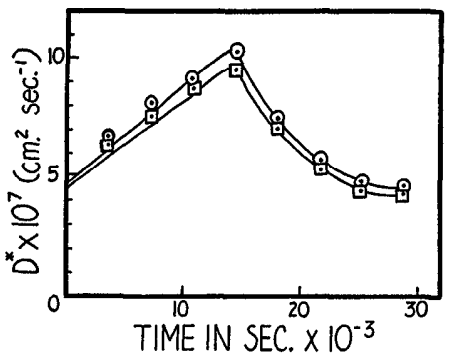


FIG. 3. Variation of the apparent diffusion constants (D^*) of ovomucoid during electrophoresis at ionic strength 0.1 and pH 3.9. Current reversed after 14,400 seconds. □ and ○ represent the experimental data for the right and the left limbs of the cell.

Heating purified ovomucoid for 1 hour at 100° at pH 3.9 in low salt concentration does shift the isoelectric point to pH 4.2, which is in good agreement with the value of 4.3 found by Longsworth, Cannan, and MacInnes, but lower than Hesselvik's value of 4.5.

The electrophoretic inhomogeneity of ovomucoid prepared by heat treatment has been previously recognized by Longsworth, Cannan, and MacInnes (13). Our purified preparations were studied quantitatively in this respect by the method of Alberty (8). In Fig. 3, the apparent

diffusion constants at 1.5° in both cell limbs, obtained in buffer of pH 3.9, ionic strength 0.1, at a potential gradient of 1.7 volts per cm., are plotted against time in seconds. The curve maxima indicate the point at which the current was reversed at 14,400 seconds. After complete electrophoretic reversal, the apparent diffusion constant was found to equal the diffusion constant obtained by the usual procedure. Since the boundary spreading was completely reversible, a heterogeneity constant was calculated. Values of 0.51×10^{-5} and 0.55×10^{-5} were found for the two limbs. Curves constructed from these values agree reasonably well with the experimental

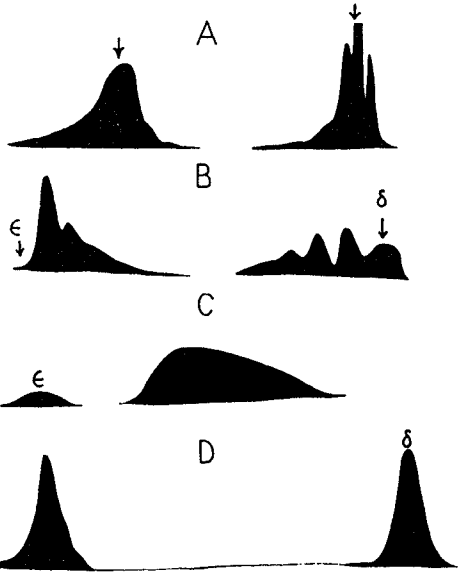


FIG. 4. Electrophoretic patterns of purified ovomucoid at ionic strength 0.01. Duration of experiments 10,800 seconds. *A*, *C*, and *D* 2.1 per cent protein; *B*, 1.1 per cent; *A*, pH 4.0; *B*, pH 4.5; *C*, descending boundary at pH 8.6; *D*, ascending boundary at pH 8.6. Arrows denote the positions of the initial boundary.

points, except during the early stages of the experiment, when the errors in the determination of the apparent diffusion constant are relatively large.

The mean heterogeneity constant of 0.53×10^{-5} is of the same order of magnitude as that found for some other protein systems (15, 16). By the use of this constant and the value of 2×10^{-5} for the slope of the mobility-pH curve of Fig. 2, the standard deviation for the distribution of the isoelectric point of ovomucoid is calculated to be 0.26 unit.

Electrophoretic patterns of purified ovomucoid in buffers of ionic strength 0.01 and different pH values are shown in Fig. 4. These experi-

ments were also carried out at a constant potential gradient of approximately 6 volts per cm. A resolution which is particularly evident on the ascending limb indicates at least five electrophoretic maxima at pH 4.5, as shown by Fig. 4. The effects noted are electrophoretically reversible. Resolution of these maxima is less complete upon electrophoresis at pH 4.0, and, at pH 8.6, ovomucoid shows only a pronounced asymmetrical broadening on the descending limb. It has been previously pointed out by Alberty (8) that heterogeneities in protein systems are more readily discerned at the lower ionic strengths. In any event, the electrophoretic experiments with ovomucoid at ionic strength 0.01 indicate the presence of at least two components in the system, whereas at ionic strength 0.1 only one is indicated. Heating ovomucoid preparations at 100° for 1 hour

