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The Composition and Proposed Subunit Structure of Egg-White β-Ovomucin

THE ISOLATION OF AN UNREDUCED SOLUBLE OVOMUCIN

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1. New preparations of reduced carboxymethylated β-ovomucin (*S*-carboxymethyl-β-ovomucin) were homogeneous by sedimentation analysis, analytical sedimentation to equilibrium in CsCl gradients, and disc electrophoresis in sodium dodecyl sulphate. 2. Degradation of *S*-carboxymethyl-β-ovomucin with either CNBr or trypsin indicated the presence of a subunit (approx. mol.wt. 112300). 3. Electron microscopy showed that *S*-carboxymethyl-β-ovomucin consisted of chains of globular units (approx. mol.wt. 103000). In 6M-guanidinium chloride *S*-carboxymethyl-β-ovomucin existed mainly as an aggregate (mol.wt. 720000). 4. *S*-Carboxymethyl-β-ovomucin contained ester sulphate (4.24%, w/w) and carbohydrate (60%, w/w), which consisted of large amounts of galactose (22%, w/w), galactosamine (8.9%, w/w) and sialic acid (10.6%, w/w). 5. An unreduced soluble fibrous component (component S_{GH}) extracted from crude ovomucin precipitate with 5M-guanidinium chloride contained β-ovomucin (approx. 70%, w/w). By using the Scheraga–Mandelkern equation the molecular weight of component S_{GH} was calculated to be 11.5×10^6.

Crude ovomucins obtained from the thick egg-white fraction of the domestic hen's egg (Robinson & Monsey, 1971) contained mainly two types of glycoprotein, a homogeneous α-ovomucin and a heterogeneous β-ovomucin. The reduced soluble derivative of the β-ovomucin fraction, which was separated from α-ovomucin by density-gradient centrifugation, contained large amounts of galactose, galactosamine, hydroxy amino acids and sialic acid, unlike other egg-white glycoproteins (Robinson, 1972). Natural liquefaction of thick egg white is accompanied by degradation of β-ovomucin, and therefore it is considered that this egg-white glycoprotein may be mainly responsible for the gel-like properties of thick egg white (Robinson & Monsey, 1972*a*,*b*).

The present paper describes first the composition and a probable subunit structure for homogeneous preparations of reduced β-ovomucin, and secondly the isolation of a very large fibrous unreduced ovomucin which contained approx. 70% (w/w) of β-ovomucin.

Experimental

Materials

Reagents for preparing polyacrylamide gels, and CNBr, were purchased from Eastman Kodak Ltd., Kirby, Liverpool, U.K. The latter was used within 1 week of receipt. Sodium dodecyl sulphate ('specially pure' grade), polyethylene glycol 20M, dansyl chloride and dansyl-amino acids were purchased from British Drug Houses Ltd., Poole, Dorset, U.K. Silica gel G manufactured by E. Merck, Darmstadt, W. Germany, was supplied by Anderman and Co., East Molesey, Surrey, U.K. Carboxypeptidase A (treated with di-isopropyl phosphorofluoridate) was purchased from Sigma Chemical Co., London S.W.6, U.K. Trypsin (treated with L-tosylamido-2-phenethyl chloromethyl ketone) was purchased from Worthington Biochemical Corp., Cambrian Chemical Ltd., Croydon, U.K. All pyridine used was redistilled from ninhydrin (Hill & Delaney, 1967). Other materials were as described by Robinson & Monsey (1971).

Preparation of a soluble unreduced ovomucin (component S_{GH})

Crude ovomucin complex was precipitated from the thick white fractions of 60 newly laid eggs (Robinson & Monsey, 1971). After extraction of the precipitate at 2°C, first with portions of water (1 litre) and secondly with KCl (2%, w/v; 1 litre) until the E_{276} of the supernatant solutions was zero, a further extraction with 5M-guanidinium chloride was made by the addition of solid guanidinium chloride to the washed precipitate of crude ovomucin (volume was

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now 400ml). After 16h at 2°C the translucent suspension of crude ovomucin in 5M-guanidinium chloride was dialysed against three changes (2 litres) of 0.02M-Tris–HCl buffer (pH7.5)–0.1M-NaCl and centrifuged at 35000*g* at 2°C. (Preliminary experiments showed that if centrifuging was carried out before the dialysis step, it was far more difficult to separate the supernatant from the precipitate.) For some physical studies the supernatant containing component S_{GH} was concentrated by dialysis against the solutions necessary for these studies.

The supernatant was dialysed against water and freeze-dried in order to isolate samples for chemical analyses and for some viscosity measurements. The dry solid obtained was reduced and *S*-carboxymethylated (Robinson & Monsey, 1971), and the solution of the product was dialysed against water, deionized by passage through a column (10cm×1cm) of Dowex 50W (X8; H^+ form) and again freeze-dried. The residue was dried over P_2O_5.

Preparation of S-carboxymethylated β-ovomucin

This derivative of β-ovomucin was isolated after reduction of the ovomucin complex and preparative equilibrium sedimentation in density gradients of CsCl containing 5M-guanidinium chloride (Robinson & Monsey, 1971). For final purification, a second centrifugation was performed at a starting density of 1.46g/ml. The product was deionized as described above and dried in a vacuum over P_2O_5 before chemical analysis.

