An Investigation of the Egg-White Mucoproteins, Ovomucoid and Ovalbumin

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Very little information is available about the structure of the mucoproteins because of the difficulties that arise when classical methods are applied. The severe difficulties in the preparation of workable quantities of pure mucoproteins has also retarded structural studies. Only the carbohydrate of ovomucoid has received a detailed structural investigation (Stacey & Woolley, 1940, 1942). This substance contains about 25% of carbohydrate, made up of D-mannose, D-galactose and 2-acetamido-2-deoxy-D-glucose units, and is readily prepared, and so forms an excellent model substance for investigation of the mucoproteins in general. Ovalbumin, although containing less than 5% of carbohydrate, composed of D-mannose and 2-acetamido-2-deoxy-D-glucose (Neuberger, 1938), may be obtained in a greater state of purity than ovomucoid. The nature of the carbohydrate peptide bond remained obscure until recent examination of the glycopeptides produced on enzymic hydrolysis of ovalbumin suggested that aspartic acid is directly linked to the carbohydrate through one of its carboxyl groups (Cunningham, Neunke & Neunke, 1957; Jevons, 1958; Johanson, Marshall & Neuberger, 1958). This paper describes some attempts to enrich the carbohydrate component of these mucoproteins by treatment with alkali and by other methods, but in no case was the pure carbohydrate isolated. Application of methods commonly employed in polysaccharide chemistry to the structural studies of mucoproteins has been studied with reference to ovomucoid and ovalbumin. As a result some new structural features of these mucoproteins have been revealed.

EXPERIMENTAL

Preparation of the mucoproteins

Ovomucoid. Powdered egg albumin (British Drug Houses Ltd.; 750 g.) was mechanically stirred for 6 hr. with water (25 l.). The addition of 10% (w/v) trichloroacetic acid (3 l.) produced a copious white precipitate which was removed on the centrifuge (approx. 600 *g*/30 min.) and the supernatant liquid was dialysed for several days. The clear-yellow liquid, obtained after filtration, was concentrated at a temperature below 35° to a smaller volume (3·5 l.). A precipitate of crude ovomucoid was obtained by the addition of methylated spirits (6·6 l.) at −10°. Several reprecipitations from aqueous solution with 2 vol. of ethanol at −10° afforded, on drying under reduced pressure, a white powder (yield, 20 g.); $[\alpha]_D^{25}$ $-62\pm3°$ in water (*c*, 1·02) [Found, sulphated ash, 2·8; N (Dumas), 13·4; P, nil; acetyl, 3·8; OMe, 0·2%].

Ovalbumin. Crystalline ovalbumin was prepared from egg albumin (British Drug Houses Ltd.) by Kekwick & Cannan's (1936) procedure and recrystallized twice. Dialysis of an aqueous solution of the crystals to remove sodium sulphate, followed by freeze-drying, yielded a white, easily water-soluble solid [Found: sulphated ash, 3·7; N (Dumas), 14·9%].

Analysis of the mucoproteins

Electrophoresis. The mucoproteins were examined in the Tiselius apparatus (Hilger and Watts Ltd.). Solutions (1%, w/v) in the appropriate buffer solution were equilibrated by dialysis against a large volume (> 2 l.) of the same buffer at 4° for 24 hr. Electrophoresis was then done at 4° with a current of 10–15 mA in one of the following buffers: phosphate, pH 6·9, *I* 0·13; acetate, pH 4·4, *I* 0·01. Electrophoresis of ovomucoid was also carried out in 0·2M-KH_2PO_4–0·1M-NaOH buffer, pH 6·8, in the presence of guanidine hydrochloride (50 g./l.) and sodium chloride (15 g./l.).

Paper electrophoresis was performed on Whatman no. 1 paper soaked in 0·025M-sodium tetraborate, pH 9, for 3 hr. at 5 mA. The mucoproteins were detected by the bromophenol blue reagent (Durrum, 1950).

Absorption spectra. The ultraviolet absorption of ovomucoid was measured at 0·1% concentration in water, with a Unicam SP. 500 spectrophotometer and a 1 cm. cell.

Paper chromatography. Paper chromatography was carried out by the descending method on Whatman no. 1 filter paper with the following solvent systems: (1) butan-1-ol–ethanol–water (40:11:19, by vol.); (2) butanol–pyridine–water (10:3:3, by vol.); (3) ethyl acetate–acetic acid–water (9:2:2, by vol.); (4) benzene–ethanol–water (169:47:15, by vol; top layer); (5) ethyl acetate–acetic acid–formic acid–water (18:3:1:4, by vol.). Aldoses and methylated aldoses were detected by *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950), lactones by the method of Abdel-Akher & Smith (1951), 2-amino-2-deoxyhexoses by spray reagents based on the Elson–Morgan test (Partridge, 1948) and polyols with the periodate–*p*-anisidine spray (Bragg & Hough, 1958).

Characterization of the monosaccharide components

Ovomucoid. Acid hydrolysis with 2N-H_2SO_4 for 48 hr. at 100° and subsequent paper-chromatographic examination indicated the presence, in unequal amounts, of galactose,

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mannose and 2-amino-2-deoxyglucose, together with amino acids. The neutralized hydrolysate ($BaCO_3$) was freed of amino acids and the amino sugar by passage down a cation-exchange column [Amberlite IR-120 (H)]. After concentration of the eluate D-mannose and D-galactose were separated by paper chromatography (solvent 3) and then characterized as the phenylhydrazone (m.p. and mixed m.p. 191–193°) and the 1-methyl-1-phenylhydrazone (m.p. and mixed m.p. 181°) derivatives respectively (Hirst, Jones & Woods, 1947).

A similar hydrolysate of ovomucoid (1 g.) was passed through a squat charcoal column (Andrews, Hough & Powell, 1956) and the column was thoroughly washed with water. The effluent and combined washings were acidified with concentrated HCl (1 ml.) and concentrated to yield 2-amino-2-deoxy-D-glucose hydrochloride, which after recrystallization from aq. ethanol had $[\alpha]_D + 70°$ in water (12 hr.; *c*, 0·43). The absence of other amino sugars in ovomucoid was shown by ninhydrin oxidation (Ruelius & Girard, 1954) of the hydrolysate, when only arabinose was found.

Ovalbumin. The mucoprotein (100 g.) was hydrolysed in 2N-H_2SO_4 (500 ml.) at 95–100° for 48 hr. After neutralization ($BaCO_3$), the solution was adjusted to pH 4 and passed through a cation-exchange column [Amberlite IR-120 (H)]. Concentration of the effluent gave a syrup which on heating with an ethanolic solution of phenylhydrazine gave D-mannose phenylhydrazone (0·5 g.) with m.p. and mixed m.p. 190–193° (Hirst *et al.* 1947). The phenylhydrazone (0·3 g.) was heated under reflux with an excess of benzaldehyde in ethanol–water (3:1, v/v) until all the crystals had dissolved. The reaction mixture was exhaustively extracted with ether to remove excess of benzaldehyde and its phenylhydrazone derivative. The aqueous solution was concentrated to a syrup (160 mg.), which had $[\alpha]_D^{25} + 8°$ in water (*c*, 3·2) and was indistinguishable from mannose on paper chromatograms.

Ninhydrin oxidation of a small quantity of the abovementioned ovalbumin hydrolysate, followed by paper chromatography, revealed arabinose as the only pentose component, in agreement with the occurrence of 2-amino-2-deoxy-D-glucose in this mucoprotein (Neuberger, 1938).

Quantitative analysis for neutral monosaccharides

Resin-hydrolysis method. Cation-exchange resin (Amberlite IR-120: 10 g.) was shaken for 30 min. with 10% HCl (40 ml.) and washed with water until the washings were free from Cl^- ions. The resin was dried by washing successively with ethanol and ether. The mucoprotein (approx. 50 mg.) was accurately weighed into a small test tube (9 cm. × 1 cm.) and water (2·5 ml.) and prepared ion-exchange resin (1 g.) were added. The tube was closed with a rubber cap and heated at 95–100° for 24–30 hr. The pressure that developed during the initial stages of heating was released by puncturing the cap with a hypodermic needle. After hydrolysis a known weight (5–10 mg.) of either ribose or xylose was added to the cooled hydrolysate, the resin was filtered off and the monosaccharides were separated on paper chromatograms (Flood, Hirst & Jones, 1948) for subsequent determination by Jones & Pridham's (1954) benzidine method.

