

A Histochemical Study of the Organic Matrix of Hen Egg-Shells

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SUMMARY

A study of the organic material of the hen egg-shell has been made by the use of recognized histochemical techniques.

It has been found that, in the so-called spongy layer of the shell, the organic matrix consists of a protein / acid mucopolysaccharide complex. The organic material of the mammillary layer appears to be more concentrated in a central core, and is somewhat different in that it contains fat and a reducing substance.

The protein / acid mucopolysaccharide complex is a collagen-like material, but is probably not collagen itself because the protein portion is low in hydroxyproline and the acid mucopolysaccharide is most likely mucoitin and not chondroitin sulphuric acid.

The results are discussed in relation to shell calcification and it is suggested that the protein / acid mucopolysaccharide complex is first firmly attached to the keratin of the membrane, but once this is accomplished it links on to material of its own kind. Calcification proceeds simultaneously, but near the points of contact with the membrane (i.e. in the core of each mammilla) calcification is not so intense as it is in the rest of the shell.

INTRODUCTION

ALTHOUGH the hen egg-shell is of fundamental importance both in the commercial preservation and marketing of eggs, and also in the development of the incubated chick, little work has been done on shell structure.

Stewart (1935) gave a good general review of what was known of the structure of the hen egg-shell at that time. He recognized the two shell membranes which lie over the albumen of the egg and which are composed of a network of fibres. Attaching the outer membrane to the shell are the mammillae, which Stewart described as knobs composed of large spherulite crystals of calcite. These knobs have organic cores and are roughly hemispherical, being continuous above with the main body of the shell, which is referred to as the spongy layer. This is badly named because it is composed of calcite crystals bound together by a sparse organic matrix. The shell is covered by a cuticle or 'bloom' which also covers the mouths of the pores. The pores are funnel-shaped tubes, round or oval in cross-section. They start from the interstices between the mammillae, pass through the main body of the shell, and finally open under the cuticle (fig. 1). More recently, Tyler (1956) has studied certain aspects of shell structure, including pore size and shape, by using plastic embedding techniques.

Moran and Hale (1936) investigated the structure of the two shell membranes, which they were able to divide into several layers. The outer mem-

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brane, i.e. the one next to the shell, is composed of three layers, the outermost consisting of keratin fibres $2-5\mu$ in diameter and the other two of mucin fibres 0.8μ in diameter. The inner membrane, which lies immediately over the egg albumen and forms the inner boundary of the air space, is composed of two rather indistinct layers of finer keratin and mucin fibres. The outer

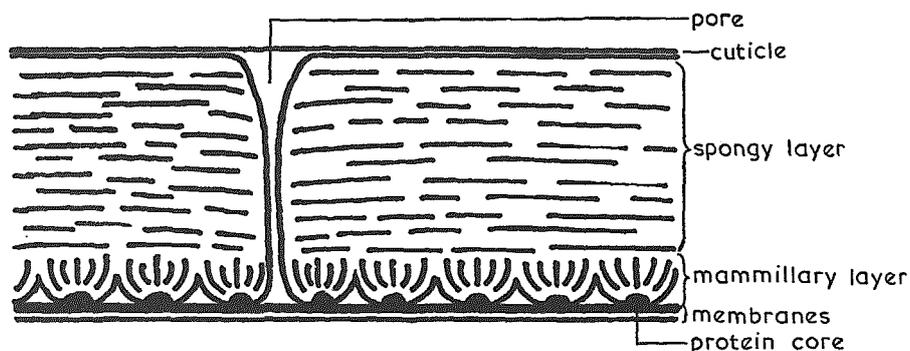


FIG. 1. Conventional drawing of a radial section of an egg-shell, showing mammillae with protein core, spongy layer, membranes, and cuticle. The section also shows a pore.

membrane is the thicker of the two. According to Calverly (1933) both membranes are composed mainly of keratin fibres.

