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A Study of the Organic Material of Hen's-Egg Shell

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The general structure of the hen's-egg shell was reviewed by Simkiss & Tyler (1957). Surrounding the egg white are two shell membranes each composed of a network of fibres. The shell is attached to the outer of these membranes by the mammillae, which are roughly hemispherical knobs composed of large spherulite crystals of calcite. Each mammilla contains a core of organic matter and merges above into the main body of the shell. Although this is known as the spongy layer it is composed of a dense mass of calcite crystals bound together by a sparse organic matrix. The shell is pierced by funnel-shaped tubes, the pores, which start from the interstices between the mammillae, pass through the shell and open on the surface. Covering the surface of the shell and the mouths of the pores is a thin, tough cuticle. The structure of the shell is shown diagrammatically in Fig. 1.

Although the general structure of the shell is established, little work has been done on the

chemical composition of the organic components. Moran & Hale (1936) studied the shell membranes histologically and were able to separate each into a number of layers. The outer membrane, i.e. that next to the shell, consisted of three layers, the outermost of keratin fibres and the other two of mucin fibres, and the inner membrane consisted of two rather indistinct layers of keratin and mucin fibres. Partial amino acid analyses (Calvery, 1933; Jones & Mecham, 1944; Munks, Robinson, Beach & Williams, 1945), together with X-ray diffraction studies (Wolken, 1951), support the view that the membranes are composed largely of keratin.

Almquist (1934) investigated the matrix chemically and concluded that the protein was a collagen. From histochemical studies Simkiss & Tyler (1957) showed that the matrix consisted of a protein-acid polysaccharide complex. A re-appraisal of the results of the staining techniques (Simkiss, 1958) indicated also the presence of neutral sugar, particularly in the mammillary cores. The mammillae stained strongly for fat whereas only very faint staining was observed in the rest of the matrix.

Dickie (1848) suggested that the cuticle was a cellular epithelium over a basement membrane. Marshall & Cruickshank (1938) stated that it was porous, but otherwise structureless, and that it thickened over the mouths of the pores forming the cuticular plaques. Both Moran & Hale (1936) and Haines & Moran (1940) considered the cuticle to be mainly mucin, whereas Conrad & Scott (1938) suggested the possibility of its formation from egg white diffusing out of the egg and Romanoff & Romanoff (1949) believed that it was similar to the egg-shell membranes. The presence of protein containing many disulphide links and free sulphydryl groups and some evidence for the presence of phospholipid was shown by Simkiss (1958).

Fig. 1 Conventional diagram of a radial section of a hen's-egg shell to show the general structure.

EXPERIMENTAL

Materials

Cuticle. Eggs from Light Sussex $\times$ Rhode Island Red hens were broken in half, the contents removed and the shells washed thoroughly in running tap water. The half-shells were immersed in 5% (w/v) EDTA (disodium salt adjusted to pH 7.5–8.0 with NaOH). After about 90 min. the cuticle could be washed from the outer surface of the shell with a jet of water. The cuticles were washed repeatedly with distilled water followed by acetone and dried at 60°.

Membrane. After removal of the cuticle the membranes were peeled off the inner shell surface and treated similarly to the cuticle.

Matrix. The true shell remaining after the removal of the cuticle and membranes was broken into small pieces and decalcified by stirring overnight with an excess of 20% (w/v) EDTA (pH 7.5–8.0). The mixture was dialysed in Visking tubing against running distilled water for 8 days and then freeze-dried. The product was lightly crushed and passed through a 100-mesh sieve.

Preparation of hexosamine-containing fractions

Hexosamine-containing fraction extracted from the matrix with potassium chloride–potassium carbonate solution. Matrix (5 g.) was suspended in 100 ml. of aq. 30% (w/v) KCl soln. containing 1% (w/v) of K_2CO_3 (Einbinder & Schubert, 1950) and the mixture stirred mechanically at room temperature for 48 hr. in the presence of a little toluene. The mixture was centrifuged and the residual matrix re-extracted 17 times, the last six extractions proceeding for 3–4 days each. The residue from the extractions was finally washed successively with water and acetone, and then dried. Each extract was dialysed against running distilled water for 3 days and then evaporated to about 10 ml. by suspending the dialysis sac over an electric fire. Evaporation was sufficiently rapid to prevent the temperature exceeding 40°. A small amount of insoluble material was removed by centrifuging before the addition of 3 vol. of ethanol containing a little LiCl. The precipitates were allowed to stand for 24 hr. at 4° and were then collected by centrifuging, washed twice with 80% (v/v) ethanol, absolute ethanol, acetone and light petroleum (b.p. 40–60°).

Hexosamine-containing fractions prepared after enzymic digestion of the matrix. Matrix (2.5 g.) was suspended in 50 ml. of water and the pH adjusted to 1.5 with 6N-HCl. Crystalline pepsin (0.1 g.) was added and the mixture incubated at 37° in the presence of toluene, the pH being adjusted every 24 hr. After 72 hr. the pH was brought to about 8.5, 0.15 g. of crystalline trypsin was added and incubation continued for a further 24 hr. The hexosamine-containing fractions were precipitated and purified as described by Meyer, Linker, Davidson & Weissmann (1953) and the calcium salts were fractionated by the step-wise addition of ethanol. Each fraction was re-dissolved and re-precipitated, smaller volumes being used. Some precipitation then occurred at lower ethanol concentrations than were initially required. Four fractions were finally collected at ethanol concentrations of 20, 30, 40 and 50% (v/v) respectively.