Viscosity measurements

A Cannon–Fenske (no. 50) viscometer supplied with a National Physical Laboratory calibration certificate was used. All solutions were passed through a no. 1 sintered-glass filter (Pyrex) to exclude dust and after dilution were left for 24h, before the measurements were made at 25°C (±0.05°C).

The soluble ovomucin, component S_{GH}, was dialysed against 0.1M-sodium acetate buffer (pH5.0) containing 6M-guanidinium chloride. Requisite dilutions were made and the intrinsic viscosity determined by extrapolation to zero concentration of the reduced-viscosity–concentration relationship. Similarly, the intrinsic viscosity of the reduced and alkylated component S_{GH} was determined.

Analytical ultracentrifugation

Velocity sedimentation in the presence of 6M-guanidinium chloride, equilibrium sedimentation (Yphantis, 1964) for determination of weight-average molecular weights and analytical sedimentation in density gradients were carried out in a Beckman model E ultracentrifuge as described by Robinson & Monsey (1971). Determinations of the sedimentation coefficients at zero concentration were carried out on both component S_{GH} and *S*-carboxymethyl β-ovomucin. The preparations were dissolved in 0.1M-sodium acetate buffer (pH5.0) containing 6M-guanidinium chloride, and dilutions were made with the same solution. The concentration range used for component S_{GH} was 0.5–7mg/ml and for *S*-carboxymethyl β-ovomucin 0.5–10mg/ml. Centrifugations in 12 or 30mm cells were carried out at 44000rev./min at 25°C by using schlieren optics. The peaks were measured in a comparator (magnification ×10). For the determination of molecular weights the speed setting was either 8000 or 10000rev./min, but the exact speed was found by timing the revolution counter.

Electron microscopy

S-Carboxymethyl-β-ovomucin (0.1mg) or component S_{GH} (0.1mg) contained in water (1ml) was mixed with saturated aq. uranyl acetate (1ml). Samples were dried on carbon-coated grids and examined in an AEI 801 electron microscope. Photographs of ovomucin were taken at a magnification of 40000, and several physical measurements (Slayter, 1969) were taken from prints further enlarged.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

S-Carboxymethyl-β-ovomucin (1mg) was heated in a boiling-water bath for 2min, in 0.2ml of 0.25M-Tris–HCl buffer (pH8.20) containing 0.065M-dithiothreitol and 2.5% (w/v) sodium dodecyl sulphate. Solutions were cooled and made 10% (w/v) with respect to sucrose. Samples (0.03ml and 0.005ml) were applied to gels to be stained with Amido Black and with periodic acid–Schiff stain respectively. Polyacrylamide gels (3%, w/v) were made by the method of Weber & Osborn (1969), modified by decreasing the volume of acrylamide solution to 4.05ml, adding 9.45ml of water and by decreasing the volume of *NNN'N'*-tetramethylethylenediamine to 0.006ml. Electrophoresis was allowed to proceed for 3.5h at 5mA per gel. Gels were immersed in Amido Black [1% (w/v) in 7% (v/v) acetic acid]. Other gels were stained with periodic acid–Schiff reagents (Zacharius & Zell, 1969). Precautions were taken to remove the sodium dodecyl sulphate before adding the periodic acid–Schiff reagents (Koblyka & Carraway, 1972).

Cyanogen bromide cleavage

S-Carboxymethyl-β-ovomucin (5mg) was suspended in 70% (v/v) formic acid (1ml) and CNBr

crystals (50mg) were added. The reaction mixture was left for 20h at 20°C, then diluted with 10vol. of distilled water and freeze-dried. Sodium phosphate buffer (0.5ml; pH6.75, 0.01M in phosphate), containing 1% (v/v) 2-mercaptoethanol and 2.5% (w/v) sodium dodecyl sulphate, was added to the dried peptides. The solution was left for 20h at room temperature and then made 10% (w/v) with respect to sucrose. Samples were applied to 10% (w/v) polyacrylamide–sodium dodecyl sulphate gels (Weber & Osborn, 1969), 0.15ml to gels to be stained with Amido Black and 0.02ml to gels to be treated with periodic acid–Schiff reagents. Electrophoresis was allowed to proceed for 2.5h at 7.5mA per gel.

Tryptic peptide 'maps'

S-Carboxymethyl-β-ovomucin (8mg) was added to 1% (w/v) NH_4HCO_3 (4ml). A solution containing 1mg of trypsin in 2.5ml of 0.001M-HCl was made; 0.2ml of this enzyme solution was added initially to the ovomucin, and after 1h a further 0.2ml. Digestion was allowed to proceed at 37°C for 5h and then the digest was freeze-dried. The dry peptides were dissolved in 0.2M-NH_3 (50μl) and 25μl was applied to a sheet (60cm×32cm) of Whatman no. 3 paper. High-voltage electrophoresis in the long direction at 70V/cm for 1.5h with redistilled pyridine–acetic acid–water (25:1:224, by vol.) at pH6.5 as solvent, described by Rees *et al.* (1970), was used to separate the peptides in the first dimension. After air-drying the paper, ascending chromatography in butan-1-ol–acetic acid–water–pyridine (15:13:12:10, by vol.) for 24h was used for the second dimension (Rees *et al.*, 1970). After drying and washing the paper in ethanol–acetone (1:1, v/v) the peptides were chlorinated (Reindel & Hoppé, 1954), and located by dipping in a freshly prepared solution containing equal volumes of KI (0.75%, w/v) and saturated tolidine in 2M-acetic acid. Peptides containing arginine were located with the Sakaguchi reagent as described by Acher & Crocker (1952).