Little work has been done on the organic matrix which binds the calcite crystals of the shell together, except that Almquist (1934) has stated, on the basis of a chemical investigation, that this protein differs from the membrane protein and may be classified as a collagen-like substance.

Dickie (1848) originally suggested that the cuticle was a cellular epithelium over a basement membrane. This view has never been directly attacked, but Marshall and Cruickshank (1938) stated that they considered the cuticle to be a somewhat porous but apparently structureless substance. These authors also introduced the concept of 'cuticular plaques', which are the thickenings of the cuticle over the mouths of the pores; they further suggested that these regions of the cuticle might act as an additional evaporating surface for the removal of water vapour from the egg. Moran and Hale (1936) and Haines and Moran (1940) considered the cuticle to be mainly mucin.

Tyler and Geake (1953) treated shells with a boiling 2.5% sodium hydroxide solution for 5 min and showed that this removed the membranes cleanly and probably some of the cuticle. Further treatment with 10% sodium hydroxide solution led to the removal of more protein, but after about 7 hours no more protein could be removed. They considered that the resistant protein was matrix protein, mechanically beyond the attack of the solvent, and that the portion removed was probably pore protein present in the pore channels.

It was felt that much remains to be done on the proteins of the shell and it was decided to use the simple staining and more complex histochemical techniques as a start. This paper describes work on the matrix and mammillae.

METHODS

Decalcification

Shells were decalcified with solutions of the sodium salts of ethylene diamine tetra-acetic acid (EDTA). The original method was to use an 8% solution of the disodium salt in water (pH 4.3–5.3) which had been brought to pH 7 with either sodium hydroxide or sodium bicarbonate. More recently, a 6% solution of the trisodium salt has been used without further treatment. This solution has a pH of about 8.

The use of a chelating agent in a slightly alkaline solution eliminates the possibility of carbon dioxide bubble formation during decalcification. This is a great advantage because the ease with which carbon dioxide is produced during acid decalcification of shells probably explains the damage to the organic structure and consequently the poor results obtained by previous workers.

Formalin was added to the decalcifying solution to a strength of 6% so that fixation would accompany, if not precede, the decalcification. Used in this way any acidity in the formalin solution was neutralized by the EDTA solution and hence did not produce bubbles of carbon dioxide from the shell. Formalin was chosen as the fixative because (a) it can be used in an approximately neutral solution, (b) it is not dependent on the presence of metallic ions which the EDTA would remove, (c) it is of wide general application both histologically and histochemically, and (d) its mechanism of fixation is at least partially understood.

Shells decalcified in this way were washed in water, dehydrated in a series of alcohols, cleared in benzene, and embedded in 52°–56° C paraffin wax. Sections were then cut at 5–10 μ for routine work.

Histological and histochemical techniques

The sections were originally stained with Mallory's triple stain and from the general structure revealed it was then possible to use more specific histochemical methods. As the work progressed the methods became centred around tests for three main types of substance, namely, mucin, protein, and fat. The methods used have been based chiefly on Pearse (1953), but references to original work are given where necessary.

Mucins. These have been investigated by using such stains as mucicarmine, toluidine blue, and alcian blue (Steedman, 1950). Hale's (1946) dialysed iron method, though less specific, was also used. The periodic acid / Schiff test (PAS) of McManus (1948) has also been used in this study and also the modification employing acetylation before the test (McManus and Cason, 1950).

The effect of calcium ions on the metachromatism produced by toluidine blue has also been studied. This technique was first described by Sobel and Hanok (1952) and Sobel and Burger (1954).

Proteins. Millon's reagent was used as a test for 'phenolic' proteins (Pollis-

ter's modification as quoted by Gomori (1952)), but a more satisfactory result has been obtained by using the tetrazotized benzidine- β naphthol (TBN) method of Danielli (1947).