Methods

Nitrogen. Nitrogen was determined by a micro-Kjeldahl method.

Sulphate. Samples were hydrolysed with 6N-formic acid at 100° for 9 hr. (Muir, 1958). The hydrolysates were evaporated to dryness *in vacuo*, dissolved in water and portions taken for determination by the method of Jones & Latham (1956).

Hexosamine. The method of Belcher, Nutten & Sambrook (1954) was used. The highest yield of hexosamine was obtained after hydrolysis in sealed tubes at 100° with 4N-HCl–90% formic acid (1:1, v/v). Insoluble material was removed by centrifuging, the hydrolysates were evaporated to dryness *in vacuo* over NaOH flakes and the residue was taken up in water.

Uronic acid. Water-insoluble samples were heated with N- H_2SO_4 at 100° for 2 hr. and a portion was taken for determination by the carbazole method of Dische (1947) with glucuronolactone as standard. The suggestions of Boas (1955) to eliminate non-specific colour formation were followed. The pretreatment was not used for the water-soluble fractions.

Total neutral sugar. The primary cysteine reaction of Dische (1955) was used with galactose as standard.

Paper electrophoresis. For qualitative work an apparatus similar to that of Foster (1952) and, for quantitative separations, one in which the paper strips were suspended horizontally, was used. The paper was either Whatman no. 1 or 3MM, both chromatography grade. 0.2M-Sodium acetate–acetic acid (pH 5.0) and 0.2M-boric acid–NaOH (pH 10.0) were the buffers mainly used (Foster, Newton-Hearn & Stacey, 1956). Neutral sugars and uronic acids were located with the *o*-aminodiphenyl reagent of Gordon, Thornburg & Werum (1956); hexosamines with ninhydrin (Smith, 1953) or with the Elson & Morgan reagent (Partidge, 1948); amino acids with ninhydrin; acid polysaccharide with the Cetavlon (cetyltrimethylammonium bromide)–bromocresol purple method of Bera, Foster & Stacey (1955); neutral polysaccharide with the periodic acid–Schiff reagent (Koiv & Gronwall, 1952); and proteins with the bromophenol blue method (Durrum, 1950).

Paper chromatography. Separations were made on Whatman no. 1 chromatography-grade paper by downward development at 20°.

Identification of sugars. In the earlier work samples were hydrolysed with 2N- H_2SO_4 for 3 hr. at 100°. The hydrolysate was neutralized with solid $BaCO_3$ and after centrifuging was passed through a column of Amberlite IR-120 (H^+) or Dowex 50 (H^+ form) to remove amino acids and peptides. Hexosamines which were also absorbed were subsequently eluted as described by Boas (1953). In later work the method of Anastasiadis & Common (1958) was used. Neutral sugars were separated with ethyl acetate–pyridine–water (8:2:1, by vol.) (Roudier, 1951) and ethyl acetate–acetic acid–water (9:2:2, by vol.) (Andrews, Hough & Jones, 1952), and hexosamines and uronic acids with ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.) (Fischer & Dorfel, 1955). Neutral sugars, tentatively identified chromatographically, were eluted from chromatograms and their mobility on electrophoresis in borate buffer was checked against authentic samples. For hexosamines the identification method of Stoffyn & Jeanloz (1954) was also used.

Identification of amino acids. Samples were hydrolysed with 6N-HCl in sealed tubes at 100° for 24 hr. and the HCl was removed by evaporation to dryness *in vacuo* over solid NaOH. Two-dimensional separations were made with

butan-1-ol-acetic acid-water (60:15:25, by vol.; solvent 1) (Smith, 1958) for 24 hr. in the first direction followed by butan-1-ol-ethyl methyl ketone-aq. NH_3 soln.-water (5:3:1:1, by vol.; solvent 2) (Wolfe, 1957) in the second.

Quantitative estimation of amino acids. The hydrolysate was applied to the centre line of a strip of Whatman 3MM paper 30 cm. long and separated into three groups of amino acids, acidic, basic and neutral respectively, by electrophoresis in 0.2M-ammonium acetate buffer, pH 5.0, at a potential of 5v cm^{-1} for 12 hr. Each group was eluted quantitatively and samples were run on chromatograms: acidic amino acids, solvent 1, 24 hr.; neutral amino acids, solvent 1, 24 hr., solvent 2, 36 hr.; basic amino acids, butan-2-ol-aq. NH_3 (aq. soln., 0.88 sp.gr., diluted 3:100, v/v) (3:1, v/v). The basic amino acids and proline were determined by direct photometry of the coloured spots produced with ninhydrin and isatin respectively. The remaining amino acids, located by the method of Cook & Luscombe (1957) and with the aid of guide strips, were determined by the method of Yemm & Cocking (1955) after elution from the paper. Interference by ammonia was eliminated by the method of Kornberg & Patey (1957). Only methionine and valine could not be measured separately by the method.