TABLE II

Sedimentation Experiments on Ovomucoid Preparations

Material	Protein concentration	Sedimentation constant s_{20w}
	per cent	*Svedberg units*
Electrophoretically separated ovomucoid	0.4	2.7
	1.2	2.8
Crude ovomucoid, alcohol precipitation	0.9	2.8
" " ammonium sulfate precipitation	0.75	2.9
Purified ovomucoid, pH 3.9	0.5	2.75
" " " 3.9	0.9	2.85
" " " 3.9	1.5	2.7
" " " 1.4	0.75	2.75
" " " 11.6	0.8	2.7
Treated by 5% trichloroacetic acid	0.5	2.8
Heat-treated	0.7	2.6

does not change their electrophoretic properties in diethyl barbiturate buffer of pH 8.6 and ionic strength 0.1.

Sedimentation Studies

The sedimentation constants of various ovomucoid preparations were determined under a variety of conditions. These results are presented in Table II. Ovomucoid shows a single symmetrical boundary, which indicates that in the systems considered the protein is substantially homogeneous. No difference in the sedimentation constant of ovomucoid prepared by fractionation methods and by electrophoretic isolation was apparent. The sedimentation constant was independent of protein concentration over a 0.5 to 1.5 per cent range and gave an average s_{20w} = 2.8 Svedberg units. Crude ovomucoid gave the same values as did the

purified material. No difference in ovomucoid precipitated from the trichloroacetate solutions by ethanol and ammonium sulfate was apparent. Moreover, ovomucoid was not affected by previous treatment with 5 per cent trichloroacetic acid. Sedimentation experiments at pH 1.4 to 11.6 likewise revealed no change. Ovomucoid solutions heated for 1 hour at 100° consistently gave a slightly lower sedimentation constant (s_{20w} = 2.6 Svedberg units) than did unheated material. A sedimentation experiment at very low salt concentration at pH 3.9 showed a single boundary. The sedimentation diagram of purified ovomucoid in 0.15 M NaCl at pH 3.9 is shown in Fig. 5.

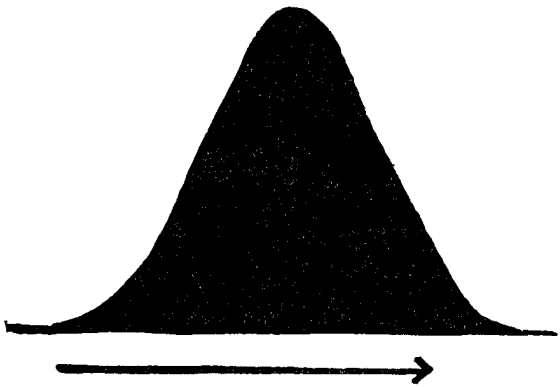


FIG. 5. Sedimentation pattern of purified ovomucoid after 160 minutes at 185,000 × g.

Diffusion Constants

Values for the diffusion constant at 1.2° at protein concentrations of 0.6 and 0.9 per cent were found to be 4.45 and 4.25 × 10^{-7} cm.^{2} sec.$^{-1}$ respectively. The mean value of these determinations, when corrected to 20°, is 8.0 × 10^{-7} cm.^{2} sec.$^{-1}$.

Apparent Specific Volumes

This constant (V_{sp}) was determined at protein concentrations of 1.60, 1.09, and 0.79 per cent respectively. The corresponding V_{sp} values were 0.684, 0.686, and 0.679. Since the last value was subject to greater errors because of the lower protein concentration, the mean of the first two values, 0.685, was used. This figure is considerably lower than the V_{sp} observed for most proteins (7), although Carter has reported 0.658 for thymus nucleohistone (17). From the values for the sedimentation and diffusion constants and the partial specific volume, a molecular weight of 27,000 is calculated. This is in agreement with the value of 28,800

reported by Lineweaver and Murray (1), obtained from osmotic pressure measurements on ovomucoid.

Viscosity Studies

The specific viscosity of ovomucoid was found to be proportional to the concentration within the limits of experimental error. As is shown in Fig. 6, heat-treated ovomucoid (100° for 1 hour) showed only a very slight increase in viscosity. It is interesting that material treated in identical fashion gave a slightly lower sedimentation constant, a change which might be due to the increased viscosity. Treatment with 8 M urea did, however, cause a small increase in viscosity. From the viscosity increment of 8, an axial ratio of 6.5, calculated as a prolate ellipsoid, is

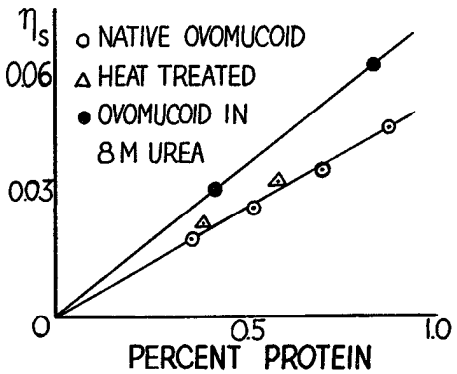


FIG. 6. Effect of heat treatment and 8 M urea on the specific viscosity of ovomucoid.

obtained from the tables of Mehl, Oncley, and Simha (18). From the sedimentation and diffusion constants a value of 1.35 is obtained for the frictional ratio. This gives an axial ratio of 6.3, which is in good agreement with the value obtained by viscosity studies. Since the degree of hydration is not known, the figures obtained are necessarily maximum values.