Acid-hydrolysis method. The mucoprotein (approx. 50 mg., accurately weighed) was hydrolysed with 2N-H_2SO_4 (5 ml.) in a sealed tube at 95–100° for 18 hr. A weighed quantity of ribose or xylose was then added and the solution was neutralized ($BaCO_3$). After filtration, the hydrolysate was slowly passed down a cation-exchange column [Amberlite IR-120 (H); 10 cm. × 1 cm.], which was then washed with water until the effluent gave a negative Molisch test. The total effluent was concentrated under reduced pressure, the monosaccharide components were separated by paper chromatography and then determined as described above. Quantitative estimates of the monosaccharide components of ovalbumin and ovomucoid are shown in Table 1.

Quantitative estimation for 2-amino-2-deoxyglucose. The mucoprotein (approx. 50 mg., accurately weighed) was hydrolysed with 2N-H_2SO_4 (2·5 ml.) at 95–100° in a sealed glass tube. Samples were removed at various times up to 48 hr. and, when cool, were neutralized with 2N-NaOH, made up to 100 ml. and amino sugar was determined by the method of either Belcher, Nutten & Sambrook (1954) or Tracey (1952). Results are shown in Table 1.

Table 1. *Carbohydrate composition of ovomucoid and of ovalbumin*

Mannose and galactose were determined by the methods described in the text, after hydrolysis of the mucoproteins with cation-exchange resin, followed by separation of the sugars by paper chromatography. The hydrolysis of each sugar was followed until a constant value was obtained.

Mucoprotein	Amount found (% of hexose or amino hexose)		Molecular ratio
Ovomucoid	D-Mannose	6·3 ± 0·3	3·7
	D-Galactose	1·7 ± 0·3	1
	2-Amino-2-deoxy-D-glucose (method of Belcher, Nutten & Sambrook, 1954)	12·3 ± 0·5	7·2
	2-Amino-2-deoxy-D-glucose (method of Tracey, 1952)	12·8 ± 1·7	7·4
Ovalbumin	Mannose	2·75 ± 0·15	2
	2-Amino-2-deoxy-D-glucose (method of Belcher *et al.* 1954)	1·34 ± 0·04	1

Structural investigations of the mucoproteins

Acid-hydrolysis studies on ovomucoid. By following the rate at which the components of a polysaccharide appear during acidic hydrolysis useful structural data can often be obtained about the monosaccharides which occupy outer and inner positions in the macromolecule, as, for example, with the blood-group polysaccharides (Kabat & Leskowitz, 1954).

A solution of ovomucoid (57 mg.) in water (5 ml.) was heated (95–100°) with ion-exchange resin [Amberlite IR-120 (H); 1 g.] in a sealed, all-glass polarimeter tube (0·535 dm. in length) and the change in optical rotation was followed to a constant value (Table 2). In a similar experiment the liberated D-mannose and D-galactose were determined quantitatively, as described above, by separation on paper chromatograms followed by estimation of the monosaccharides by the benzidine method (Jones & Pridham, 1954) (see Table 2).

In another experiment ovomucoid (60 mg.) was heated at 100° with 2N-H_2SO_4 (2·5 ml.) and at various intervals of time the appearance of reducing sugar in the hydrolysate was measured (Somogyi, 1945) and the corresponding liberation of 2-amino-2-deoxy-D-glucose determined by the method of Belcher *et al.* (1954) (Table 3).

Table 2. *Rate of appearance of* D-*mannose and* D-*galactose, and the change in optical rotation, when ovomucoid was heated in aqueous solution with cation-exchange resin*

The sugars were determined colorimetrically (Jones & Pridham, 1954) after quantitative separation on paper chromatograms.

Duration of heating (hr.)	Mannose (%)	Galactose (%)	Optical rotation (α°)
0	—	—	−0·29
1	2·1	1·6	−0·29
3	2·6	1·6	—
6	2·3	1·6	—
10·5	—	—	−0·04
12	5·0	1·6	—
24	5·2	1·6	+0·02
30	—	—	+0·03
48	6·0	1·6	+0·03
70	—	—	+0·04

Table 3. *Change in reducing power and liberation of 2-amino-2-deoxy-*D-*glucose during acid hydrolysis of ovomucoid*

Ovomucoid was hydrolysed for various times at 100° with 2N-H_2SO_4 and the liberated 2-amino-2-deoxyglucose (Belcher *et al.* 1954) and increase in reducing power (Somogyi, 1945) were determined.

Duration of hydrolysis (hr.)	Reducing power (% of hexose)	2-Amino-2-deoxy-D-glucose (%)
1	17·4	8·6
3	22·2	11·4
6	21·2	12·1
12	23·2	12·8
24	21·0	12·0
48	—	10·1

Since monosaccharides which are linked in the furanose form are readily hydrolysed by 0·01N-H_2SO_4 at 80°, whereas pyranosides are not so susceptible (Andrews *et al.* 1956), the hydrolysis products of ovomucoid at pH 2·0 and 80° were investigated on paper chromatograms, but no monosaccharides were rapidly liberated under these conditions.

The isolation and characterization of disaccharides and other oligosaccharides from partial hydrolysates of mucoprotein can afford conclusive evidence of the interglycosidic linkages. This type of linkage analysis was applied to ovomucoid. Difficulties due to the presence of large quantities of peptides and amino acids, and to low yields of oligosaccharides, were encountered. Ovomucoid (6 g.) was heated at 95–100° with N-H_2SO_4 (200 ml.) for 6 hr.: previous examination of a similar hydrolysate had suggested that this time gave the optimum yield of oligosaccharides. The neutralized [$Ba(OH_2)$] liquid was filtered, concentrated to about 100 ml. and separated by adsorption on to a squat charcoal column followed by fractional elution with ethanol–water mixtures of increasing ethanol content (Andrews *et al.* 1956).

An amorphous disaccharide (11·8 mg.; R_{Gal} 0·39, solvent 5) was obtained on elution with aq. 5·0–7·5% (v/v) ethanol. It had $[\alpha]_D + 16°$ in water (*c*, 1·18), contained traces of a contaminating amino acid and gave only mannose on acid hydrolysis. Oxidation of this crude disaccharide with sodium metaperiodate in unbuffered solution and at pH 8·0 (Hough & Perry, 1956) gave 0·57 mole (8 hr.) and 0·9 mole (5 hr.) of formaldehyde respectively.

Partial hydrolysis of ovalbumin with acid. A suspension of denatured ovalbumin (80 g.) in water (2 l.) was taken to pH 1 by addition of trichloroacetic acid and heated at 80° for 12 hr. with continuous stirring; the pH had then risen to 7. After filtration, the clear solution was evaporated to a solid, which was extracted with 80% (v/v) methanol–water. The extract was examined on paper chromatograms but no free sugars were detected. The residue was soluble in water and had $[\alpha]_D - 26°$ (*c*, 1·04) (Found: sulphated ash, 50·2; N, 7·1%). Paper ionophoresis in 0·05M-sodium tetraborate for 13 hr. at 3 mA showed the presence of a major component which had moved 6·0 cm. towards the anode and gave a positive reaction with the periodate spray reagent; on paper chromatograms it remained on the starting line. Acid hydrolysis with N-H_2SO_4 followed by paper chromatography revealed the presence of mannose (4·7%), 2-amino-2-deoxy-D-glucose (5·3%) and amino acids, including glutamic acid and aspartic acid, glycine and alanine. An *N*-terminal end-group analysis (Partridge & Davis, 1955; Blackburn & Lowther, 1951) showed only *N*-dinitrophenylalanine on paper chromatograms.

Periodate oxidation of the mucoproteins

Valuable evidence for carbohydrate structures has been obtained by periodate oxidation and exhaustive methylation, and in the vast majority of cases these methods have provided mutually confirmatory results. The periodate method has the advantage that it can be applied directly and requires very mild conditions. However, the presence of protein can interfere with the determination of the formic acid produced from the carbohydrate (Anderson, Greenwood & Hirst, 1955) and the uptake of periodate (Bragg & Hough, 1958). Consequently the main use of this method when applied to mucoproteins is to determine quantitatively which monosaccharide units are attacked by periodate.