Absorptiometric and reflectance measurements. A Hilger Spekker H760 instrument was used for absorptiometric measurements and, for reflectance measurements, an EEL reflectance spectrophotometer.

Infrared spectra. The dried hexosamine-containing fractions were examined with the KBr pressed-disk technique with a Grubb Parsons S3A double-beam spectrophotometer fitted with a rock-salt prism.

RESULTS

Composition of the hen's-egg-shell matrix

The dry matrix contained 7.92% of ash, sulphate being present but phosphate absent. Amino acids and sugars were found after hydrolysis.

Galactose, mannose, fucose, galactosamine, glucosamine and glucuronic acid were identified by chromatographic and electrophoretic techniques. Analytical values are given in Table 1. The ratio of galactose to mannose, determined by a reflectance method (McCready & McComb, 1954) after location of the spots on chromatograms with aniline-trichloroacetate, was found to be 1.7:1, but fucose was present in too low a concentration to be estimated. If a 1:1 molar ratio of sulphate:hexos-

amine is assumed, the molar ratio sulphate:hexosamine:uronic acid is 1:1:0.65 and residual hexosamine:neutral sugar is 1:0.94.

The amino acid composition of the matrix is given in Table 2. No methionine was detected on two-way chromatograms either with ninhydrin or with the platinum iodide reagent of Toennies & Kolb (1951), and, after alkaline hydrolysis of the matrix, no tryptophan was found with the Ehrlich reagent (Jepson & Smith, 1953). Tests for hydroxyproline with ninhydrin, isatin and the sensitive isatin-Ehrlich reagent (Jepson & Smith, 1953) were negative. The quantitative results are low, for only 66% of the non-hexosamine nitrogen was recovered as amino acid nitrogen. Amide nitrogen was not estimated and destruction of amino acids is to be expected during the hydrolysis of protein in the presence of carbohydrate (Block & Bolling, 1951). However, it seems probable that losses occurred during the separation and estimation of the amino acids greater than were expected from experiments performed during the development of the method, when recoveries of leucine and glutamic acid ranged from 90.1 to 101.7% (mean 96.9%).

The matrix contains no hydroxyproline, about twice as much dicarboxylic amino acids as basic amino acids and is low in aromatic and sulphur-containing amino acids. There is considerable similarity between the composition of this protein and of the non-collagenous protein associated with chondroitin sulphate in cattle cartilage (Partridge & Davis, 1958) and pig cartilage (Muir, 1958) (Table 2).

Hexosamine-containing fraction extracted from hen's-egg-shell matrix with potassium chloride-potassium carbonate solution

Even after prolonged extraction with repeated changes of $\text{KCl-K}_2\text{CO}_3$ solution the matrix still retained 48.4% of its hexosamine. The rate of extraction had become very low and the ratio of hexosamine nitrogen to α -amino nitrogen had fallen from 0.29 to 0.14. This incomplete removal of hexosamine may only indicate the inadequacy of the method rather than that the unextracted

Table 1. *Analytical values for the hen's-egg-shell matrix, membranes and cuticle*

Values in parentheses are moles/ 10^4 g.

	Matrix (% of sulphated organic matter)	Membranes (% of organic matter)	Cuticle (% of organic matter)
Nitrogen	15.01	15.54	15.94
Hexosamine nitrogen	0.46	0.11	0.24
Non-hexosamine nitrogen (by difference)	14.55	15.43	15.70
Hexosamine	5.83 (3.25)	1.45 (0.81)	3.06 (1.71)
Neutral sugar (as galactose)	3.57 (1.98)	1.97 (1.09)	2.87 (1.59)
Uronic acid (as glucuronic acid)	1.45 (0.75)	0	0
Ester sulphate	1.10 (1.15)	Trace	0

Table 2. *Amino acid composition of hen's-egg-shell matrix compared with that of the non-collagenous protein associated with chondroitin sulphate in cattle and pig cartilage*

	Egg-shell matrix (g. of N/ 100 g. of protein N)	Non-collagenous protein		Egg-shell matrix (g. of amino acid/100 g. of organic matter)
		Cattle cartilage* (Partridge & Davis, 1958) (g. of N/100 g. of protein N)	Pig cartilage† (Muir, 1958) (g. of N/100 g. of protein N)	
Aspartic acid	4.8	5.71	5.58	6.7
Glutamic acid	7.4	8.80	7.57	11.5
Arginine	11.6	5.57	9.73	5.3
Lysine	3.0	5.04	3.27	2.3
Histidine	1.1	3.67	2.87	0.6
Cystine	1.4	—	Trace	1.8
Methionine	0.0	0.87	—	0.0
Glycine	6.9	4.79	7.07	5.4
Serine	4.0	2.64	6.38	4.4
Alanine	5.4	4.79	5.64	5.0
Threonine	4.1	2.86	3.35	5.2
Valine	4.3	4.05	4.46	5.3
Isoleucine	6.3	2.77	2.99	8.7
Leucine		6.15	6.26	
Tyrosine	0.3	2.60	1.87	0.6
Phenylalanine	2.3	4.67	3.31	4.0
Proline	3.1	6.94	1.07	3.8
Hydroxyproline	0.0	0.00	0.00	0.0
Total	66.0	71.92	72.24	70.6

* Protein prepared by alkaline hydrolysis of protein-chondroitin sulphate extracted with KCl-K₂CO₃ soln.