Other Studies

Purified ovomucoid was found to contain 13.2 per cent nitrogen, in agreement with the results of Lineweaver and Murray (1). The ash content was less than 0.05 per cent. No trichloroacetate ion was bound by the purified ovomucoid. The sample showed 9.7 per cent mannose as determined by the orcinol method. The carbazole method of Dische (11) gave a carbohydrate value of 9 per cent when the extinction at 540 mμ was compared with that of pure mannose.[2] These results are in fair

[2] We wish to thank Miss Margaret E. Marshall for carrying out this determination.

agreement with those reported by Sörensen (19). An average of four determinations gave a glucosamine value of 17 per cent, which is somewhat higher than that reported by most workers (4). The nitrogen, glucosamine, and mannose content of the various ovomucoid preparations of Fig. 1 showed no apparent differences.

The antitryptic activities of the various ovomucoid fractions were essentially identical. The samples gave a value of 8 antitryptic units per mg. of protein, compared to the value of 1 unit per mg. of dried egg white as defined by Lineweaver and Murray (1). Our results are quite consistent with these values. Failure to discern any difference in activity between the various fractions assayed may well be due to the limitations of the method used. Attempts to crystallize ovomucoid from concentrated ammonium sulfate solutions were unsuccessful.

DISCUSSION

Ovomucoid is a mucoprotein of unusual stability. The viscosity data indicate that no denaturation is apparent after the protein is heated for 1 hour at 100°. However, biological inactivation in the form of loss of antitryptic activity has been shown by Lineweaver and Murray (1) to occur under such conditions. The shift in the isoelectric point likewise shows that undefined changes have occurred. This emphasizes the danger of using heating procedures to prepare mucoproteins, despite their unusual stability. In 8 M urea solution, only small changes appear in specific viscosity.

At ionic strength 0.01 and pH 4.5, ovomucoid shows the presence of at least five electrophoretic maxima. In sedimentation analysis under the same conditions there is a single boundary. Thus it would appear that the electrophoretic results are not due to a dissociation of this protein into smaller molecular fragments. Our present knowledge of electrophoretic phenomena occurring at low ionic strength does not permit an explanation of these results. A somewhat similar situation has been previously noted by Sharp *et al.* (20) for horse serum albumin. The possibility of anomalies arising from the binding of buffer ions may be a factor in the apparent complexity of the electrophoretic behavior in this system.

If the heat stability of ovomucoid is due to its high carbohydrate content, it would appear that the various protein molecules present are mucoproteins, since the system showed such extreme resistance to denaturation. The high density of this protein (proteins) may be related to its carbohydrate content, which, in terms of mannose and glucosamine, constitutes approximately 27 per cent of ovomucoid.

The electrophoretic behavior in buffers of ionic strength 0.1, while

showing ovomucoid to be relatively heterogeneous, does not disclose the complexity seen at ionic strength 0.01. In order to evaluate this property of ovomucoid more fully, extensive fractionation of this system will be required. It might be well to point out the inconsistency of interpreting the results of fractionation experiments carried out at low ionic strengths in terms of electrophoretic analyses at ionic strength 0.1. Thus, upon electrophoresis at the latter ionic strength, one is led to the conclusion that a fairly homogeneous protein has been separated, while great complexity may be indicated in buffers of ionic strength 0.01. Since the subfractionation used in preparing so called purified ovomucoid was carried out at an ionic strength of approximately 0.01, one might suggest that in terms of the electrophoretic diagram shown in Fig. 4, *B* the possibilities for further separation are indicated.

SUMMARY

Ovomucoid has been prepared under conditions of fractionation which are less rigorous than those usually employed, giving a substance which is substantially monodisperse. A combination of data on sedimentation, diffusion, viscosity, and partial specific volume for this very stable mucoprotein gives a molecular weight of 27,000 and an axial ratio of approximately 6.4.

In buffers of ionic strength 0.1 the ovomucoid behaves as a single electrophoretic component with a heterogeneity constant of 0.53×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$.

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