N-Terminal amino acid analysis

S-Carboxymethyl-β-ovomucin (3mg) was treated with dansyl chloride in 8M-urea–0.1M-$NaHCO_3$ solution as suggested by Beale (1969). The insoluble dansylated protein was dialysed first against aq. 50% (v/v) acetone, then against water and was finally freeze-dried. After hydrolysis in constant-boiling HCl (1ml) for 20h at 110°C in a sealed ampoule the hydrolysate was dried by rotary evaporation and extracted with acetone–0.1M-acetic acid (3:2, v/v). The extract was applied to a thin layer (0.25mm) of silica gel G (20cm×20cm). Appropriate markers were added and the plate was developed in the first dimension with methyl acetate–propan-2-ol–NH_3 (sp. gr. 0.88) (9:7:4, by vol.) and in the second dimension with chloroform–methanol–acetic acid (15:4:1, by vol.). The fluorescent dansyl-amino acids were observed under u.v. light.

C-Terminal amino acid analysis

S-Carboxymethyl-β-ovomucin (10mg) was treated with carboxypeptidase A by the method of Ambler (1967). The enzyme was solubilized by Method 1 and the ratio of substrate to enzyme was 75:1 (w/w). Incubation was carried out at 37°C. Half of the digest was withdrawn after 2h, the pH of which was adjusted to 3.0 with acetic acid, and the digest dried by rotary evaporation. Analysis for free amino acids was carried out on a Beckman 120C amino acid analyser. The other half of the sample was digested for 22h and treated as described above. A blank, containing enzyme but without ovomucin, was put through the same procedure.

Other chemical analyses

Hexoses were determined by a g.l.c. method, hexosamines and amino acids by using the Beckman 120C amino acid analyser and sialic acid by the thiobarbituric acid procedure as previously described (Robinson & Monsey, 1971). Ester sulphate was determined after hydrolysis in 6M-formic acid for 9h at 110°C (Jones & Lethan, 1956).

Results

Extraction of the suspension of insoluble crude ovomucin complex with 5M-guanidinium chloride followed by dialysis of the supernatant against water or buffer solutions yielded a clear viscous solution of a soluble non-reduced form of ovomucin. For clarity this ovomucin is referred to as component S_{GH}. The yields of component S_{GH} accounted for approx. 0.02% (w/w) of the thick egg-white fraction, or alternatively approx. 10% (w/w) of the crude ovomucin complex.

Ultracentrifugation

Sedimentation-velocity ultracentrifugation of either component S_{GH} or *S*-carboxymethyl-β-ovomucin in the presence of guanidinium chloride showed the presence of single schlieren peaks. The relationships between the sedimentation coefficients of component S_{GH} and *S*-carboxymethyl-β-ovomucin and concentration are shown in Fig. 1. By using a computer program for regression analysis the following relationships between sedimentation coefficients in guanidinium chloride and concentration (c, mg/ml) have been calculated. For component S_{GH} $S =$

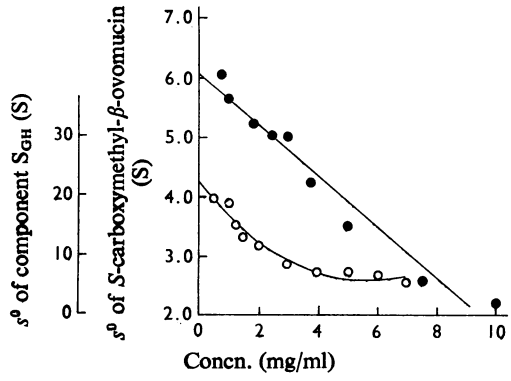


Fig. 1. *Determination of s^0 of component S_{GH} and S-carboxymethyl-β-ovomucin*

Both were dissolved in 0.1M-sodium acetate buffer, pH5.0, containing 6M-guanidinium chloride. The rotor speed was 44000rev./min and the temperature was 25°C. ○, Component S_{GH}; ●, *S*-carboxymethyl-β-ovomucin.

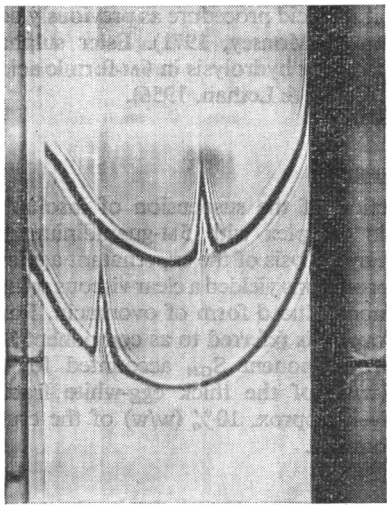


Fig. 2. *Sedimentation-velocity experiment with component S_{GH}*

The concentration of component S_{GH}, dissolved in 0.05M-Tris–HCl buffer (pH7.5) containing 5M-guanidinium chloride, was 0.23% (w/v). Half of the solution was made 1% (w/v) with respect to dithiothreitol by adding the reducing agent as a solid. Two 30mm cells were used. The frame was photographed after 64min at 44000rev./min. The temperature was 25°C. The direction of sedimentation was from left to right. Top schlieren peak, component S_{GH}; bottom schlieren peak, reduced component S_{GH}.