† Protein present in chondroitin sulphate complex extracted with CaCl₂ soln.

hexosamine is combined in a different form from that extracted.

The extracted material was bulked, dissolved in water and precipitated with Cetavlon. After removal of the Cetavlon, galactosamine, a trace of glucosamine, glucuronic acid, galactose and mannose were identified in the precipitate together with a protein moiety. Quantitatively the preparation contained 19.74 % of hexosamine, 16.24 % of uronic acid, 4.6 % of neutral sugar and 8.24 % of ester sulphate. Assuming a 1:1 molar ratio of sulphate:hexosamine, the molar ratio sulphate:hexosamine:uronic acid is 1:1:0.97 and of residual hexosamine to neutral sugar is 1:1.05. The amino acid composition of the protein moiety of the hexosamine-containing fraction was qualitatively similar to that of the total matrix protein.

After electrophoresis on paper in borate buffer (pH 10) or acetate buffer (pH 5), staining for acid polysaccharide showed that most of the complex migrated as a single spot with a similar mobility in both buffers. A faint stain was observed at the origin which was a little stronger in the acetate buffer. Since protein staining only gave a positive reaction at the origin it is probable that a little of the acid polysaccharide in the preparation remained combined with a larger proportion of protein than the rest. Staining for neutral sugar also gave a positive reaction only at the origin. In

both buffers chondroitin sulphate (L. Light and Co. Ltd.) migrated a little faster than the mobile acid polysaccharide fraction, but the viscosity of this commercial sample, in aq. 0.1 % solution, was less.

After reprecipitation with Cetavlon in the presence of 0.2M-Na₂SO₄ (Scott, 1956), addition of ethanol to the dialysed supernatant gave only a slight cloudiness, indicating that little or no un-sulphated acid polysaccharide was present. The sulphated acid polysaccharide fraction was converted into its calcium salt and ethanol was added in a stepwise manner. Slight cloudiness occurred at ethanol concentrations of 20 and 30 % (v/v) and was removed by centrifuging, but recoverable precipitates were formed only at 40 and 50 % (v/v) respectively.

The infrared spectra of the hexosamine-containing fraction as extracted (1) and after purification in aqueous solution with Cetavlon (2), and of the two preparations obtained by precipitating the calcium salts at ethanol concentrations of 40 and 50 % (v/v) (3 and 4 respectively), are given in Fig. 2. The four spectra are of the same general shape but they differ in the relative intensities of different bands in the same spectrum. The absolute intensities of the same band in different spectra cannot be compared as there were differences in the amount of each sample taken, particularly between

Fig. 2. Infrared spectra of the hexosamine-containing fraction extracted from hen's-egg-shell matrix with $\text{KCl-K}_2\text{CO}_3$ soln. (1), the fraction after purification with Cetavlon (2) and the sub-fractions obtained by precipitation of the calcium salts at ethanol concentrations of 40 and 50% (v/v) (3 and 4 respectively).

the first three and the fourth. Treatment with Cetavlon caused a decrease in the intensity of the bands at 1639 and 1558 cm^{-1} , particularly of the latter, compared with the ester sulphate band at 1235 cm^{-1} and with the broad carbohydrate OH band centred near 1050 cm^{-1} . This change is due to the removal of protein. In general all four spectra resemble those given by Orr (1954) and Mathews (1958) for chondroitin sulphates. Mathews (1958) distinguishes the three isomeric chondroitin sulphates on the basis of their absorption in the region $1000\text{--}700\text{ cm}^{-1}$. The A isomer shows three bands, at 928 , 852 and 725 cm^{-1} ; the B isomer shows four bands, at 928 , 855 , 840 and 712 cm^{-1} ; the C isomer shows three bands at 1000 , 820 and 775 cm^{-1} . The spectrum of the hexosamine-containing fraction as extracted (1) shows bands at 926 , 853 and 833 cm^{-1} , and then absorption increases to a maximum at 714 cm^{-1} . The 833 cm^{-1} band is of similar intensity to the 853 cm^{-1} band but in the spectrum of the pure B isomer (Mathews, 1958) the 840 cm^{-1} band exceeds the 855 cm^{-1} band. Spectra (2), (3) and (4) correspond closely to that of chondroitin sulphate A. It is probable that a small amount of the B isomer was present initially but that it decreased to a negligible amount by mechanical losses during the subsequent reprecipitations.

Incubation of the hexosamine-containing fraction at 37° with testicular hyaluronidase in 0.9% NaCl soln. caused a marked drop in the viscosity of the solution. The mixture was dialysed and the

residual solution treated with Cetavlon, when only a faint cloudiness was formed. To confirm the depolymerization of most of the hexosamine-containing fraction, it was incubated in solution with albumin and testicular hyaluronidase (Meyer & Rapport, 1952). On subsequent acidification only a faint cloudiness was produced compared with the dense precipitate in the absence of hyaluronidase.

