P. C. M. Simons

Ultrastructure of the hen eggshell and its physiological interpretation

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Dit proefschrift met stellingen van Petrus Cornelis Maria Simons, landbouwkundig ingenieur, geboren te Helvoirt op 17 juni 1937, is goedgekeurd door de promotor, drs. A. M. Frens, hoogleraar in de dierfysiologie, waarin begrepen de stofwisselingsleer van mens en dier.

De Rector Magnificus van de Landbouwhogeschool

J. M. Polak

Wageningen, 26 april 1971

P. C. M. Simons

Central Institute for Poultry Research 'Het Spelderholt', Beekbergen

Ultrastructure of the hen eggshell and its physiological interpretation

Proefschrift
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doctor in de landbouwwetenschappen,
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The coverpicture: Surface of fresh hen-egg

Abstract

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Financial losses from egg breakage have increased over the years. To seek any relation with shell strength, the structure of eggshell and shell membranes were examined by light microscope, transmission electron microscope and scanning electron microscope. They were taken from eggs laid by laying hens, from eggs removed from the oviduct and from eggs laid prematurely, and occasionally from incubated hen eggs and eggs of guinea-fowl, duck, turkey, emu and ostrich.

The results with different methods of measurement showed how shell membranes vary in thickness, how some structural properties of shell may influence shell strength, how the shell and shell membranes form and how they are destroyed during incubation and what factors influence the cuticular surface of the eggs.

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STELLINGEN

I

De gangbare methodieken ter bepaling van de sterkte van de eischaal zijn niet bevredigend; het verdient derhalve aanbeveling meettechnieken te ontwikkelen, die beter te interpreteren informatie verschaffen.

Dit proefschrift.

II

Het is opmerkelijk, dat Balch en Cooke op grond van de aanwezigheid van hydroxyproline in de schaalvliezen niet concluderen, dat collageen of elastine in de schaalvliezen voorkomt.

Balch, D. A. & R. A. Cooke, 1968. Symposium on the physiology of egg shell and albumen formation, Grignon, Frankrijk.
Dit proefschrift.

III

De veronderstelling van Simkiss en Tyler dat aan de afzetting van anorganische stof in de schaal een afzetting van organische stof voorafgaat, is juist gebleken.

Simkiss, K. & C. Tyler, 1958. Q. Jl microsc. Sci. 99 : 5-13.
Dit proefschrift.

IV

De organische matrix in de schaal speelt gedurende het laatste deel van de broedperiode een rol bij het transport van calcium uit de schaal naar het zich ontwikkelende embryo.

Dit proefschrift.

V

Uit de vorige eeuw zijn vele waardevolle literatuurgegevens over de eischaal beschikbaar. Hiervan is door vele onderzoekers te weinig gebruik gemaakt.

Dit proefschrift.

VI

Het wassen van consumptieëieren dient, onder bepaalde voorwaarden, te worden toegestaan.

Haines, R. B. & T. Moran, 1940. J. Hyg., Camb. 40 : 453-461.

VII

Het percentage eieren dat op batterijbodems wordt beschadigd, kan worden verlaagd door een betere constructie van deze bodems.

Carter, T.C., 1970. Wld's Poult. Sci. J., 26 : 549-561.

VIII

Verdere verbetering van landbouwhuisdieren vraagt meer aandacht voor de fysiologische processen die met de produktie samenhangen.

IX

Naar analogie van het onderwijs aan de praktijkscholen voor de pluimveeteelt en de varkenshouderij is vakonderwijs op de overige gebieden binnen de landbouw gewenst.

X

Meer onderzoek naar de bruikbaarheid van kippemest als voederbestanddeel voor herkauwers is van belang.

XI

In ontwikkelingslanden kan de pluimveehouderij meer bijdragen tot de voorziening in dierlijk eiwit.

XII

Het is onbevredigend dat pluimveevlees niet als vlees wordt aangemerkt in de vleeskeuringswet, noch in de warenwet.

Vleeskeuringswet: algemene bepalingen
Warenwet : vlees- en vleeswarenbesluit.

Proefschrift van P. C. M. Simons
Wageningen, 28 juni 1971.

Woord vooraf

Dit proefschrift is het resultaat van een onderzoek bij het Instituut voor de Pluimveeteelt 'Het Spelderholt' te Beekbergen en het Laboratorium voor Fysiologie der Dieren van de Landbouwhogeschool te Wageningen.

Professor Frens dank ik voor zijn bereidwilligheid als promotor op te treden. Ik ben hem zeer erkentelijk voor de leerzame discussies en de prettige wijze waarop hij mij bij het werken aan dit proefschrift heeft geadviseerd.