Fats. Sudan black B in propylene glycol (Chiffelle and Putt, 1951) or in 70% alcohol, Sudan III, Fettrot red 7 B (Ciba), and Nile blue sulphate (Cain 1947) have been used along with the performic acid / Schiff test (PFAS) of Pearse (1951). Since this last test gives a positive result with either keratin or unsaturated fats it was necessary to use it in conjunction with other tests such as control slides stained with Sudan black B or the use of methylene blue in veronal acetate buffer at pH 2.6 after performic acid treatment. The former will detect fat and the latter will distinguish between the oxidation-products of the two kinds of material.

The above methods for fat have been employed before and after attempting the extraction of fat from sections. The attempt was made by treating consecutively with cold acetone, hot acetone, hot ether, and hot chloroform / alcohol (24 h in each), or alternatively pyridine at 60° C for 24 h.

Enzyme treatments. Hyaluronidase (Benger's 'hyalase') has been used in a 1 mg/ml solution in 0.85% saline in an attempt to 'digest' mucins. Pepsin and trypsin solutions were also tried with the appropriate tests applied before and after the enzyme treatment. Crystalline pepsin was used at a strength of 2 mg/ml in 0.02 N.HCl to give a pH of 1.6, and the slides were incubated for 2-3 h at 37° C. Purified trypsin was used at 0.1 mg/ml in 0.03 M phosphate buffer at pH 6.0, and the slides were incubated at 37° for 15-60 min.

Diastase has also been used in 1% aqueous solution for 30 min at room temperature as a test for glycogen.

Basiphilia. The methylene blue extinction test (MBE) was used over a range of pH values.

Reducing compounds. The ferri-ferricyanide test of Chevrement and Frederic (1943) was used, being tried before and after the blocking of sulphhydryl groups with phenyl mercuric chloride in butanol.

Vital staining

A laying hen was injected with 5 ml of a 1.25% solution of Evans blue stain. This stain was forced about 10 cm. up the cloaca and oviduct of the hen with a syringe about 4 h after laying. A second hen was similarly treated. In both cases much of the stain was forced out by peristalsis when the syringe was removed, but sufficient almost certainly remained in the tract. Each hen laid the next day at the expected hour and the shells were examined.

The hen used in the first experiment also had 10 ml Evans blue stain injected intravenously into each wing vein; again this was done 4 h after an egg had been laid and the bird laid next day as expected.

It is of interest to note that 3 weeks after these treatments the hens were killed and the liver, ovaries, oviduct, and cloaca were well stained, indicating that the injections had reached their objective.

RESULTS

Matrix

The TBN reaction showed clearly that the matrix contains some protein; other tests indicated mucin substances, whilst the diastase test showed glycogen to be absent.

The matrix stained red with mucicarmine, indicating that the blue staining with Mallory's triple stain was caused by a mucin substance, whilst Hale's dialysed iron method gave a positive result, suggesting acid mucopolysaccharide; alcian blue gave a result strongly supporting this. The degree of basiphilia was tested by the MBE test. Sections left in the solution for 24 h and viewed without dehydration showed that the matrix could bind the dye at pH 5, though it was usually unable to do so at pH 4. This suggests that the material is acidic.

The PAS test was strongly positive and furthermore the reaction was blocked by a prior treatment of the sections with acetic anhydride. The effect of acetylation was then removed by alkali and the test became positive once more. This indicates that two hydroxyl groups or one hydroxyl and one amino group are present on adjacent carbon atoms, possibly as carbohydrate residues, in the matrix. Toluidine blue staining was also tried on the acetylated material and the metachromatism was unaffected. It would thus appear that the two tests detect different aspects of the same molecule, the PAS test detecting the groups on adjacent carbon atoms and the toluidine blue the polymerized and/or acidic nature of the molecule. Direct toluidine blue staining either in very dilute (0.001%) or in acidic buffered (pH 3.6) solutions at 0.01% showed that the matrix was strongly γ -metachromatic.