Table 4. *Periodate oxidation of ovomucoid at different* pH *values*

Ovomucoid (100 mg.) was dissolved in water or buffer solution (100 ml.). Portions (5 ml.) were removed at various times and the consumption of periodate (Neumüller & Vasseur, 1953) and production of acid (Anderson, Greenwood & Hirst, 1955) were determined.

Time of reaction (hr.)	Consumption of periodate (g. of ovomucoid/mole of $NaIO_4$)			Acid production (g. of ovomucoid/equiv. of acid)	
	Unbuffered	pH 6·96	pH 3·5	Indicator	Potentiometer
0·5	—	1580	1359	—	—
1	1033	1295	1045	40 300	41 700
1·5	—	1154	—	—	—
2	1009	1095	847	20 750	41 700
3	916	1067	847	—	—
4	—	—	788	10 075	—
5	790	—	—	—	—
6	—	1040	—	—	—
7	720	—	663	8 060	41 700
9	630	710	—	—	—
24	438	418	494	4 030	10 410
48	353	—	400	—	—

Table 5. *Composition of the products after periodate oxidation of ovomucoid and ovalbumin*

Ovomucoid (or ovalbumin) was oxidized by periodate for 2 hr. in the dark. Residual periodate was then destroyed with excess of ethylene glycol. Ions were removed by dialysis (2 days) and the product was hydrolysed with 2N-H_2SO_4. The liberated monosaccharides were determined by the methods described in the text.

Aldose	Found (%)
Ovomucoid	
D-Mannose	3·2
D-Galactose	0
2-Amino-2-deoxy-D-glucose	5·9
Ovalbumin	
D-Mannose	0·85
2-Amino-2-deoxy-D-glucose	0·92

Table 6. *Consumption of periodate by ovalbumin at* pH 3·6

Ovalbumin (430 mg.) was dissolved in acetate buffer (pH 3·6; 50 ml.), 0·3M-sodium metaperiodate (10 ml.) was added and the solution made up to 100 ml. with water. Portions (5 ml.) were removed for the determination (Neumüller & Vasseur, 1953) of periodate consumption.

Time of reaction (hr.)	Consumption of periodate (g. of ovalbumin/mole of $NaIO_4$)
0·5	1270
1	1162
2	1195
3	1180
6	985
17	686
42	489

The oxidations were investigated at both pH 3·6 and pH 6·96 to diagnose any over-oxidation of the carbohydrate, since this can have structural significance. Intermediary formyl esters formed by oxidative cleavage of the $C_{(1)}$–$C_{(2)}$ bond of the reducing end group are relatively stable at pH 3·6 but labile at pH 6·96, and consequently lead to further oxidation at the latter pH through the oxidation of malondialdehyde and other derivatives (Hough & Perry, 1956; Hough, Perry & Woods, 1957; Hough, Taylor, Thomas & Woods, 1958; Cantley, Hough & Pittet, 1959). The course of the periodate reaction was also followed in unbuffered solution to determine the acid liberated in relation to the amount of periodate that had reacted.

Ovomucoid. Ovomucoid (100·9 mg.) was dissolved in water (90 ml.) and 0·3M-sodium metaperiodate solution (5 ml.) was added. The solution was made up to 100 ml. and transferred to a brown bottle (Potter & Hassid, 1948). A blank was prepared by omission of the ovomucoid. At various times, portions (10 ml.) were transferred into a mixture of 0·2M-Na_2HPO_4–0·13M-KH_2PO_4 buffer (pH 7·0; 20 ml.) and 20% KI (3 ml.) (Neumüller & Vasseur, 1953). The liberated iodine was titrated with 0·01N-$Na_2S_2O_3$ with 1% starch as indicator and from this figure the periodate uptake was determined.

Similar determinations at different pH values were carried out by dissolving the mucoprotein in sodium phosphate buffer (pH 6·96; 25 ml.) and acetate buffer (pH 3·5; 25 ml.) respectively and then proceeding as described above by dilution to 100 ml. after addition of 0·3M-sodium metaperiodate solution (5 ml.). The results are shown in Table 4. The release of formic acid and formaldehyde was then investigated; ovomucoid (0·201 g.) was dissolved in water (100 ml.) containing 0·3M-sodium metaperiodate (5 ml.) and transferred to a brown bottle. A blank was prepared by omission of the mucoprotein. Samples (10 ml.) were mixed with ethylene glycol (5 ml.) and, after keeping for 10 min., the acid was titrated with 0·01N-NaOH, either potentiometrically to pH 6·25 (Anderson *et al.* 1955) or with methyl red–methylene blue indicator (0·1%; 4:1, v/v) (Table 4). Tests for formaldehyde, by using Schryver's (1910) reagents on the final reaction solutions, gave negative results.

To determine which monosaccharides had been attacked by periodate, ovomucoid was allowed to react for 2 hr. with periodate, under the unbuffered conditions described above, and then the excess of periodate was destroyed by the addition of an excess of ethylene glycol. After dialysis for 2 days to remove sodium iodate, formaldehyde, formic acid and excess of glycol, the oxidized mucoprotein was isolated by evaporation under reduced pressure and hydrolysed with acid, and the liberated monosaccharides were determined as

described above for the mucoprotein. The results are in Table 5.

Ovalbumin. The methods employed were similar to those used for ovomucoid. No ammonia and a negligible quantity of acid was produced during the oxidation under unbuffered conditions. For quantitative results see Tables 5 and 6.

Exhaustive methylation of the mucoproteins and examination of the components of the methylated products

Whilst the methylation technique has been widely applied in the structural determination of polysaccharides and the mucopolysaccharides, the only mucoproteins so investigated have been ovomucoid (Stacey & Woolley, 1940, 1942) and frog-spawn mucin (Folkes, Grant & Jones, 1950). In both cases extensive degradation was encountered during the reaction with dimethyl sulphate and strong aqueous alkali (as a consequence of the presence of protein moiety and the amino sugar). As a preliminary to this investigation alternative techniques were examined. However, sodium in liquid ammonia, followed by treatment with methyl iodide (Freudenberg, Plankenhorn & Boppel, 1938; Hodge, Karjala & Hilbert, 1951), and another method involving treatment with thallous hydroxide followed by methyl iodide (Campbell, Hirst & Jones, 1948), were both unsuccessful. Consequently recourse was had to a modification of the dimethyl sulphate method described by Stacey & Woolley (1940, 1942).

Methylation of ovomucoid. A mixture of ovomucoid (25 g.), water (100 ml.), carbon tetrachloride (300 ml.) and dimethyl sulphate (100 ml.) was stirred whilst 35% (w/v) sodium hydroxide solution (200 ml.) was added dropwise over 2 hr. After another hour, dimethyl sulphate (100 ml.) and 35% (w/v) sodium hydroxide solution (200 ml.) were added simultaneously and dropwise over a period of 2 hr. The reaction mixture was stirred for a further 24 hr., when the insoluble material (A) was filtered off, the filtrate was neutralized with acetic acid, concentrated to dryness and the residue was exhaustively extracted with methanol. After evaporation of the methanol, the residue was dissolved in aq. 50% (v/v) acetone (400 ml.), mixed with material A and methylated twice as before. The reaction mixture was neutralized with acetic acid, evaporated to dryness and continuously extracted with acetone for 24 hr. The acetone extract was concentrated, the residue was extracted with chloroform and the soluble material isolated as a thick syrup by evaporation of the solvent. The product (4·6 g.) had $[\alpha]_D^{25} - 8\cdot3^\circ$ in water (*c*, 1·21) (Found: sulphated ash, 8·4; N, 6·0; OMe, 19·3%).

This methylated product was treated with acetic anhydride for 15 hr. to acetylate any free amino groups, and then, after removal of acetic anhydride by distillation, was heated under reflux with methyl iodide in the presence of silver oxide (Bray, Henry & Stacey, 1946). Finally, the reaction mixture was filtered, H_2S was passed through the solution to remove Ag^+ ions, the mixture was filtered again and the filtrate evaporated to dryness. The resultant syrup (2·9 g.) had $[\alpha]_D^{25}$ 0° in acetone (*c*, 0·78) (Found: sulphated ash, 10·8; N, 5·2; OMe, 7·9%).