Het eerste deel van dit onderzoek is verricht onder de deskundige leiding van Professor Brouwer aan wie ik veel dank verschuldigd ben voor zijn wijze lessen.

De directeur van 'Het Spelderholt', Ir J. Folkerts, diens voorganger, Ir P. Ubbels, alsook het hoofd van de afdeling Produktie, Ir A. R. Kuit betuig ik mijn oprechte dank voor het feit dat zij mij in de gelegenheid hebben gesteld mijn onderzoek uit te werken.

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Een groot deel van het experimentele werk is verricht bij de afdeling Elektronenmicroscopie van de Stichting Technische en Fysische Dienst voor de Landbouw te Wageningen. Met name de heer S. Henstra dank ik voor zijn goede raadgevingen en technische bijstand, Mej. E. van Lohuizen en Mej. D. Quartel voor hun medewerking bij het vervaardigen van preparaten, en de heer H. G. Elerie voor zijn aandeel in het fotografisch werk.

Ik heb het zeer op prijs gesteld dat Professor Tyler van het Laboratorium voor Fysiologie en Biochemie van de Universiteit te Reading (Engeland) mij de gelegenheid heeft geboden tijdelijk in zijn laboratorium te werken.

Met waardering memoreer ik de medewerking van Professor Pfefferkorn van het Instituut voor Medische Fysica van de Universiteit te Münster en van de heer T. Takeuchi, directeur van 'Jeolco (Europe) S.A.' te Parijs, die het raster-elektronenmicroscopisch onderzoek hebben mogelijk gemaakt.

Alle medewerkers van 'Het Spelderholt' die mij terzijde stonden, dank ik voor hun aandeel in deze dissertatie. In dit verband wil ik met name Dr G. Beuving, Ir W. F. van Tijen, Mevr. M. Th. Kisjes-Linthorst en de heren D. de Boer, G. van Dijk jr., F. A. T. Jansonius, A. Oosterwoud en G. P. Teunis noemen.

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aan mijn ouders
aan Trees
Cris en Bas

Samenvatting

De structuur van de eischaal en de schaalvliezen die in Fig. 1 schematisch is weergegeven, werd bestudeerd met behulp van een lichtmicroscoop, een transmissie-elektronenmicroscoop en een raster-elektronenmicroscoop. Het onderzoek betrof voornamelijk eischalen en schaalvliezen van kippeëieren, afkomstig van lichte legrassen en premature kippeëieren. Incidenteel werden eischalen en schaalvliezen van bebroede kippeëieren en eieren van enige andere vogelsoorten bestudeerd. Aan de hand van de hiervan verkregen resultaten kon een nader inzicht worden verkregen in: de variabiliteit van de dikte van de schaalvliezen, de structuurfactoren in de schaal die de schaalsterkte beïnvloeden, de vorming van de schaal en de schaalvliezen, de ontkalking van de schaal tijdens het broedproces en de factoren die de structuur van de oppervlakte van de organische cuticula beïnvloeden.

Drie methoden voor het meten van de schaalvliesdikte van kippeëieren werden kritisch vergeleken. Schaalvliezen die van de schaal losgemaakt en gedroogd waren, bleken duidelijk dunner te zijn dan schaalvliezen in radiale ontkalkte preparaten en slijpplaatjes. Dit komt waarschijnlijk omdat het onmogelijk was de schaalvliezen volledig van de schaal te verwijderen. Daarom mag gesteld worden dat de feitelijke dikte van de droge schaalvliezen slechts gemeten kan worden in radiale doorsneden.

De structuurfactoren in de schaal die de schaalsterkte beïnvloeden zijn bestudeerd bij eischalen van kippen en parelhoenders. Parelhoeneischalen zijn onderzocht omdat deze duidelijk sterker zijn dan die van kippeëieren, zelfs als rekening wordt gehouden met de verschillende schaaldikte. Uit dit onderzoek is naar voren gekomen dat de schaalsterkte zou kunnen worden beïnvloed door: de schaalvliezen, de verdeling van de mammillen over de schaal, de gemiddelde diameter van deze mammillen, de gemiddelde diameter van de kolommen, de verdeling van de organische matrix, de grootte van de blaasvormige gaatjes in de matrix, het aantal gaatjes per oppervlakteëenheid, de kristallijne structuur in de kolommen en de aanwezigheid van de organische cuticula. Breuken in de schaal vertonen in de kegellaag een strikt radiaal verloop maar plaatselijk kunnen ze in de palissadelaag afbuigen naar het tangentiale vlak. In beide gevallen volgen de breuken meestal laagjes organische stof.