The main hexosamine-containing substance extracted from the matrix with $\text{KCl-K}_2\text{CO}_3$ soln. is chondroitin sulphate A with a small amount of chondroitin sulphate B. However, precipitation of the chondroitin sulphate complex, in aqueous solution, with Cetavlon failed to remove all neutral sugars and there was also a small excess of hexosamine present. To be precipitated by Cetavlon these sugars must be linked with molecules containing acid groups. It is unlikely that the neutral sugars are linked directly with the chondroitin sulphate molecule, which has often been found free from them (e.g. Muir, 1958). The ester sulphate could be accounted for by the chondroitin sulphate, and only a trace of glucosamine was found so that it is unlikely that keratosulphate or a similar substance is present. The electrophoretically immobile component stained for neutral sugar, protein and acid polysaccharide; so that it seems probable that the neutral sugar was precipitated because it was attached to the same protein moiety as the chondroitin sulphates.

Hexosamine-containing fractions prepared from hen's-egg-shell matrix after degradation with pepsin and trypsin

Since only some 50% of the hexosamine was extracted with $\text{KCl-K}_2\text{CO}_3$ soln., hexosamine-containing fractions were prepared after degradation of the matrix with pepsin and trypsin, when only a small residue remained undissolved. The ethanol fractionation was arbitrary as the amount of material being precipitated at a given ethanol concentration also depended on the concentration of hexosamine-containing material present.

All four fractions obtained contained galactose, mannose, glucuronic acid and galactosamine. Galactose was always present in greater concentration than mannose. In the 20 and 30% ethanol-precipitated fractions there were only small amounts of glucuronic acid. A second uronic acid was present, in greater concentration, which was identified chromatographically as iduronic acid. The galactosamine in the 40 and 50% ethanol-precipitated fractions was accompanied by smaller amounts of glucosamine. Two other unidentified spots reacting with the Elson & Morgan reagent were found on chromatograms developed with the Fischer & Dorfel solvent, one at R_f 0.35 in all four

Table 3. *Analytical values for the ethanol-precipitated hexosamine-containing fractions prepared after digestion of the hen's-egg-shell matrix with pepsin and trypsin*

Column headings represent the ethanol concentrations (v/v) used for precipitation. Values in parentheses: moles/ 10^4 g.

	Values (% of dry matter)			
	20%	30%	40%	50%
Hexosamine	16.39 (9.15)	16.32 (9.11)	24.38 (13.61)	20.72 (11.56)
Uronic acid (as glucuronic acid)	9.94 (5.12)	11.81 (6.08)	23.51 (12.11)	15.99 (8.24)
Neutral sugar (as galactose)	3.91 (2.17)	3.89 (2.16)	3.93 (2.18)	8.14 (4.52)
Ester sulphate	—	6.25 (6.51)	5.25 (5.46)	3.89 (4.05)

fractions and the other at R_f 0.45 in the 40 and 50 % fractions only (R_f = rate of movement relative to phenylalanine, R_f glucosamine-HCl 0.79, R_f galactosamine-HCl 0.73).

Analytical values for the fractions are given in Table 3. The colour yield per mole of iduronic acid by the carbazole method is less than that for glucuronic acid, which, as the lactone, was used as the standard in this work (Hoffman, Linker & Meyer, 1958). Since iduronic acid was found in the 20 and 30 % fractions, the uronic acid values for these fractions and for the intact matrix will be low. Analysis of the matrix and of the chondroitin sulphate complex extracted with KCl- K_2CO_3 soln. showed that with a 1:1 molar ratio of hexosamine: sulphate, the molar ratio of residual hexosamine to neutral sugar was close to unity. To fit the results in Table 3 to this picture, some desulphation during the preparation of the fractions must be postulated. Assuming from the earlier analyses a 1:1 molar ratio of hexosamine:neutral sugar, then the molar ratios of hexosamine:uronic acid in the 40 and 50 % fractions are 1:1.06 and 1:1.17 respectively. These values are similar to those given by Hoffman *et al.* (1958), who found molar ratios of 1:1.12 and 1:1.18 for chondroitin sulphates A and C respectively when uronic acid was estimated with the carbazole method.

The amino acid composition of the 20 and 40 % fractions was qualitatively similar to that of the matrix.

After electrophoresis in borate or acetate buffer, staining for acid polysaccharide showed that all four fractions migrated as a single component with a mobility similar to that of the mobile fraction extracted with KCl- K_2CO_3 soln. No acid polysaccharide was detected at the origin and staining for protein was negative. Staining for neutral sugars gave a faintly positive reaction at the origin for the 40 % fraction and a strongly positive reaction for the 50 % fraction. There was also a positive reaction for neutral sugars in the mobile portion of the latter fraction.