Methylation of ovalbumin. Denatured ovalbumin (300 g.) was methylated by repeated treatments with dimethyl sulphate and 35% (w/v) NaOH solution, in the same manner as described for ovomucoid, to give an acetone-soluble product (30 g.) (Found: sulphated ash, 38·2; N, 3·7; OMe, 11%). After *N*-acetylation with acetic anhydride and subsequent treatment with silver oxide and methyl iodide, an aqueous solution of the product (21 g.) was deionized (Bio-Deminrolit) and evaporated to give a thick syrup (6·5 g.) which had $[\alpha]_D^{25} - 14^\circ$ in water (*c*, 0·98) (Found: sulphated ash, 3·8; N, 10·3; OMe, 12·5%).

Examination of the methylated sugars produced on hydrolysis of the methylated ovomucoid fraction. A solution of the methylated ovomucoid fraction (2·8 g.) in methanolic 4% (w/v) HCl was heated under reflux for 5 days. The solution was neutralized (Ag_2CO_3) and Ag^+ ions were precipitated

Table 7. *Fractionation of hydrolysate of methylated ovomucoid*

The hydrolysate of methylated ovomucoid (2·8 g.) was adsorbed on a charcoal column. The methyl sugars were eluted by fractional elution with ethanol–water mixtures.

Concn. of ethanol (%)*	Wt. (mg.)	$[\alpha]_D$ (water)	Solvent I R_F	Solvent I R_G	Comments	Methylated sugars present
Water	360	—	—	—	Amino acids present	—
2·5	248	—	—	—	Amino acids present	—
5	170	—	0·54 0·36	0·64 0·54	Contained amino acids	Di-*O*-methylmannose; mono-*O*-methylmannose
10	176	—	0·54 0·36	0·64 0·54	Contained amino acids	Di-*O*-methylmannose; mono-*O*-methylmannose
15	109	+32°	0·63 0·57	0·76 0·69	Faster sugar predominates	Tri-*O*-methylhexoses
20	76	+43°	0·70	0·84	80% of faster sugar	2:3:4:6-Tetra-*O*-methyl-D-galactose
			0·61	0·74	—	Tri-*O*-methylhexose
25	98	+36°	0·81	0·97	Slower sugar predominated (3:1)	2:3:4:6-Tetra-*O*-methyl-D-mannose
			0·74	0·84	—	2:3:4:6-Tetra-*O*-methyl-D-galactose
30	30	—	0·81	0·97	—	2:3:4:6-Tetra-*O*-methyl-D-mannose
40	31	+48°	0·75	0·92	—	—

* Each fraction was eluted with 1 l. of solvent.

with H_2S. After filtration the solution was concentrated to a syrup containing the methyl glycosides, which were hydrolysed for 3 hr. with N-H_2SO_4 at 95–100°. The hydrolysate was neutralized ($BaCO_3$) and the mixture of methylated sugars fractionated by adsorption on to a squat column of acid-washed charcoal (4 cm. × 10 cm.; British Drug Houses Ltd.) followed by fractional elution with increasing concentrations of ethanol in water (Andrews *et al.* 1956). The results are summarized in Table 7. This technique was favoured to partition chromatography on a cellulose column since experience had shown that the charcoal column gave fractions which were less contaminated by amino acids. Each fraction (1 l.) from the column was deionized (Bio-Deminrolit) before evaporation, since they were slightly acidic, and then was examined on paper chromatograms. The water washings from the column were carefully examined for mannose but none was found.

Paper chromatography of the fractions eluted with aq. 5 and 10% (w/v) ethanol suggested that they contained the same two methylated sugars. The fractions were combined and the two components separated on paper chromatograms (solvent 3). Both methylated sugars yielded mannose on de-*O*-methylation with hydrobromic acid (46%, w/v) at 100° for 6 min. (Hough *et al.* 1950). Their rates of movement on paper chromatograms were typical of a mono-*O*-methylmannose and a di-*O*-methylmannose respectively. An attempt to isolate a phenylosazone derivative of the mono-*O*-methylmannose (19 mg.) was unsuccessful. The faster-moving di-*O*-methyl component (21 mg.) had $[\alpha]^{25}_D + 13°$ in water (*c*, 0·35). This reducing sugar was oxidized with sodium metaperiodate for 5 hr., as described by Hough, Powell & Woods (1956), and the reaction mixture was examined for formaldehyde but none was found. Similarly, no formaldehyde was detected after reduction of the di-*O*-methylmannose with sodium borohydride to a di-*O*-methylmannitol derivative followed by periodate oxidation (Hough *et al.* 1957). This evidence suggests that one of the components from hydrolysed methylated ovomucoid is 2:6-di-*O*-methyl-D-mannose.

The fraction eluted with aq. 15% (v/v) ethanol was obtained as a syrup (109 mg.) (Found: OMe, 40·6; N, 0·5. Calc. for tri-*O*-methylhexose: OMe, 41·8%). Oxidation of this syrup with bromine–water gave a mixture of lactones, as revealed by paper chromatography (solvents 1 and 4), which we failed to characterize.

The fraction eluted with aq. 20% (v/v) ethanol was obtained as a syrup (76 mg.) (Found: OMe, 45·1; N, 0·7. Calc. for tetra-*O*-methylhexose: OMe, 52·5%), which was heated under reflux for 3½ hr. with redistilled aniline (34 mg.) in ethanol (5 ml.). Evaporation of the reaction mixture yielded needle-shaped crystals with $[\alpha]_D - 78° \rightarrow + 35°$ (equil.) in acetone (*c*, 0·4) and m.p. and mixed m.p. with authentic 2:3:4:6-tetra-*O*-methyl-D-galactose anilide, 188° [Found: OMe, 35·9. Calc. for $C_{12}H_{12}ON(OMe)_4$: OMe, 39·9%].

The 25% (v/v) ethanol fraction contained a mixture of tetramethyl sugars (Found: OMe, 48·9; N, 0·8. Calc. for tetramethylhexose: OMe, 52·5%), which were separated by paper chromatography (solvent 1) and converted into 2:3:4:6-tetra-*O*-methyl-D-mannose anilide, m.p. and mixed m.p. 147°, and 2:3:4:6-tetra-*O*-methyl-D-galactose anilide, m.p. and mixed m.p. 188° respectively. Present in the 30% (v/v) ethanol fraction was a tetra-*O*-methyl sugar, which was converted, as described above, into the anilide, which had $[\alpha]_D - 10°$ (equil.) in methanol (*c*, 0·1) and m.p. and mixed m.p. with authentic 2:3:4:6-tetra-*O*-methyl-D-mannose anilide, 147–148° [Found: OMe, 38·3. Calc. for $C_{12}H_{12}ON(OMe)_4$: OMe, 39·9%].

The fraction eluted by aq. 40% (v/v) ethanol consisted largely of methyl glycosides, since hydrolysis with 2N-HCl for 10 hr. at 100° produced a mixture of methyl sugars consisting largely of tetra-*O*-methylmannose, with tetra-*O*-methylgalactose and an unidentified tri-*O*-methylhexose as minor components.

Examination of the methylated sugars produced on hydrolysis of the methylated ovalbumin fraction. The methods employed are the same as those used above for methylated ovomucoid. The methylated ovalbumin (6·5 g.) was heated under reflux in methanolic 4% (w/v) HCl (170 ml.) for 3 days and the liberated methyl glycosides were then hydrolysed for 3 hr. at 95–100° with N-H_2SO_4 (25 ml.). After neutralization ($BaCO_3$), the neutral solution was evaporated to a syrup (4·8 g.), which was extracted by heating under reflux with chloroform and with methanol to enrich the methylated sugars. The combined extracts were evaporated to a syrup (1·43 g.), which was fractionated on a column of acid-washed charcoal (10 cm. × 5 cm.; British Drug Houses Ltd.) by fractional elution with water–ethanol mixtures (Table 8).