Sobel and his co-workers (Sobel and Burger, 1954; Sobel and Hanok, 1952) investigated the effect of calcium ions on the metachromatism of rachitic bone, part of which had not been calcified. The present writers had, naturally, to be content with decalcified shells. In the first experiment with formalin-fixed paraffin sections, solutions of 0.01% toluidine blue in glass-distilled water gave, with various concentrations of calcium, the following degrees of metachromatism as assessed visually:

Ca ⁺⁺ <i>M.eq. per litre</i>	<i>Metachromatism</i>
0	++++
5	++++
20	++++
40	++
83	+
250	Trace
500	0

Staining a similar section in toluidine blue containing 250 m.eq. per litre sodium ions had no effect on the metachromatism (i.e. ++++), but when the slide was transferred to a solution with 250 m.eq. per litre calcium ions the metachromatism disappeared. These results are almost identical with

those obtained by Sobel, and indicate competition between toluidine blue and calcium ions for combination with the matrix substance. The experiments were repeated with unfixed frozen gelatine-embedded material, instead of formalin-fixed paraffin sections, but substantially the same results were obtained. Thus the decalcified unfixed material gives the same results as those reported by Sobel and Burger (1954) for fixed rachitic bone. The process of calcification of the shell in itself may therefore produce some change in the matrix.

A further experiment was tried with formalin-fixed paraffin sections which had been digested for 3 h with pepsin. These sections showed only a faint metachromatism and it was thought that the protein may have been removed, causing the liberated mucopolysaccharide to undergo some degree of depolymerization and hence to show a low level of metachromatism. To follow up this point toluidine blue with various concentrations of calcium was tried on slides after pepsin treatment. There was, however, no increase in metachromatism, but only a falling off at 100 m.eq. per litre as previously. It would appear, therefore, that the reduced metachromatism found is caused by a small part of the matrix which has not been completely digested, i.e. most of the protein is removed and the liberated mucopolysaccharide lost in the solution. This was confirmed by the PAS and TBN tests on sections digested by pepsin.

When trypsin was tried on formalin-fixed paraffin sections, no effect was shown structurally or in their response to toluidine blue. Hyaluronidase was then tried for 3 h and the result was similar to that for trypsin; however, there was possibly some reduction in metachromatism after 18 h. It was found in other experiments with formalin-fixed paraffin sections after 3 h in hyaluronidase that if a slide was stained in dilute toluidine blue (0.01%) and viewed immediately it showed no metachromatism, but if left for 30–60 min the full colour developed. This was further investigated by placing slides treated with hyaluronidase either in glass-distilled water, or in a solution of 15 m.eq. per litre calcium ions for 1 h. The slides were then washed and stained simultaneously in 0.005% toluidine blue, metachromatism being scored against time. The following results were obtained as means of three experiments:

<i>Time (min)</i>	<i>Treatment</i>	
	<i>Water</i>	<i>Ca solution</i>
1	—ve	$\frac{1}{2}$ +
3	—ve	++
5	—ve	++++
10	+	++++
15	+	++++
20	++	++++
30	++++	++++

These results are similar in their interpretation to those obtained by Sobel and Burger (1954) for unfixed rachitic bone. It appears that formalin fixation does not stop depolymerization by hyaluronidase, but anchors the depoly-

merized mucopolysaccharide on to the protein remaining on the slide. The depolymerized mucopolysaccharide is then easily repolymerized by treatment with calcium ions. Toluidine blue also does this, but much more slowly at the concentration used in this experiment.

Mammillae

The mammillary 'knobs' are essentially the inner part of the shell and in its staining properties their organic material is very similar to the organic matter of the matrix with which it is continuous. Thus the mammillae stained with Hale's dialysed iron and mucicarmine and gave a stronger PAS reaction than the main part of the matrix. They differed from the matrix in that toluidine blue stained orthochromatically and Mallory's triple stain showed a red core within the base of each one. Staining with Sudan black B showed that there is a large amount of fat present in the mammillae, whereas the matrix only stains very faintly.