The infrared spectra of the four fractions are given in Fig. 3. In general shape, they resemble those obtained from the chondroitin sulphate com-

Fig. 3. Infrared spectra of the hexosamine-containing fractions liberated from hen's-egg-shell matrix by pepsin and trypsin and subsequently precipitated as the calcium salts at ethanol concentrations of (a) 20%, (b) 30%, (c) 40% and (d) 50% (v/v) respectively.

plex extracted with KCl- K_2CO_3 soln., but the relative intensity of the ester sulphate band at 1240 cm^{-1} was less and it decreased with increasing ethanol-solubility of the fractions. The shape of the strong carbohydrate OH band, at 1050 cm^{-1} , of the 20 and 30 % fractions differed from that of the 40 and 50 % fractions, especially on the high-frequency side, and the relative intensity of this band was greater in the latter two fractions. The major differences between the spectra were again in the $1000\text{--}700\text{ cm}^{-1}$ range. The 20 and 30 % fractions resembled chondroitin sulphate B (Mathews, 1958) but had an additional band at 994 cm^{-1} . A band close to this region is present in the spectrum of chondroitin sulphate C (Orr, 1954; Mathews, 1958), where it is accompanied by strong absorption at 820 and 775 cm^{-1} . Both these bands are absent from the spectra of the 20 and 30 % fractions. Since the 1000, 820 and 775 cm^{-1} bands in chondroitin sulphate C are all attributed to the ester sulphate group, the band at 994 cm^{-1} in the 20 and 30 % fractions is probably due to a different

group. Further, although some glucuronic acid was present in these fractions, the ethanol-solubilities of the fractions make it more likely that small amounts of the A isomer, rather than the C isomer, would be present with the B isomer (Meyer, Davidson, Linker & Hoffman, 1956).

In the 40 and 50 % fractions absorption at 994 and 833 cm^{-1} had disappeared and the band close to 928 cm^{-1} had been replaced by one at about 952 cm^{-1} in the 40 % fraction and at 942 cm^{-1} in the 50 % fraction respectively. The band at 852 cm^{-1} was reduced in intensity in the 40 % fraction and to a trace in the 50 % fraction, and a new band at 787 cm^{-1} had appeared in both. A second new band, present only as a trace in the 40 % fraction, appeared at 898 and 889 cm^{-1} in these two fractions respectively. When chondroitin sulphate A is depolymerized to the tetrasaccharide the 852 cm^{-1} band remains stationary, but that at 928 cm^{-1} shifts to 940 cm^{-1} . Further, on desulphation, the normal bands in the 1000–700 cm^{-1} range for chondroitin sulphates A and C disappear and are replaced by a weak band at 788 cm^{-1} (Hoffman *et al.* 1958). The shift of the 928 cm^{-1} band to higher frequencies coupled with the decrease in intensity of the 852 cm^{-1} band and the appearance of a band at 787 cm^{-1} confirm that during preparation the 40 and 50 % fractions had undergone depolymerization and desulphation, a conclusion already deduced on the basis of the low intensity of the 1240 cm^{-1} band and the analytical values given in Table 3. The spectra of the 40 and 50 % fractions confirm the presence of chondroitin

sulphate A. It seems unlikely that the C isomer was present, but in view of the extent of the desulphation which had occurred, the possibility that it may have been present originally cannot be definitely ruled out. The origin of the weak band at 889 cm^{-1} in the 50 % fraction is not known.

The protein-precipitation test showed that testicular hyaluronidase did not depolymerize the 20 and 30 % fractions. The 40 % fraction was completely depolymerized and the 50 % fraction largely so.

After degradation of the matrix with pepsin and trypsin, more hexosamine-containing material was recovered than was obtained by extraction with $\text{KCl-K}_2\text{CO}_3$ soln., but greater degradation of the chondroitin sulphates occurred. The presence of both chondroitin sulphates A and B was confirmed. The finding of neutral sugar in the mobile component of the 50 % fraction on electrophoresis in borate buffer again raises the question of the possible presence of keratosulphate. Although protein was known to be present in this fraction, no protein was revealed on the electrophoretograms. It is most likely that the chondroitin sulphate and neutral sugar were again linked to the same protein moiety but that the staining method was not sufficiently sensitive to show up the protein.

Composition of hen's-egg-shell membranes

The egg-shell membranes contained 1.85 % of ash, and, on an ash-free basis, 15.54 % of nitrogen. The nitrogen value is close to that (15.7 %) found by Jones & Mecham (1944) but differs from the

Table 4. *Amino acid composition of hen's-egg-shell membranes and cuticle*

	Membranes (g. of N/100 g. of protein N)	Cuticle (g. of N/100 g. of protein N)	Membranes (g. of amino acid/ 100 g. of organic matter)	Cuticle (g. of amino acid/ 100 g. of organic matter)
Aspartic acid	6.4	6.1	9.4	9.0
Glutamic acid	9.1	7.8	14.8	12.8
Arginine	9.6	12.7	4.6	6.2
Lysine	3.0	6.4	2.4	5.2
Histidine	4.9	1.4	2.8	0.8
Cystine	6.2	3.1	8.2	4.2
Methionine*	4.7	5.3	6.0	6.9
Valine				
Glycine	4.5	11.1	3.7	9.3
Serine	4.7	4.9	5.4	5.8
Alanine	2.7	3.6	2.6	3.6
Threonine	3.7	4.4	4.8	5.8
Isoleucine	1.0	3.1	1.5	4.5
Leucine	2.3	2.9	3.3	4.2
Tyrosine	3.3	5.2	2.9	4.6
Phenylalanine	2.1	1.6	3.9	3.0
Proline	5.2	3.3	6.6	4.3
Hydroxyproline	0.0	0.0	0.0	0.0
Total	73.4	82.9	82.3	90.2