Premature eieren werden ongeveer 20½ uur voor de geschatte legtijd uit de isthmus (vliesvormend gedeelte van de eileider, voorafgaande aan de uterus) gehaald. De schaalvliezen van deze eieren werden vergeleken met schaalvliezen

van normaal gelegde eieren om een beter inzicht te krijgen in de vorming. Zowel het binnenste als het buitenste schaalvlies bij eieren uit de isthmus was dunner dan bij normaal gelegde eieren. Het binnenste schaalvlies werd volledig afgezet in de isthmus en niet later geheel of ten dele uit de eiïnhoud gevormd. De kernen van de vliesvezels werden eerder afgezet dan de mantelsubstantie. Eischaalvliezen van 12, 8 en 4 uur prematuur gelegde eieren leken gelijk aan die van normaal gelegde eieren. Het is dan ook erg waarschijnlijk dat tijdens het 'plumping'-proces (de eerste vijf uren van de schaalvorming, wanneer een waterige oplossing aan het wit van het ei wordt toegevoegd) de vezels meer uit elkaar werden getrokken door het groter worden van het ei. Hierdoor konden meer en grotere mazen in de vliezen ontstaan. De vele verbindingen (vliezen en draden) die aanwezig zijn tussen de verschillende schaalvliesvezels van de eieren uit de isthmus zouden gedurende dit proces kunnen worden verbroken. De diameter van de vezels onderging daarbij geen verandering.

De wijze waarop de verschillende lagen van de schaal werden afgezet (respectievelijk van binnen naar buiten: de kegellaag met de mammillen, de palissadelaag, de oppervlaktekristallaag, de organische cuticula en de bij sommige vogelsoorten voorkomende deklaag) is beschreven. Tussen de kristallieten in de schaal waren hogere concentraties organische stof aanwezig dan binnen de kristallieten en ook aan de buitenkant van premature eieren kwam steeds een dun homogeen laagje van deze organische stof op de schaal voor. Dit houdt verband met de rol die de organische stof speelt bij de verkalking van de schaal. De afzetting van de organische stof ging vooraf aan die van de anorganische. Een deel van de afgezette organische stof nam tijdens de schaalvorming anorganisch materiaal op terwijl een ander deel waarschijnlijk voor de kristallieten uitgeduwd werd, waardoor tenslotte een laagje organische stof tussen twee tegen elkaar groeiende kristallieten terecht kwam.

Vele putvormige gaatjes met kleine kristallen erin zijn waargenomen in de kegel- en de palissadelaag. De samenstelling van deze kristallen en de oorzaak van hun ontstaan zijn nog niet bekend.

Het kalium- en magnesiumgehalte in de schaal nam van binnen naar buiten toe. Het is niet duidelijk hoeveel van deze mineralen in de oppervlaktekristallaag voorkomt.

Gedurende de broedperiode onttrekt het ontwikkelende embryo calcium aan de schaal voor de beenvorming. Er ontstaat dan in iedere mammil van de schaal een grote holte, die waarschijnlijk zijn oorsprong vindt in het kristallisatiecentrum. Een breuk in de schaal evenwijdig aan het oppervlak treedt tegen het einde van de broedperiode op door deze grote holten en de beschreven ringen van Sajner. De wijze van ontkalking en de daarmee gepaard gaande structurele veranderingen in de schaal tijdens de bebroeding zijn uiteengezet. Het was duidelijk dat de ontkalking van de schaal langs ophopingen van organisch materiaal tussen de kristalindividuen en de kristallieten plaats vond. Het verloop van de ontkalking werd beïnvloed door de structuur van de organische matrix in de mammil.

Het oppervlak van een vers kippeëi vertoonde normaliter de vesiculaire bouw van de organische cuticula met veel stervormige barstenstelsels. De grootste hiervan lagen in het oppervlak van de porieproppen. De oppervlakte werd bij het ouder worden van het ei vlakker en compacter tengevolge van het krimpen van de blaasjes van de organische cuticula; de barsten in de porieproppen werden daarbij groter.

Water met een temperatuur beneden 40° C loste de organische cuticula niet op. Water boven 40° C veranderde de oppervlaktestructuur van het ei. Wassen met water waaraan een wasmiddel (Nusan) was toegevoegd, veranderde de oppervlaktestructuur van het ei duidelijk en loste waarschijnlijk een deel van de organische cuticula op. Deze bleek veel resistenter te zijn dan algemeen werd aangenomen; zelfs vijf minuten koken in 10 % KOH loste deze laag niet geheel op.

Het optreden van streepjes op het ei, die zijn ontstaan tengevolge van beschadiging van de organische cuticula kort na het leggen van het ei, is beschreven. Deze streepjes kwamen veel vaker voor bij eieren die op batterijen gelegd zijn dan bij nesteieren.