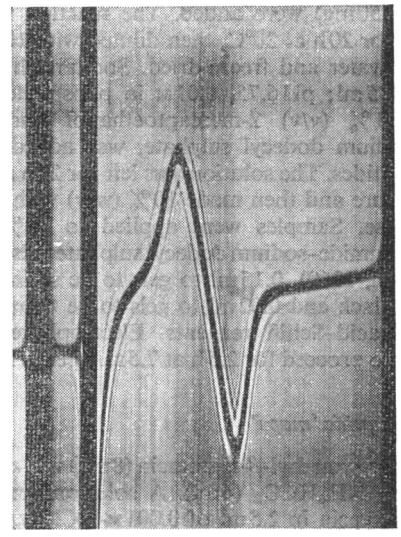


Fig. 3. *Equilibrium centrifugation of reduced S-carboxymethyl-β-ovomucin in a gradient of CsCl*

S-Carboxymethyl-β-ovomucin (4.5mg) was dissolved in 0.05M-Tris–HCl buffer (pH7.5) containing 5M-guanidinium chloride. CsCl crystals were added, until the density of the solution was 1.49g/ml. A 30mm cell was used. The rotor speed was 40000rev./min and the temperature was 25°C. The frame was photographed after 28h by using schlieren optics.

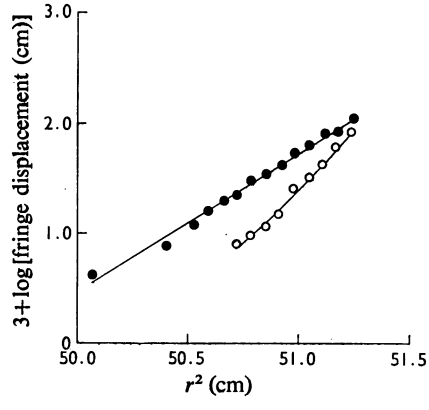


Fig. 4. *Sedimentation-equilibrium plots of S-carboxymethyl-β-ovomucin*

S-Carboxymethyl-β-ovomucin (1mg) was dissolved in 1ml of 0.1M-Tris–HCl buffer (pH8.2) containing 6M-guanidinium chloride and 10mM-dithiothreitol. Centrifugations, at 22°C, were carried out at (●) 8000rev./min and (○) 10000rev./min. Calculations were made from interference photographs taken after 67h.

Table 1. *Physical constants of β-ovomucin*

	Component S_{GH}	*S*-Carboxymethyl-β-ovomucin
Sedimentation coefficient s^0 (in 6M-guanidinium chloride at 25°C)	22.08S	6.09S
Partial specific volume* (ml/g)	0.674	0.662
Intrinsic viscosity in 6M-guanidinium chloride		
Before reduction	598.2	
After reduction	108.1	
Molecular weight sedimentation equilibrium		
(8000rev./min)		720000
(10000rev./min)		523000–743000
By Scheraga–Mandelkern equation†	11.5×10^6	

* The values of $\bar{v}$ were calculated from the chemical composition and no correction has been applied for any effect of solvent interactions.

† β parameter calculated to be 3.3×10^6 (see the text).

$-(5.695+0.92)c+22.09$. For *S*-carboxymethyl-β-ovomucin, $S=-(0.4304+0.03)c+6.09$.

The sedimentation coefficients and other physical constants are shown in Table 1. After reduction with dithiothreitol (1%, w/v) of a solution of component S_{GH} (0.23%, w/v) in 0.05M-Tris–HCl buffer (pH7.5) containing 5M-guanidinium chloride a slower sedimenting component similar to *S*-carboxymethyl-β-ovomucin (Fig. 2) was observed. Equilibrium ultracentrifugation in density gradients of CsCl containing 5M-guanidinium chloride showed both component S_{GH} and *S*-carboxymethyl-β-ovomucin (Fig. 3) to be homogeneous and free of the lower-density β-ovomucins. The buoyant densities of component S_{GH} and *S*-carboxymethyl-β-ovomucin were observed to be 1.46 and 1.48g/ml respectively.

High-speed equilibrium sedimentation in the presence of 5M-guanidinium chloride showed that the preparations of *S*-carboxymethyl-β-ovomucin were composed mainly of molecules with a molecular weight of approx. 720000 (Table 1). However, although the relationship for log (fringe displacement) against r^2 at 8000rev./min was linear (Fig. 4), a similar plot of data obtained at 10000rev./min showed a slight curvature (Fig. 4). As shown in Table 1 a minimum molecular weight of 523000 was calculated from the data shown in Fig. 4.