All fractions were observed to contain amino acids. Evaporation of the aq. 10% (v/v) ethanol fraction gave a syrup (40 mg.) which had the mobility of a di-*O*-methylmannose on paper chromatograms. No formaldehyde was produced on periodate oxidation of the sugar either before

Table 8. *Fractionation of hydrolysate of methylated ovalbumin*

The hydrolysate was adsorbed on a charcoal column followed by fractional elution with ethanol–water mixtures.

Concn. of ethanol (%)*	Wt. of fraction (mg.)	Solvent I R_F	Solvent I R_G	Methylated sugar present
Water	14	—	—	—
2·5	17	—	—	—
5	50	0·29	—	—
—	—	0·33	—	—
10	40	0·57	0·68	Di-*O*-methylmannose
20	51	0·71	0·81	Tri-*O*-methylmannose
30	72	0·71	0·81	Tri-*O*-methylmannose
40	83	0·82	0·98	2:3:4:6-Tetra-*O*-methyl-D-mannose
50	66	0·82	0·98	2:3:4:6-Tetra-*O*-methyl-D-mannose

* Each fraction was eluted with 1 l. of solvent.

Table 9. *Composition of the fractions formed by the action of barium hydroxide on ovomucoid*

See text for details of preparation of the fractions and method of analysis.

Fraction	$[\alpha]_D$ (water)	N (%)	Sulphated ash (%)	2-Amino-2-deoxy-D-glucose (%)	Mannose (%)	Galactose (%)	Ratio of mannose:amino sugar
I*	13±2°	4·2	0·2	21·2	+	+	—
I†	—	—	—	9·2	17	0	—
IA	—	—	1·9	17	19	10	1·1
IB	—	—	0·8	22·5	28	38	1·25
II	16±4°	—	8·8	12	12	5	1·0
III	10°	—	0·7	0·9	1·2	1·8	1·3
IV	—	—	3·1	6·0	7·0	5·0	1·2

* Acetyl absent. † After oxidation with periodate.

or after reduction with sodium borohydride (Hough *et al.* 1957). This evidence suggested that the sugar was 2:6-di-*O*-methyl-D-mannose.

The syrup (79 mg.) obtained by evaporation of the aq. 40 and 50% (v/v) ethanol fractions was indistinguishable from 2:3:4:6-tetra-*O*-methylmannose on paper chromatograms. It had $[\alpha]_D^{25}$ +25·5° in methanol (*c*, 0·47) and was converted into the crystalline anilide, which had m.p. and mixed m.p. 145–147° with authentic 2:3:4:6-tetra-*O*-methyl-D-mannose anilide.

Attempts to enrich the carbohydrate moiety of the mucoproteins

Several authors have claimed to have isolated carbohydrate fractions from mucoproteins by the action of aq. $Ba(OH)_2$ (Levene & Mori, 1929; Fränkel & Jellinek, 1927; Neuberger, 1938; Stacey & Woolley, 1940). It was claimed that the protein was degraded by alkaline hydrolysis, but that the carbohydrate was, in the main, stable to alkali. However, useful structural information has been obtained by studying the action of aqueous alkali on polysaccharides (Kenner, 1955), since 1 → 3-linked and 1 → 4-linked hexose polymers undergo stepwise degradation from the reducing end group along the polysaccharide chain, with the formation, and peeling off, of a saccharinic acid unit from each hexose unit, whereas 1 → 2 or 1 → 6 linkages impede this degradation process. The action of aq. $Ba(OH)_2$ on ovomucoid and ovalbumin was studied further to determine if there had been any loss of carbohydrate by this chain-degrading process.

Ovomucoid. A mixture of ovomucoid (10 g.), 10% (w/v) $Ba(OH)_2$ soln. (150 ml.), ethanol (15 ml.) and octan-2-ol (10 ml.) was heated under reflux for 17 hr. in a stream of nitrogen. After neutralization with H_2SO_4, the filtered solution was evaporated to approx. 40 ml. and poured into an excess of methylated spirits. The light-brown precipitate was centrifuged off, dissolved in water, reprecipitated with ethanol and dried. The supernatant ethanol was found to contain only amino acids, and no carbohydrates could be detected even after hydrolysis (2N-H_2SO_4). The precipitated material was non-reducing to Fehling solution and, on hydrolysis with N-H_2SO_4, gave amino acids, mannose, galactose and 2-amino-2-deoxyglucose. In a quantitative experiment there were found 9·5% of mannose, 7·6% of galactose and 7% of 2-amino-2-deoxyglucose.

In a large-scale experiment ovomucoid (70 g.) was degraded as described above and the cooled solution acidified with H_2SO_4 and then neutralized ($BaCO_3$). After filtration, the solution was concentrated almost to dryness and the thick syrup poured into ethanol (1 l.). The precipitated solid was isolated by centrifuging, dissolved in water (200 ml.) and ethanol (200 ml.) was added. The precipitate which formed was removed by centrifuging. The supernatant liquid was poured into ethanol (1·6 l.) to yield a light-brown solid (9 g.) (Found: sulphated ash, 32·1; N, 7·0%). Hydrolysis of a small portion revealed galactose, mannose, 2-amino-2-deoxyglucose and small amounts of amino acids. A solution of the solid was deionized by passage through a column of ion-exchange resin [Amberlite IR-120 (H)] to give an acidic solution, which was poured into ethanol (10 vol.) and the white precipitate was dried (fraction I; 1·14 g.). The product was non-reducing to Fehling solution. The supernatant solution was concentrated under reduced pressure at room temperature to about 150 ml. and then neutralized by stirring with $BaCO_3$. After filtration, the solution was poured into ethanol (1 l.) to yield a light-brown precipitate (fraction II; yield, 2 g.).

The cation-exchange resin used in the procedure above to deionize the original solution was shaken for several hours with aq. NH_3 soln. (sp.gr. 0·88; 100 ml.). The resin was filtered off and the filtrate concentrated under reduced pressure to a small volume. Addition of excess of ethanol yielded a precipitate (fraction III; yield 1·61 g.). Fraction IV (0·36 g.) was obtained by repeated treatment of the ion-exchange resin with aq. NH_3 soln. The compositions of the fractions are shown in Table 9.

Properties of fraction I. Paper chromatography revealed a single periodate-reacting spot on the starting line (solvent 3). Paper ionophoresis in 25 mM-sodium tetraborate at 3 mA indicated that the material was homogeneous and moved round the cathode (5·5 cm./3 hr.). After hydrolysis, 2-amino-2-deoxyglucose, galactose and mannose were detected. An equivalent weight of 454 was found by titration with 0·01N-NaOH with phenolphthalein as indicator.

The consumption of periodate, under unbuffered conditions and at pH 3·5, is shown in Table 10. During this oxidation no formaldehyde was detected with the Hough *et al.* (1956) technique, but 1 mole of NH_3 was liberated from 770 g. of fraction I. The composition of fraction I before and after periodate oxidation is shown in Table 9.

An attempt was made partially to hydrolyse fraction I under the same conditions as Levene (1941) had used for the preparation of a disaccharide from a similar fraction. Fraction I (0·99 g.) was kept in 10N-HCl (10 ml.) for 45 hr. at 25°. Excess of HCl was removed by allowing the solution to evaporate in a vacuum desiccator over P_4O_{10} and KOH pellets. The residue was extracted successively with hot

Table 10. *Consumption of periodate by fraction I*

Consumption of periodate and the acid produced during oxidation were determined by the methods of Neumüller & Vasseur (1953) and Anderson *et al.* (1955) respectively.