* Methionine and valine were not quantitatively separated and the combined values are calculated as valine.

values of 13.16% and 16.57% given by Plimmer & Rosedale (1925) and Calvery (1933) respectively. The amino acid composition is given in Table 4; 73.4% of the non-hexosamine nitrogen and 82.3% of the organic matter were recovered as amino acids. Both methionine and valine were identified on two-way chromatograms but, as they are not separated by the quantitative method, both have been calculated as valine. The shell-membrane protein is distinguished from the matrix and cuticle proteins by its high content of cystine, histidine and proline. The analysis supports the view that the egg-shell membranes are keratin.

Even after prolonged washing with water the membranes retained some carbohydrate material (Table 1). Hexosamine and galactose were found with probably some mannose, but the low concentration of sugars in the presence of high concentrations of amino acids made identification difficult. Neither fucose nor uronic acid was found. A trace of precipitate was formed on testing for sulphate but the amount was too small for estimation.

Composition of hen's-egg-shell cuticle

The egg-shell-cuticle preparation contained 3.49% of ash, and, on an organic-matter basis, 15.94% of nitrogen. The amino acid composition (Table 4) shows that the cuticle protein differs from the membrane and matrix proteins in having a higher content of lysine, glycine and tyrosine and a cystine content midway between the high content of the membrane and the low content of the matrix. Analysis for sugars demonstrated the presence of galactose, mannose, fucose and hexosamine, but no uronic acid was found. The molar ratio hexose: neutral sugar (Table 1) was 1:0.93.

DISCUSSION

Egg-shell matrix

Tyler & Geake (1953, 1958) found a variation in the nitrogen content of the true shell, i.e. the shell without membranes and cuticle, of hen's eggs of from 0.210 to 0.306%. Therefore, on the basis of nitrogen content $\times 6.25$, from 1.3 to 1.9% of the true shell consists of an organic matrix. This remains as an organized structure on decalcification of the shell with EDTA. The matrix was found to consist of a protein-polysaccharide complex, thus confirming the histochemical work of Simkiss & Tyler (1957). At least 70% of the matrix is protein and there is about 11% of polysaccharide. Simkiss & Tyler (1957) found some fat to be present, especially in the mammillae, but fat has not been estimated in the present study.

Almquist (1934) recognized that the matrix protein differed from the protein of the shell

membranes and classified it as a collagen. This classification must now be abandoned, for the matrix protein contains no hydroxyproline. Visual examination of two-way chromatograms showed no marked difference between the amino acid composition of the matrix and that of the protein moiety of the hexosamine-containing fractions extracted with potassium chloride-potassium carbonate solution or liberated by digestion with enzymes. It is therefore probable that the matrix protein is homogeneous.

Chondroitin sulphates are present in the matrix. The presence of a non-collagenous protein associated with chondroitin sulphate in cartilage was established by Shatton & Schubert (1954), and amino acid analyses have been given by Muir (1958) working with pig cartilage and Partridge & Davis (1958) with bovine cartilage. There is considerable similarity between the amino acid composition of the matrix protein and of these non-collagenous cartilage proteins.

The matrix contains galactosamine, glucosamine, glucuronic acid and iduronic acid, galactose, mannose and fucose. This spectrum of sugars is widely distributed throughout the animal kingdom (Bettelheim-Jevons, 1958). Simkiss & Tyler (1957), using histochemical methods, demonstrated the presence of acid polysaccharide. The present study has established that chondroitin sulphates A and B are present. Chondroitin sulphates have been isolated with a sulphate:galactosamine molar ratio 1.3:1 (shark cartilage, see Suzuki, 1960) and 1:3 (head fraction of bovine trachea, Bettelheim & Philpott, 1960). Although no preparation from egg-shell matrix of chondroitin sulphate A free from neutral sugars was obtained in the present work, the purified chondroitin sulphate complex extracted under mild alkaline conditions had a molar ratio sulphate:hexosamine:uronic acid of 1:1:0.97 on the basis of a 1:1 sulphate:hexosamine ratio. Assuming that all the ester sulphate of the matrix is derived from chondroitin sulphate, then 35% of the total polysaccharide in the matrix is present in this form. The remainder is polysaccharide containing galactosamine, glucosamine and neutral sugars. Although the analytical values indicate that the molar ratio hexosamine:neutral sugar is close to 1:1, no evidence is yet available about the manner in which the sugars are linked together nor about the number of polysaccharides present.