Viscosity

For component S_{GH} dissolved in 6M-guanidinium chloride a linear relationship between concentration and reduced viscosity was observed (Fig. 5*a*). The intrinsic viscosity $[\eta]$ of component S_{GH} after reduction with 0.3M-mercaptoethanol in the presence of 6M-guanidinium chloride decreased from 598.2ml/g to 108.1ml/g (Fig. 5*b*). By using the relationship between $[\eta]$ and the number of residues (Tanford *et al.*, 1967) in a denaturing solvent and assuming an average residue weight for amino acids of 118.62, the molecular weight of the protein moiety of reduced component S_{GH} was calculated to be 238000.

Electron microscopy

Although electron micrographs of component S_{GH} showed the presence of large aggregates of randomly dispersed fibres (Plate 1*a*) those of preparations of *S*-carboxymethyl-β-ovomucin showed that this derivative of ovomucin was composed of aggregates containing globular units joined end to end to form small chains (Plate 1*b*). Measurements, standardized by using crystalline catalase, carried out on enlarged photomicrographs showed that the chains contained up to 10 globular units each 60nm in diameter. By substitution in the equation for spherical molecules $M=4/3\,\pi r^3\rho N$ (Slayter, 1969), where r = radius, ρ = density of solute and N = Avogadro's number, the molecular weight of the unit was calculated to be 103000.

Electrophoresis

Staining with either the periodic acid–Schiff reagents or Amido Black of 3% (w/v) polyacrylamide gels after disc electrophoresis in the presence of 1% (w/v) sodium dodecyl sulphate showed that *S*-carboxymethyl-β-ovomucin had migrated approx. 5mm into the gel as a single band (Plate 2). Unfortunately, suitable high-molecular-weight reference compounds are not available for a determination of the molecular weight of *S*-carboxymethyl-β-ovomucin by this method. Attempts to carry out disc electrophoresis in the presence of urea, or without prior treatment of *S*-carboxymethyl-β-ovomucin with sodium dodecyl sulphate, were unsuccessful because the ovomucin did not penetrate the gel.

Chemical analysis

Both component S_{GH} and *S*-carboxymethyl-β-ovomucin contain large equimolar amounts of galactosamine and ester sulphate (Table 2). However, component S_{GH} contains much more mannose and appreciably more protein than *S*-carboxymethyl-

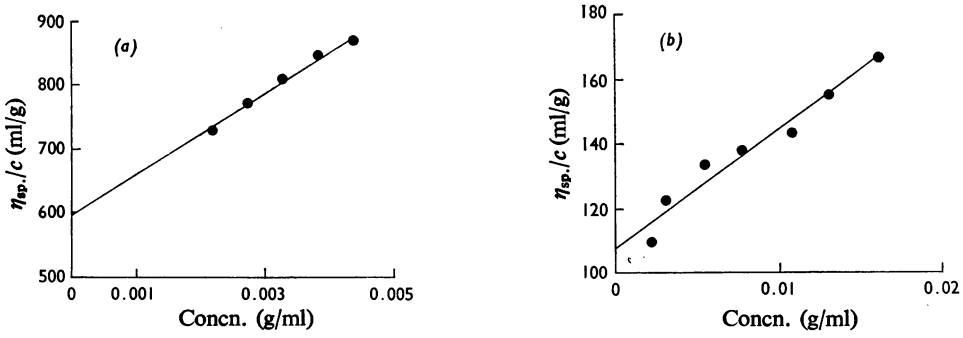


Fig. 5. *Determination of the intrinsic viscosities of component S_{GH} and reduced component S_{GH}*

Preparations were dissolved in 0.1 M-sodium acetate buffer (pH 5.0) containing 6 M-guanidinium chloride. A Cannon–Fenske glass capillary viscometer was used at 25°C. (*a*) Component S_{GH}; (*b*) reduced and carboxymethylated component S_{GH}.

Table 2. *Chemical composition of component S_{GH} and S-carboxymethyl-β-ovomucin*

Amino acids were determined by using anhydrous molecular weights. Component S_{GH} was reduced and carboxymethylated.