Time of oxidation (hr.)	Consumption of periodate (g. of fraction I/mole of $NaIO_4$)		Acid production (g. of fraction I/ mole of acid)
	pH 3·5	Unbuffered	
0·5	293	339	0
1	304	315	1210
3	226	221	808
6	211	205	808
24	163	163	808

methanol and ethanol, and the extracts were evaporated to a solid (0·54 g.), which was dissolved in water (20 ml.). The precipitates obtained by the addition of 4 and 9 vol. of ethanol to this solution were collected (fractions IA and IB respectively; Table 7). The supernatant liquid was concentrated to a syrup (350 mg.), which was shown by paper chromatography to contain amino acids, mono- and oligosaccharides. Fractionation of the mixture on a squat column of acid-washed charcoal (British Drug Houses Ltd.; Andrews *et al.* 1956) yielded, on elution with aq. 5% (v/v) ethanol, a syrup (17 mg.), which gave on paper chromatograms only one spot (R_{Gal} 0·40; solvent 5). This material showed a pink colour with the Elson–Morgan spray reagent (Partridge, 1948) and a purple colour with ninhydrin. Hydrolysis, followed by paper chromatography, revealed the presence of equal amounts of mannose and 2-amino-2-deoxyglucose, but after reduction of this component with potassium borohydride followed by acid hydrolysis only mannose could be detected with *p*-anisidine. The periodate–*p*-anisidine spray revealed an additional polyol (R_{Gal} 1·21; solvent 2), corresponding to 2-amino-2-deoxyglucitol.

Ovalbumin. When similar procedures were applied to ovalbumin no enrichment of the carbohydrate was found. Thus the fraction obtained after treatment with baryta was found to contain 3·7% of mannose and 1·8% of 2-amino-2-deoxyglucose.

Other attempts to isolate the carbohydrate moiety. Holdsworth (1952) cleaved the cell-wall mucoprotein of *Corynebacterium diphtheriae* into carbohydrate and protein by reaction with boiling aqueous picric acid. Investigation revealed that ovomucoid was stable to this treatment.

It was thought that the glycopeptide linkages might be acid-labile. Hence, ovomucoid heated at 80° for 16 hr. at pH 2·0 (maintained by addition of 0·01 N-H_2SO_4) yielded an electrophoretically homogeneous, dialysable fragment which contained mannose, galactose, glucosamine and amino acids; little enrichment of the carbohydrate was evident.

Carbohydrate liberation from ovomucoid did not occur with the following enzymes: pepsin, papain, hyaluronidase (Schering, Berlin), pectinase (Carter and Co. Ltd., Coleford), 19 A.P. (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), bacterase (Norman Evans and Rais, Manchester) and liver enzymes prepared from *Helix pomatia* (Howe & Kabat, 1953).

RESULTS AND DISCUSSION

Ovomucoid

Physical properties. Ovomucoid was prepared by ethanol fractionation of the supernatant liquid resulting from the precipitation of the other egg-white proteins with trichloroacetic acid. This method was preferred to that of Neuberger & Yuill (1940), in which heat coagulation was used to precipitate the other proteins, since, during heating, ovomucoid may be modified with change in the isoelectric point, sedimentation constant and viscosity (Fredericq & Deutsch, 1949) and with partial loss of anti-tryptic activity (Lineweaver & Murray, 1947). Although electrophoresis revealed a single peak at pH 6·9 and *I* 0·13, spreading of the peak suggested heterogeneity. At low ionic strengths (0·01) and at pH 4·4 at least two peaks were obtained during electrophoresis. Fredericq & Deutsch (1949) and Bier, Terminiello, Duke, Gibbs & Nord (1953) have made similar observations but the fractionation reported by the latter group of authors was probably incomplete since anti-tryptic activity was found in all of their fractions. Evidence of heterogeneity was also obtained by Jutisz, Kaminski & Legault-Démare (1957), who resolved ovomucoid into two components by zone electrophoresis on a column of cellulose; only one of the components possessed anti-tryptic activity. Rhodes, Azari & Feeney (1958) have recently described a promising method for the fractionation and purification of egg-white proteins by ion-exchange chromatography on carboxymethylcellulose. Their ovomucoid fraction had a specific activity similar to that prepared by the trichloroacetic acid method. Ovomucoid showed a weak absorption at 277·5 mμ, no other absorption peak being detected, thus showing the absence of flavoprotein. No further purification of the ovomucoid preparation was attempted in view of difficulties involved in preparing quantities sufficiently large for structural investigations. Ovomucoid appears to be homogeneous by ultracentrifuge-sedimentation analysis (Fredericq & Deutsch, 1949), and ionophoresis of ovomucoid on paper impregnated with borate buffer at pH 9 gave a single band.

Identification of the monosaccharide components. Early workers (Fränkel & Jellinek, 1927; Levene & Mori, 1929; Sørensen & Haugaard, 1933) found D-mannose and 2-amino-2-deoxyglucose. D-Galactose was first characterized by isolation of the tetramethyl ether from a methylated fraction of ovomucoid (Stacey & Woolley, 1942). We were able to characterize D-galactose and D-mannose as the 1-methyl-1-phenylhydrazone and phenylhydrazone derivatives respectively from an acid hydrolysate of ovomucoid. 2-Amino-2-deoxy-D-glucose was

isolated as the hydrochloride from a hydrolysate which had been passed through a charcoal column. The amino sugar is believed to exist as the *N*-acetyl derivative (Stacey & Woolley, 1940). The presence of 2-amino-2-deoxygalactose in ovomucoid (Masamune & Yoshizawa, 1950) was not verified with a sensitive ninhydrin-oxidation method (Ruelius & Girard, 1954).

Quantitative analysis of the carbohydrate. The quantitative analysis of the monosaccharide components of mucoproteins is complicated for various reasons. In general, difficulties arise from the presence of several closely related sugars which can react with amino acids, with loss of carbohydrates, to give products which interfere with the estimations of both amino sugars and other monosaccharides (Immers & Vasseur, 1952; Rondle & Morgan, 1955). It is not surprising that previous investigations of the composition of ovomucoid (Needham, 1927; Karlberg, 1936; Fredericq & Deutsch, 1949; Bier *et al.* 1953; Dixon, 1955; Gottschalk & Ada, 1956) have shown considerable variations. Thus the ratio of 2-amino-2-deoxy-D-glucose:hexose has varied from 1:1 (Dixon, 1955) to 2:1 (Fredericq & Deutsch, 1949).

Cation-exchange resins (H^+ form) have been used with considerable advantage for the hydrolysis of mucoproteins (Glegg & Eidinger, 1954; Dixon, 1955) since sugar–amino acid interactions are minimized by the removal of amino sugar and amino acids on the resin whilst the neutral sugars remain in solution. Resin hydrolysis of ovomucoid was found to be complete in 30 hr. The method appeared to be superior to any other for the quantitative estimation of neutral monosaccharides present in a mucoprotein. 2-Amino-2-deoxy-D-glucose was determined by two independent methods (Belcher *et al.* 1954; Tracey, 1952). With these methods we have found the ratio 2-amino-2-deoxy-D-glucose:D-mannose:D-galactose to be 7·3:3·7:1 (Table 1). The total reducing power (21–23% of hexose; Table 3), as determined by Somogyi's (1945) method, agrees well with the totalled averages (20–21%) of the monosaccharides determined separately.

Rate of hydrolysis of the carbohydrate components. No monosaccharides were liberated on heating an aqueous solution of ovomucoid, adjusted to pH 2·0, at 80°, thus suggesting the absence of furanosyl units.

The liberation of monosaccharides during the stronger acid hydrolysis of ovomucoid was followed since the results could afford useful structural evidence. In the presence of cation-exchange resin (H^+ form), D-galactose was completely liberated within 1 hr., during which time only one-third of the total D-mannose appeared free; the remaining mannose required 48 hr. for complete liberation. The resin-hydrolysis data are thus compatible with the situation in outer positions of the mucoprotein molecule, of all the D-galactopyranosyl units and of about a third of the D-mannopyranosyl units, the remaining D-mannopyranosyl units occupying inner positions.

During acid hydrolysis of 2-acetamido-2-deoxy-D-glucose with 2N-H_2SO_4, the total reducing sugar and amino sugar appeared at similar rates; the hydrolysis was complete in 12 hr. (Table 3). During the acid hydrolysis of galactomannan polysaccharides, which are composed of D-galactopyranosyl end groups in attachment with chains of D-mannopyranosyl units, the galactose also appeared first, followed by mannose (Henderson, Hough & Painter, 1958). Likewise the fucose units present in non-reducing end-group positions in the blood-group mucopolysaccharides A and O were recognized by their preferential liberation during hydrolysis with acid (Kabat & Leskowitz, 1954). De-*N*-acetylation of 2-acetamido-2-deoxy-D-glucopyranosyl units during acidic hydrolysis of the mucoprotein (Foster, Horton & Stacey, 1957) would impede the liberation of these units and also of mannose if it were linked to the reducing group of the amino sugar.