There is more neutral sugar than uronic acid in the matrix and yet, while uronic acid-rich fractions, with up to a 15-fold increase in uronic acid content, have been isolated, there was no fraction correspondingly rich in neutral sugars. Small amounts of neutral sugars have been present in all the preparations, but only one, the 50% ethanol-precipitated fraction obtained after enzyme

degradation of the matrix, had even double the concentration of neutral sugars originally present. Failure to obtain a fraction rich in neutral sugars after mild alkaline treatment could be explained if these sugars were combined with protein in a form more stable to alkali than the protein-chondroitin sulphate bond. Treatment of the matrix with enzymes, however, caused almost complete degradation and solution of the tissue and very little protein was subsequently precipitated from this solution with the Sevag treatment. If the molecular size of the polymers containing neutral sugars were similar to that of the chondroitin sulphate polymers, precipitation with ethanol would be expected to give fractions considerably enriched with neutral sugars. As this was not found and as the small enrichment which did occur was in the most ethanol-soluble fraction, it is possible that the neutral sugars occur in short-chain polymers which may either have been lost during dialysis or which may have escaped precipitation with ethanol. A similar picture of carbohydrate present in a glycoprotein as many prosthetic groups of relatively small size has been given for the viral haemagglutination inhibitor present in urine (Gottschalk, 1952), and for a salivary glycoprotein (Gottschalk, 1960).

Simkiss & Tyler (1958) showed that the decalcified hen's-egg-shell matrix can act as a chelating agent and suggested that it was the acid polysaccharide containing hexosamine which was responsible for this effect. There was some evidence that the chelating power of the mammillary layer was less strong than that of the spongy layer. In the thicker shells of the ratite birds, Tyler & Simkiss (1959) found a marked difference between the mammillary and spongy layers (of ostrich, emu, cassowary and rhea egg shells) in both chelating power and staining reactions. To explain the difference, they suggested that two acid 'mucopolysaccharide complexes' were present and that the mammillary-layer complex contained proportionately more neutral sugar and less uronic acid residues than the spongy-layer complex. The chemical analysis of hen's-egg-shell matrix has shown that besides the chondroitin sulphates there is also at least one other hexosamine-containing polysaccharide. Although the latter may contain neutral sugars, it has no ester sulphate or uronic acid groups. However, it might also contain sialic acid which would react with histochemical stains similarly to other acid groups of the tissue (Montreuil, Defretin, Clay & Caenen, 1959). If the chemical composition of ostrich, emu, cassowary and rhea egg-shell matrices is similar to that of the hen's-egg shell, then the differences between the spongy and mammillary layers observed by Tyler & Simkiss (1959) could be explained if the chon-

droitin sulphates were concentrated in the former and the other polysaccharide(s) in the latter.

Egg-shell membranes and cuticle

Analysis of the egg-shell membranes confirmed the results of earlier workers (Calvery, 1933; Wolken, 1951) that they are mainly composed of keratin. A small amount of hexosamine and neutral sugars remains firmly attached to the membrane even after prolonged washing. In the intact egg, the membranes are in intimate contact with egg-white glycoproteins. However, galactose was present in membrane preparations in greater amount than mannose, whereas in egg-white glycoproteins the concentration of mannose exceeds that of galactose. Further, the molar ratio of hexosamine to neutral sugar found for the membrane differs considerably from the ratios for egg-white glycoproteins quoted by Bettelheim-Jevons (1958). It is therefore unlikely that the membrane polysaccharide is due to contamination of the preparations with egg white.

Simkiss (1958) observed membrane fibres passing right into the mammillary cores, which are rich in polysaccharide, and microscopical examination of membrane preparations showed that small amounts of mammillary core material remained adhering to them. Whether all the membrane polysaccharide is accounted for by the mammillary core residues or whether some forms part of the membrane proper is not known.

The cuticle also consists mainly of protein, but the protein differs from that of both the membranes and the matrix. This is contrary to the suggestions made by earlier workers but supports the histochemical results of Simkiss (1958). Again hexosamine and neutral sugars were present, but no ribose or deoxyribose was found, thus confirming the view of Marshall & Cruickshank (1938) that the cuticle does not have a cellular structure.

SUMMARY

1. Hen's-egg-shell matrix, cuticle and membranes have been prepared separately by decalcification of the shell with ethylenediaminetetraacetic acid.
2. The matrix consists mainly of a protein-polysaccharide complex containing 11% of polysaccharide and at least 70% of protein.
3. The matrix protein is characterized by the absence of hydroxyproline, the low concentration of aromatic and sulphur-containing amino acids and the presence of about twice as much dicarboxylic amino acids as basic amino acids.
4. Chondroitin sulphates A and B are present in the matrix and comprise 35% of the total polysaccharide.

5. Some galactosamine is present in a form other than chondroitin sulphate, along with glucosamine, galactose, mannose and fucose.

6. The concentration of galactose is greater than that of mannose, and fucose is present in trace amounts. The molar ratio hexosamine:neutral sugar is close to 1:1, but no information is available as to the form(s) in which they are combined.

7. The membranes are mainly keratin with a small amount of polysaccharide containing hexosamine, galactose and probably mannose. Part or all of the polysaccharide may originate from the mammary cores.

8. Some 90 % of the cuticle is protein, which is distinguished from the membrane and matrix proteins by its content of tyrosine, glycine, lysine and cystine. Hexosamine is present together with galactose, mannose and fucose.

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