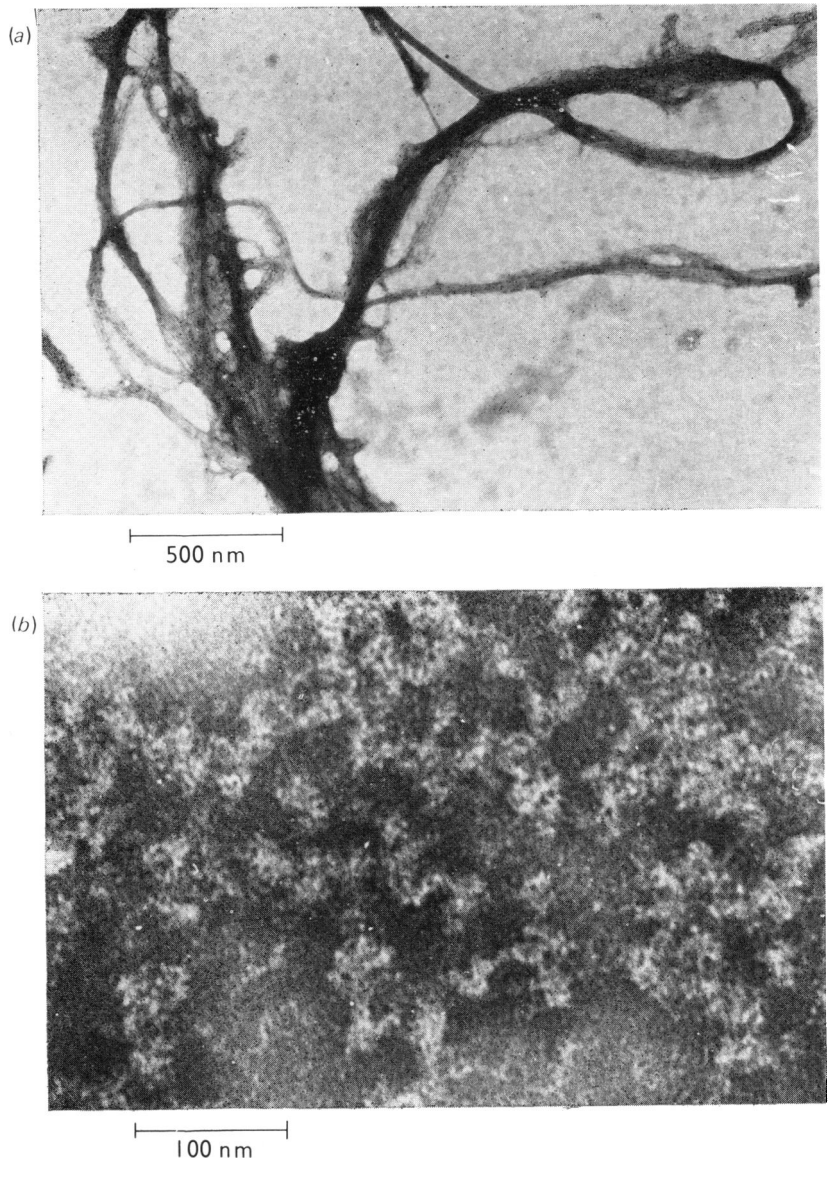
	Content			
	(g/100 g of dry sample)		(mol/10^5 g of total protein)	
	Component S_{GH}	*S*-Carboxymethyl-β-ovomucin	Component S_{GH}	*S*-Carboxymethyl-β-ovomucin
Lysine	2.61	1.73	45.6	50.9
Ammonia	1.16	1.60		
Histidine	0.83	0.63	13.4	17.3
Arginine	2.56	0.92	36.7	22.2
Aspartic acid	4.07	1.41	79.2	46.4
Threonine*	4.38	4.35	96.8	162.8
Serine*	4.13	3.73	106.1	162.0
Glutamic acid	4.61	2.30	79.8	67.4
Proline	2.68	2.22	61.8	86.6
Glycine	1.35	0.54	52.9	35.6
Alanine	1.68	0.94	52.5	50.0
Valine	2.32	1.22	52.4	46.6
Cysteine (half)†	2.61	0.92	36.2	33.9
Methionine	1.00	0.50	17.0	14.4
Isoleucine	1.69	1.06	33.3	35.4
Leucine	3.37	2.00	66.5	66.7
Tyrosine	1.72	0.99	23.6	23.0
Phenylalanine	1.97	0.99	29.9	25.4
Total protein	44.74	28.05		
Glucosamine	7.63	12.30	95.2	244.9
Galactosamine	5.35	8.86	66.8	176.5
Galactose	16.68	22.00	207.0	435.3
Mannose	3.16	0.70	39.1	13.9
Total sialic acid	9.53	10.59	69.1	122.3
Sulphate	2.91	4.24	67.7	157.6
Total	90.00	86.74		

* Corrected for destruction (threonine 5.7% loss; serine 9.2% loss).
† Determined as *S*-carboxymethylcysteine.

β-ovomucin does. Serine and threonine were the major amino acids present in both component S_{GH} and *S*-carboxymethyl-β-ovomucin (Table 2), although component S_{GH} contains proportionally more aspartic acid and glutamic acid per 10^5 g of total protein.

Chemical cleavage of S-carboxymethyl-β-ovomucin

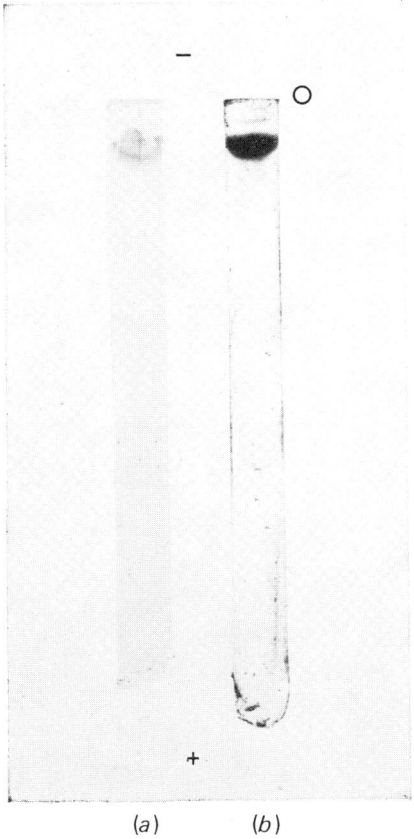
Treatment of *S*-carboxymethyl-β-ovomucin with CNBr produced five or six major peptides and one oligosaccharide or glycopeptide of apparent high molecular weight which did not stain with Amido