Periodate-oxidation studies. Although a number of mucopolysaccharides have been studied by the periodate-oxidation technique (Jeanloz & Forchielli, 1950, 1951; Aminoff & Morgan, 1951; Holdsworth, 1952), mucoproteins have not been examined in this way. Ovomucoid was oxidized with sodium metaperiodate in unbuffered solution, at pH 6·96 and at pH 3·5, all reactions being carried out in the dark and at room temperature (Table 4). In all three cases there was little difference in the reaction with periodate and consequently it is not complicated by over-oxidation processes involving the progressive removal of units from the reducing end of a chain of monosaccharide units by oxidation through malonaldehyde and formyl ester intermediates (Hough *et al.* 1956, 1957, 1958).

The oxidations occurred in two stages, consisting of an initial rapid uptake of periodate over about 2 hr., followed by a much slower oxidation over about 48 hr. The first stage can probably be accounted for mainly by oxidation of carbohydrate residues, since in general amino acids and peptides are oxidized slowly (Bragg & Hough, 1958). The second stage is undoubtedly due to over-oxidation processes involving oxidation of the carbohydrate and the amino acid residues. Vasseur (1952) found that cystine, cysteine, tyrosine and tryptophan residues of the protein jelly-coat substance of *Echinocardium cylindrica* were attacked by periodate, and Maekawa & Kushibe (1954) observed a similar destruction of amino acids during periodate oxidation of ovalbumin.

The presence of *N*-acetyl groups on the 2-amino-2-deoxy-D-glycosyl residues limits the consumption of periodate to, at most, 1 mole of periodate/amino sugar residue, since oxidation between an acetamido group and an adjacent hydroxyl group would be negligible (Aminoff & Morgan, 1951; Hough & Taha, 1956). The non-reducing end groups of D-galactopyranose and D-mannopyranose would react with 2 moles of periodate with elimination of 1 mole of formic acid. Reliable estimates of formic acid cannot be obtained in the presence of large amounts of protein. Anderson *et al.* (1955) oxidized mixtures of starch and protein and found that, provided that the protein content was less than 23% and not removed from the reaction solution, it had little effect on the determination of formic acid. With ovomucoid, however, the protein content is much higher and is probably responsible for the low yield of formic acid (2–3 moles/mole of ovomucoid).

Since the periodate uptake had suggested that certain monosaccharide units in ovomucoid were not oxidized it was of interest to investigate the composition of the product at the end of the initial rapid reaction. Paper chromatography of a hydrolysate of the oxidized product revealed the presence of D-mannose and 2-amino-2-deoxy-D-glucose but D-galactose was absent. Quantitative determinations showed that 46–48% of the 2-acetamido-2-deoxy-D-glucose residues and about 50% of the mannose residues which were originally present in ovomucoid had remained unoxidized (Table 5). On the structure proposed by Stacey & Woolley (1942) only a third of the mannose units would have been oxidized. The periodate-oxidation data are in agreement with the rate of hydrolysis results and confirm that D-galactopyranosyl and D-mannopyranosyl units occupy terminal positions in the mucoprotein molecule. Verification of this conclusion was sought by methylation of the mucoprotein.

Methylation studies on ovomucoid. Stacey & Woolley (1940, 1942) have, on the basis of methylation studies, described a structure for the carbohydrate moiety of ovomucoid. They isolated 2-acetamido-2-deoxy-3:4:6-tri-*O*-methyl-D-glucose (7 moles), D-mannose (2 moles), 3:4:6-tri-*O*-methyl-D-mannose (1 mole) and 2:3:4:6-tetra-*O*-methyl-D-galactose (1 mole). Since their method involved the use of strong alkali with dimethyl sulphate, we investigated two alternative methods of methylation, namely the action of methyl iodide and sodium upon ovomucoid in liquid ammonia (Freudenberg *et al.* 1938) and the action of methyl iodide on a thallous complex of ovomucoid (Campbell *et al.* 1948). Unfortunately, both methods produced negligible methylation (OMe, $<5\%$) and consequently a slight modification of Stacey & Woolley's (1940) procedure was employed. Since the methylated products contained polypeptidic material it was not possible to ascertain from the total methoxyl content of the product whether or not the carbohydrate component was fully methylated. To ensure that the carbohydrate was fully methylated, the product was then acetylated and treated with silver oxide and methyl iodide but this treatment lowered the methoxyl content of the product. A hydrolysate of the methylated product was fractionated on a charcoal column with fractional elution with ethanol–water mixtures, a procedure which not only separated the methylated sugars, but also freed them from substantial amounts of amino acids. The isolation of 2:3:4:6-tetra-*O*-methyl-D-galactose and 2:3:4:6-tetra-*O*-methyl-D-mannose, characterized as their respective anilides, confirmed the previous suggestions that D-galactose and D-mannose occupy the non-reducing end-group positions in the carbohydrate moiety of ovomucoid. D-Mannose must also occur at other positions in the molecule, in agreement with periodate-oxidation data, since unidentified tri-*O*- and di-*O*-methyl derivatives were detected. The di-*O*-methylmannose gave no formaldehyde either before or after reduction with sodium borohydride and hence it is probably the 2:6-di-*O*-methyl derivative. Methylated derivatives of 2-amino-2-deoxy-D-glucose were not detected, which may have been due to the severity of the methylating conditions or to *N*-methylation of the amino sugar during the reaction with methyl iodide and silver oxide, and subsequent complex formation with silver oxide (Stacey & Woolley, 1942). Bray and James (quoted by Folkes *et al.* 1950) were unable to find any 2-amino-2-deoxyhexose derivatives in a hydrolysate of methylated frog-spawn mucin, a mucopolysaccharide which originally contained 2-amino-2-deoxy-D-glucose and -D-galactose. No free D-mannose was found in our methylated product, in disagreement with the results of Stacey & Woolley (1942).

Attempted enrichment of the carbohydrate moiety of the mucoprotein. Enrichment of the carbohydrate components of the egg-white mucoproteins has been claimed by treatment with barium hydroxide solution at 100° for varying periods of time to hydrolyse the protein (Fränkel & Jellinek, 1927; Levene & Mori, 1929; Suzuki, 1941; Stacey & Woolley, 1940). Fränkel & Jellinek (1927) obtained a product in which the acetamido groups were largely retained, whereas Levene & Mori (1929), who increased the time of heating from 3 to 7 hr., found that complete de-*N*-acetylation of the acetamido sugar had occurred. Determination of the carbohydrate composition of the products formed from ovomucoid by treatment with barium hydroxide in the presence of oxygen-free nitrogen has now revealed that degradation of some of the carbohydrate units had occurred. This result was

not surprising since it is well established that poly- and oligo-hexosaccharides containing 1:4- and 1:3-linkages are rapidly degraded in alkali from the reducing end of the molecule with the formation of saccharinic acids (Kenner, 1955; Kenner & Corbett, 1955). Furthermore, any 2-amino-2-deoxy-D-glucose at the reducing end-group positions would be rearranged and degraded (Stanley, 1953; Knox & Morgan, 1954), although Levvy & McAllen (1959) have found that the amino sugars are more stable than is generally believed.

Fractional precipitation of the alkali-stable products from ovomucoid, followed by passage through a cation-exchange resin, yielded two acidic fractions (I and II; Table 7), which suggested the formation of saccharinic acids from the reducing end of the carbohydrate chain. Two basic fractions (III and IV) were isolated from the cation-exchanger by elution with ammonia solution. In an attempt to isolate disaccharides from fraction I, it was treated with 10N-HCl at room temperature to give fractions IA and IB (Table 7). In no case was a preparation completely devoid of amino acids, although it was significant that the acidic fractions (I, IA, IB) contained markedly increased carbohydrate contents whereas the basic fractions (II, III, IV) did not. Each fraction contained mannose, galactose and 2-amino-2-deoxyglucose, but the composition varied considerably. However, the ratio mannose: 2-amino-2-deoxyglucose was found to vary only slightly between 1·0 and 1·3. In every case the galactose content was enriched in comparison with the other monosaccharides, in agreement with its occurrence as non-reducing end groups, which will be unaffected by alkali. Thus the overall effect of alkali on ovomucoid is to cause hydrolysis of the protein and to degrade the carbohydrate, probably by stepwise removal of some alkali-labile 2-amino-2-deoxy-D-glucose and D-mannose units until a branching point is reached (e.g. a $1 \rightarrow 2$ or $1 \rightarrow 6$ linkage), so arresting the degradation process. In this way the D-galactose units and those D-mannose units which occupy non-reducing end-group positions will be preserved.