The ferri-ferricyanide test showed that the mammillae contain some compound which gives a positive reaction. This test was then repeated on alcohol-fixed material with the same result. Furthermore, the reaction was not prevented by a previous treatment of up to 14 days with phenyl mercuric chloride in butanol, which indicates that sulphhydryl (-SH) groups were not responsible for the reaction. Substances, other than sulphhydryl groups, which are capable of reducing ferricyanides are therefore present in the mammillae. The matrix itself did not give a positive result with the ferri-ferricyanide test.

The TBN reaction was strongly positive in the mammillae, showing these to be rich in protein, but the matrix itself gave a weaker reaction. This was confirmed with Millon's reagent.

The mammillae, therefore, although part of the shell, give some reactions which indicate that they contain organic compounds which are different from those in the rest of the shell.

Gersh and Catchpole (1949) have expressed certain ideas on the relationship between polysaccharide complexes and reactions with calcium. They consider that water-soluble unpolymerized acid mucopolysaccharides would copolymerize with calcium, and were important in calcification. This unpolymerized material was also more reactive towards the PAS test. It was, therefore, decided to investigate this point with regard to the mammillae, which are probably the first sites of shell calcification. Shells were decalcified for several days in aqueous EDTA solutions without any fixative. Water-soluble components were thus given ample time to dissolve and diffuse away, provided they were not affected by the EDTA. This material was then dehydrated, sectioned from paraffin blocks, and compared with alcohol and formaldehyde EDTA decalcified material from the same shell. The material was examined in tangential section so that the mammillary region could be studied over a greater area. Mallory's triple stain was used on all these sections and the aqueous decalcified unfixed material showed no loss by solution of either the red core or the blue outer covering of the mammillae.

In the vital staining experiments it was hoped that depolymerized polysaccharide material would show up if present. Thus, if it is in the blood, the intravenous injection should have caused the dye to be deposited in the shell. If, on the other hand, the polysaccharide was not depolymerized until after secretion on to the membrane, then the uterine injection should have stained the shell. The experiments were negative. Of course, it has to be assumed that sufficient dye reached the uterus in one case and that the dye molecule can pass through the wall of the uterus in the other. However, when taken in conjunction with the water extraction experiment, the results suggest that depolymerized water-soluble acid mucopolysaccharide is not present in the shell.

DISCUSSION

The matrix contains some protein as shown by the protein tests and also by treatment with enzymes. Trypsin had no effect but pepsin appeared to remove the protein and liberate the acid mucopolysaccharide. Trypsin tends to attack peptide links associated with the basic amino-acids arginine and lysine, whilst pepsin attacks the peptide links of dicarboxylic amino-acids such as aspartic and glutamic acids. It is, therefore, of interest to note that Balch (1956) found both aspartic (medium reaction) and glutamic acid (strong reaction) in a hydrolysate of matrix with 'pore' protein run qualitatively on a paper chromatogram.

The various reactions with mucin stains, PAS test, and metachromatism with toluidine blue strongly suggest the presence of an acid mucopolysaccharide. The question, however, arises whether the material is chondroitin or mucoitin sulphuric acid and it is here that the experiments with hyaluronidase offer some help.

Hyaluronidase, according to Stacey (1946), first separates the protein from the polysaccharide, then rapidly depolymerizes the polysaccharide; there is then a partial or complete hydrolysis of it, which results in the liberation of acetyl glucosamine and glucuronic acid.

In experiments on the shell matrix, hyaluronidase appears to depolymerize, but not to hydrolyse the mucopolysaccharide, because it has been shown that the matrix can be repolymerized with calcium ions back to its original state. This suggests mucoitin sulphuric acid. The strong reaction to the PAS test, and the secretion of shell from the oviduct, which contains mucin-secreting cells, also tend to favour the idea of mucoitin sulphuric acid.