EXPLANATION OF PLATE 1

Electron microscopy of component S_{GH} and S-carboxymethyl-β-ovomucin

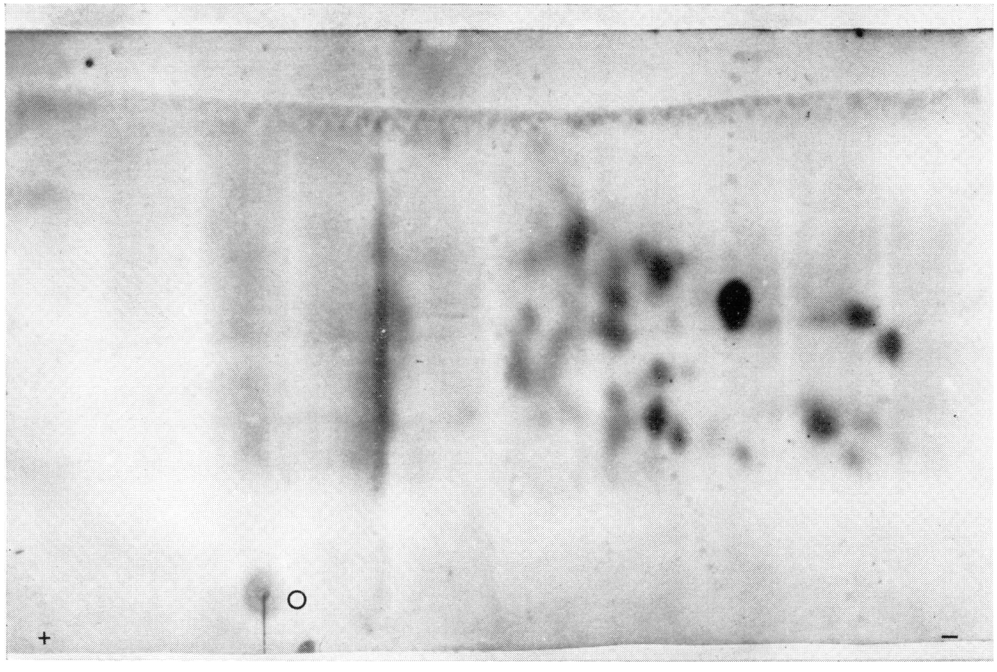
(*a*) Electron micrograph of component S_{GH} showing fibrous nature of material. (*b*) Electron micrograph of *S*-carboxymethyl-β-ovomucin in which the individual globular units are seen. Both preparations were negatively stained with uranyl acetate as described in the text.



EXPLANATION OF PLATE 2

Disc electrophoresis of S-carboxymethyl-β-ovomucin

For this 3% (w/v) polyacrylamide gels containing sodium dodecyl sulphate were used. The origin is marked 'O'. (*a*) Gel showing slight staining with Amido Black; (*b*) gel showing heavy staining with the periodic acid–Schiff reagents.



EXPLANATION OF PLATE 3

Two-dimensional map of peptides of S-carboxymethyl-β-ovomucin produced by trypsin

S-Carboxymethyl-β-ovomucin (4mg) was digested as described in the text and applied at the origin (marked 'O'). Electrophoresis was carried out in the horizontal direction and ascending chromatography in the vertical direction. The peptides were located by the chlorination method.

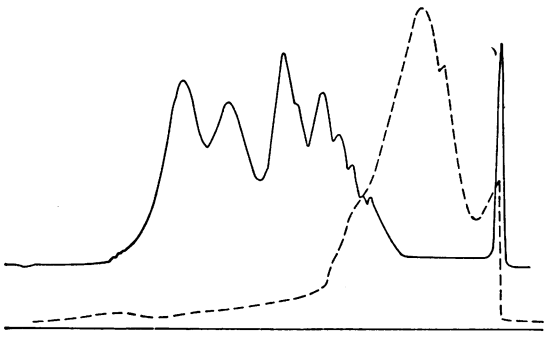


Fig. 6. *Densitometric traces of S-carboxymethyl-β-ovomucin after treatment with CNBr and electrophoresis*

The peptides were separated on a 10% (w/v) polyacrylamide–sodium dodecyl sulphate gel. ——, Gel stained with Amido Black; ----, gel stained with periodic acid–Schiff reagents. Direction of electrophoresis was from right to left. A Joyce–Loebl (Chromoscan mark 2) densitometer was used.

Black but reacted with the periodate–Schiff reagents, (Fig. 6). Consequently, it seems that *S*-carboxymethyl-β-ovomucin may contain four or five methionine residues per subunit.

N-Terminal analysis of S-carboxymethyl-β-ovomucin

By using the dansyl method, the *N*-terminal amino acid appeared to be threonine.

C-Terminal analysis of S-carboxymethyl-β-ovomucin

The weights of the released amino acids after being corrected for those released from the enzyme itself and then expressed as mol of amino acid showed that the order of release was leucine, threonine, valine and lysine.

Trypsin digestion

Plate 3 shows a peptide 'map' obtained with a mixture of peptides produced by tryptic digestion of *S*-carboxymethyl-β-ovomucin at 37°C for 5h. The 23 or 24 major spots observed when stained by the chlorination technique were highly reproducible for various preparations of *S*-carboxymethyl-β-ovomucin. A similar preparation showed seven peptides that produced a pink colour characteristic of arginine residues when treated with the Sakaguchi reagent.