The acidic fraction I was non-reducing and oxidation with periodate destroyed all of the galactose (Tables 7 and 8), in agreement with galactose end groups. Partial hydrolysis of fraction I, followed by charcoal chromatography, gave in small yield a product which from its behaviour on paper chromatograms appeared to be a disaccharide composed of mannose and 2-amino-2-deoxyglucose. Treatment of the disaccharide with sodium borohydride reduced only the amino sugar component, thus suggesting that mannose is glycosidically linked to 2-amino-2-deoxyglucose. Levene (1941) isolated from ovalbumin a disaccharide with the properties of a 2-amino-2-deoxyglucopyranosylmannose.

Partial acid hydrolysis of ovomucoid followed by fractionation on a squat charcoal column afforded a disaccharide composed only of mannose. Insufficient quantity of this material was obtained for characterization but, since formaldehyde was liberated on periodate oxidation at pH 8, the absence of a $1 \rightarrow 5$ or $1 \rightarrow 6$ linkage was suggested (Hough & Perry, 1956).

Attempts to cleave the protein–carbohydrate bond. The type of bonding between protein and carbohydrate in ovomucoid is unknown but appears to be different from the polar interactions between amino, sulphate and carboxylic acid groups which constitute the major form of bonding in mucopolysaccharide–protein complexes. The available evidence suggests that it is of a covalent nature. That it does not involve highly ionized groups was revealed by potentiometric titration. In a corneal mucopolysaccharide, Woodin (1954) was able to show a protein–carbohydrate bond between sulphuric acid ester and amidine groups by the partial dissociation of the bonds during electrophoresis in the presence of guanidine hydrochloride. Under these conditions ovomucoid gave a single peak in the Tiselius apparatus.

Holdsworth's (1952) picric acid method, treatment with dilute sulphuric acid and incubation with various enzymes failed to yield protein-free carbohydrate fractions.

Ovalbumin

Composition. Previous studies on the electrophoresis of ovalbumin have shown that it is not a single substance, either in the crystalline form or in egg white, and that the mucoprotein occurs in the diphosphate, monophosphate and free forms (Cann, 1949; Durieux & Kaminski, 1956). The diphosphate is usually the major component and the other two components are formed by progressive loss of phosphate (Perlmann, 1950). Crystalline ovalbumin was examined (pH 9; I 0·13) in the Tiselius electrophoresis apparatus, but, although it gave a single peak, this was not symmetrical, which suggested the presence of closely related substances. Steven & Tristram (1958) separated ovalbumin into three components by starch-gel electrophoresis in borate buffer (pH 8·6), but they found only slight differences in electrophoretic mobility.

Ovalbumin has an average molecular weight of 45 000 and contains about 3–4% of carbohydrate, composed of approximately 5 units of D-mannose and 3 units of 2-acetamido-2-deoxy-D-glucose (Levene & Mori, 1929; Sørensen, 1938; Neuberger, 1938; Johansen *et al.* 1958). Examination of an acid hydrolysate of ovalbumin confirmed the presence of D-mannose (2·75%) and 2-amino-2-deoxy-D-glucose (1·4%) in the molecular proportions of 2:1 respectively.

Structural studies. As with ovomucoid the periodate oxidation of ovalbumin was also a two-stage reaction at pH 3·6, but the amount of oxidant consumed at the end of the first stage (1 mole/1180 g. of ovalbumin) was far in excess of that required for the complete oxidation of the carbohydrate content (3·9 %) and thus revealed that extensive oxidation of amino acids was also occurring. Maekawa & Kushibe (1954) found that periodate oxidation completely destroyed cysteine and tryptophan in ovalbumin. Quantitative analysis of the carbohydrate remaining in the product after the initial rapid oxidation revealed that two residues of both mannose and 2-acetamido-2-deoxyglucose were unoxidized. It follows therefore that four mannose units and one 2-acetamido-2-deoxyglucose unit had been destroyed during the oxidation.

Exhaustive methylation of ovalbumin by a similar method to that used for ovomucoid yielded a product in which amino sugar derivatives were not detectable. After fractionation of an hydrolysate of the methylated product on a charcoal column, 2:3:4:6-tetra-*O*-methyl-D-mannose was characterized, thus suggesting that it occurs as non-reducing end groups in the carbohydrate portion of the mucoprotein. Uncharacterized di- and tri-*O*-methyl-D-mannoses were also present. These results are in accord with the periodate-oxidation experiments.

Ovalbumin has been degraded by the use of a variety of proteolytic enzymes to give oligosaccharides and glycopeptides (Cunningham *et al.* 1957; Jevons, 1958; Johansen *et al.* 1958). Examination of the glycopeptides has suggested that one of the carboxyl groups of aspartic acid is directly linked to the carbohydrate, thus affording the first evidence of the carbohydrate–peptide bond.

An attempt was made to enrich the carbohydrate moiety by heating denatured ovalbumin in 10 % (w/v) barium hydroxide solution in the presence of oxygen-free nitrogen (Levene & Mori, 1929), but in agreement with Neuberger (1938) little enrichment was obtained because of the alkaline degradation of the carbohydrate. The product (2 % yield) contained 3·7 % of D-mannose and 1·8 % of 2-amino-2-deoxy-D-glucose.

Partial hydrolysis of ovalbumin by heating at 80° in a dilute solution of trichloroacetic acid for 12 hr., during which time the pH has risen from 1 to 7, was more successful. The water-soluble component (sulphated ash, 50·2 %), which was insoluble in aq. 90 % methanol, contained 4·7 % of mannose and 5·3 % of 2-amino-2-deoxyglucose; considering the high ash content, this result shows that a glycopeptide in which a high proportion of the carbohydrate was present had been released. Since the glycopeptide contained approximately equal amounts of mannose and amino sugar, whereas in ovalbumin the ratio is 2:1 respectively, it would appear that hydrolysis had cloven the carbohydrate moiety of the mucoprotein, leaving a mannose-rich fragment in attachment. A *N*-terminal end-group analysis of the glycopeptide revealed only dinitrophenylalanine.

SUMMARY

1. A semi-micro analytical procedure has been developed for the analysis of the carbohydrates present in mucoproteins. Ovomucoid was found to contain 2-amino-2-deoxy-D-glucose (12·3–12·8 %), D-mannose (6·3 %) and D-galactose (1·7 %), whereas ovalbumin contained D-mannose (2·75 %) and 2-amino-2-deoxy-D-glucose (1·34 %).

2. Specific cleavage of the protein–carbohydrate in ovomucoid and ovalbumin did not occur on treatment with picric acid, barium hydroxide or 0·01 N-sulphuric acid.

3. Various carbohydrate-enriched fractions were obtained by the action of barium hydroxide on ovomucoid. It is suggested that in this process the carbohydrate portion of the mucoprotein was also degraded from the reducing end with the loss of some mannose and amino sugar units and consequent enrichment of the galactose component.

4. The partial acid hydrolysis of ovomucoid and ovalbumin was studied. Two disaccharides, a mannobiose and a D-mannosyl-2-amino-2-deoxy-D-glucose, were isolated in very small amounts from partial hydrolysates of ovomucoid. A glycopeptide was isolated from ovalbumin.

5. The action of periodate on ovomucoid and ovalbumin was a two-stage reaction involving oxidation of both carbohydrate and protein.

6. D-Galactose and D-mannose were shown to occur as non-reducing end groups in ovomucoid by using controlled hydrolysis, periodate oxidation and methylation techniques.

7. Methylation of ovomucoid and ovalbumin yielded products in which 2-amino-2-deoxy-D-glucose derivatives could not be detected.

8. D-Mannose was found to occur as non-reducing end groups in the methylated carbohydrate of ovalbumin.

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