The combination of an acid mucopolysaccharide and a protein agrees essentially with the concept suggested by Almquist (1934), namely, that the shell matrix is a substance resembling collagen. However, it is important to recognize that the protein moiety might be quite different from collagen itself and, as already stated, the polysaccharide is probably mucoitin sulphuric acid. Support for this again comes from the qualitative chromatographic tests of Balch (1956), who showed that there was no evidence of the large amounts of hydroxyproline to be expected if the protein were collagen.

At this stage some comparison may be made with the work of Gersh and Catchpole (1949). They presented a table showing the effects of various solvents and enzymes on the reactive groups of the ground substance (glycoprotein = acid mucopolysaccharide) of mouse skin in frozen-dried material. Apart from differences in the result with trypsin, possibly explained by a difference in technique, the results with shell matrix are the same. Gersh and Catchpole consider that chemically the ground substance is relatively inert, at least as far as the carbohydrate-protein complex is concerned; a state which is consistent with the polymerized form of the material. They also believe that depolymerization of the polysaccharide complexes may result in the liberation of increasing numbers of reactive groups, some of which may combine with calcium. This depolymerization may thus play a part in the calcification of cartilage. Such a concept is obviously of great interest in the present study of the shell, where calcification is the major process in its formation. However, experiments with shells decalcified in aqueous solutions showed no indication of depolymerization. Similarly, experiments with living hens, designed to yield information similar to that provided by the injection experiments of Gersh and Catchpole, failed to reveal the presence of any water-soluble depolymerized polysaccharide, either coming from the blood or formed in the developing shell.

Recently, a more likely and better-substantiated mechanism of calcification has been put forward. Levine and others (1949) studied the disease *calcinosis universalis* and suggested that decalcification removes inorganic materials, thus making available acid groups which then stain metachromatically with toluidine blue. These acid groups cannot react with toluidine blue whilst they are blocked by elements such as calcium and thus calcified material stains orthochromatically. Later Rubin and Howard (1950) found acid mucopolysaccharide in association with calcification in bone, in calcified cartilage, and in *calcinosis universalis* and other similar conditions. They naturally suggested that the calcium-binding capacity was due to the acid mucopolysaccharide. Sobel and Burger (1954) then showed that toluidine blue, like inorganic cations, could produce a reversible inactivation of the calcification mechanism, showing that toluidine blue competes with the calcium ions for combination with something, which, in the case of ossifying cartilage, was assumed to be chondroitin sulphuric acid. An earlier paper by Sobel and Hanok (1952) had shown that the chondroitin sulphuric acid could bind much more calcium if it was coupled with a protein to give a material resembling collagen.

In the present experiments the results obtained with toluidine blue and calcium solutions were almost identical with those of Sobel, who used rachitic bones. These results therefore suggest that both the acid mucopolysaccharide and the protein components of the egg-shell matrix are vitally important to the calcification mechanism.

The mammillae appear to be basically similar to the shell matrix but to contain more protein and fat. Two of their properties, namely, the orthochromatic staining of the core with toluidine blue and a stronger reaction

with the PAS test, may be explicable on the basis of a more compact organic structure and possibly a greater content of protein. However, these two properties are also the ones to be expected from a substance which is not polymerized. Thus, if calcium ions are in fact to some extent responsible for polymerization, the mammillae may be partly laid down before calcification starts and a central core may remain unpolymerized.

Acid mucopolysaccharides have been found condensed with keratin in the enamel of teeth and it may be that the firm attachment of the mammillae to the membrane is brought about first by a condensation of acid mucopolysaccharide with membrane keratin.

It is possible that this suggested keratin-mucopolysaccharide complex is less polymerized than true mucopolysaccharide, in which case it might give the tests for unpolymerized material, but, because of its combination with keratin, it might not be water-soluble. This situation could explain the difference between the present results of tests based on the work of Gersh and Catchpole and those of Gersh and Catchpole themselves. However, even if this is so, the concept of calcification put forward by Sobel and Burger (1954) is the more acceptable.

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