Discussion

The preparation of *S*-carboxymethyl-β-ovomucin seems homogeneous, by disc electrophoresis in the presence of sodium dodecyl sulphate or by ultracentrifugation. The use of a CsCl solution of greater density than that previously used during the final stage of the purification by preparative equilibrium sedimentation is clearly of considerable value. The new preparations of *S*-carboxymethyl-β-ovomucin contain more glucosamine, galactosamine, galactose and ester sulphate, but considerably less mannose and slightly less sialic acid, than did the heterogeneous preparations of *S*-carboxymethyl-β-ovomucin previously described (Robinson & Monsey, 1971).

Fully reduced β-ovomucin in dissociating solvents (Table 1) is a surprisingly large molecule (mol.wt. 720000). Assuming that the protein moiety was not shielded by the large proportion of carbohydrate from either enzymic attack by trypsin or chemical attack by CNBr, then it seems that *S*-carboxymethyl-β-ovomucin may contain a single protein moiety with approx. 23 residues susceptible to attack by trypsin and four or five residues of methionine respectively. From the results of amino acid analysis (Table 2), the molecular weight of this protein moiety containing four or five methionine residues is calculated to be 27800 or 34700 respectively. The minimum molecular weight of a protein moiety containing 16 lysine and 7 arginine residues is calculated to be 31500. End-group analysis indicated that threonine and leucine were the *N*- and *C*-terminal amino acids respectively. Assuming that β-ovomucin contains only one type of protein, then, as *S*-carboxymethyl-β-ovomucin contained 28% (w/w) protein, the molecular weight of the whole subunit is calculated to be approx. 112300. This molecular weight calculated for the subunit agrees very well with the less accurate molecular weight (mol.wt. 103000) calculated from the dimensions of the globular units observed in the electron microscope. Consequently, it seems probable that *S*-carboxymethyl-β-ovomucin, even in the presence of 6M-guanidinium chloride and excess of reducing agent, is probably an aggregate of smaller subunits. As *S*-carboxymethyl-β-ovomucin only penetrated 3% (w/v) polyacrylamide gel after severe treatment with sodium dodecyl sulphate, it is possible that the subunits of the aggregate might be held together by hydrophobic bonds.

Because component S_{GH} contained considerable amounts of galactosamine, galactose, sialic acid and particularly ester sulphate, which is a substance present in only β-ovomucin and not the other egg-white glycoproteins (Robinson, 1972), it is concluded that component S_{GH} contained approx. 70% (w/w) of β-ovomucin. However, it does seem that component S_{GH} was not composed entirely of β-ovomucin, because analysis showed that it contained appreciable amounts of mannose and proportionally more protein than *S*-carboxymethyl-β-ovomucin. Nevertheless, as only a single schlieren peak, which corresponded to *S*-carboxymethyl-β-ovomucin, was observed after

the reduction of component S_{GH}, and as the intrinsic viscosity of S_{GH} was less after reduction, it is proposed that it was made up mainly of β-ovomucin aggregates (mol.wt. 720000) joined end to end through disulphide bonds, the aggregates themselves being composed of the smaller globular subunits (mol.wt. 103000) as seen in the electron microscope.

For component S_{GH} the viscosity increment, ν, has been calculated from the intrinsic viscosity and partial specific volume (Table 1) from the relationship of Cohn & Edsall (1943) and found to be 900. From this value of ν the axial ratio of an assumed unhydrated prolate elipsoid (Yang, 1961) is calculated to be approx. 130:1. Thus component S_{GH} is found to be highly asymmetric, which is substantiated by the results of the electron microscopy. The molecular weight of such a large molecule as component S_{GH} is impossible to determine by equilibrium-sedimentation methods, but can be calculated from the Scheraga & Mandelkern (1953) equation. From s^0 and $[\eta]$, and a β function of 3.3×10^6 obtained from the relationship between axial ratio and β function (Yang, 1961), a molecular weight of approx. 11.5 million is obtained. A similar method of calculation for the molecular weight of filamentous aggregates of Tamm–Horsfall glycoprotein, but using an assumed value of 3.6×10^6 for the β function, has been used by Stevenson & Kent (1970). Alternatively, from the relationship for random coils between sedimentation coefficients and molecular weights in denaturing solvents (O'Malley & Weaver, 1972) it is calculated that S_{GH} was approx. 13 times as large as *S*-carboxymethyl-β-ovomucin (mol.wt. 720000) which corresponds to a molecule with an average molecular weight of approx. 9.36 million. Further, the ratio relationship for random coils between intrinsic viscosities and the number of residues (Tanford *et al.*, 1967), which is a better measure of hydrodynamic volumes, is calculated to be 13.37.

It is concluded that S_{GH} was mainly a soluble non-reduced macromolecular form of β-ovomucin aggregates and may represent one of the states in which β-ovomucin exists in the thick egg-white gel. However, as crude ovomucin precipitates contained approx. 30% (w/w) of β-ovomucin (Robinson & Monsey, 1972*a*,*b*) and component S_{GH} only accounted for approx. 10% (w/w) of the crude ovomucin, egg white may also contain other forms of β-ovomucin which cannot be extracted with concentrated solutions of guanidinium chloride.

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