Contents

1 Introduction

Egg production per hen has shown a marked increase since World War II, but breeding for shell quality has not kept pace with this development. Over the years egg breakages with consequent financial loss have increased on poultry farms, at the wholesale, retail and consumer level. The use of improved packaging materials and machines has reduced this breakage but not solved the problem.

In recent years there has been no extensive research into the breakage of eggs on commercial poultry farms in the Netherlands. Conditions in the Netherlands and other countries differ but it is estimated under present conditions that about 10 % of all the eggs break or are damaged on the way from the producer to the consumer, and about half this percentage on the poultry farms.

The total loss is difficult to calculate, since not all damaged eggs are useless but then some may soil other eggs. In 1968 there were 15,550,000 laying hens in the Netherlands which laid 3,969 million eggs. Using the losses in other countries as a guide, the annual loss in the Netherlands due to eggshell damage is estimated at least 15 million guilders. This is calculated on a loss of about 4 % of the eggs. In the future when production is stepped up a greater number of eggs of poor shell quality is expected so the percentage loss will be higher still.

The resistance of the eggshell to breakage during transit is indicated by shell strength. The main influences in the life of a hen on shell strength are the genetic constitution, diet, climate, housing and age. To measure strength many laboratory techniques which have their disadvantages have been developed. One of the most widely used routine methods of determining shell quality favoured by breeders of laying stock is to measure the specific gravity of the whole egg (van Tijen, 1963). Specific gravity is influenced by the evaporation of water through the shell during storage. Shell thickness is also often used as a parameter of shell strength. Besides shell thickness, egg size, curvature of the shell and shell structure all contribute to the strength. However the influence of the membrane thickness has not been proved (Bokx, 1962).

Little is known of the effect that structural factors in the shell have on the resistance of the egg against breakage, or strength. Pieces of eggshells with the same thickness and curvature may still differ in strength, which probably can be attributed to the differences in shell structure.

Our investigation on the variations in shell structure and its relation to shell strength was started by studying radial and tangential sections of the shells of hen eggs using a light microscope. With the aid of polarized light a relation

between the crystalline structure of the eggshell and its strength was established. The work with the light microscope indicated that the structure of the organic matrix intermingled with the calcareous shell might be related to the strength of the shell.

Progress was restricted by the limit of resolution in the light microscope so decalcified ultrathin sections of the complete shell together with its membranes were examined with a transmission electronmicrocope. Special attention was paid to the compactness and the distribution of the organic matrix. Efforts to examine the surface of fractures through the calcified part of the shell by the replica technique with the transmission electron microscope were not completely successful because of difficulties in removing the replicas untorn from large areas of the fractured surface. Fortunately we had the opportunity to do some research with a scanning electron microscope. With this apparatus the surface structure of rather bulky specimens can be studied at a wide range of magnifications, with a large depth of focus and a relatively high resolution, without much preliminary preparation.

It seemed to be possible to obtain some information about the infuence of some minerals in the shell on the strength using ground sections of the shell and an electron probe X-ray microanalyser.

During these structural studies many interesting physiological questions came up, which could be easily studied with the help of the various microscopes. From a comparative examination of preparations of normal and premature eggshell membranes an impression was formed about the change of the membrane fibres during shell deposition. Many photographs were taken of the contact between shell and membranes and how the first calcified layer was deposited, is discussed. Also the formation of the other calcified layers of the shell will be described from photographs of normal and premature egg shell preparations.

The most important source of calcium for the embryo is the eggshell. There is still controversy about the way shell calcium dissolves during incubation (Simkiss, 1967). Previously little examination of the structure of the shell and membranes of incubated eggs has been made using a light microscope and electron microscopists have not studied at all the decalcification during the incubation period. Therefore work in this field seemed justified. The relation between the shell structure, its decalcification, and the separation of shell membranes with some calcified material from the rest of the shell during incubation will be discussed.

The organic outer layer of the shell protects the egg contents against penetration of micro-organisms by closing the pores leading through the calcareous shell (Romanoff & Romanoff, 1949). Cleaning techniques such as washing possibly remove part of this organic cuticular layer and might cause further spoilage. As no agreement has yet been reached about the stability of the outer organic layer and its resistance against cleaning it was decided to study the influence of various factors on egg surface.

Most of the research was done in close co-operation with the Laboratory of

Animal Physiology of the Agricultural University. The Electron Microscopy Section of the Technical and Physical Engineering Research Service at Wageningen gave technical guidance and produced many of the electron micrographs.

2 Materials and methods

This study may be broadly divided into light microscopy and electron microscopy, depending on the techniques employed. Some examination of minerals in the eggshell was carried out by X-ray microanalysis. Material obtained by means of chemical and mechanical treatments was further histological treated or compared with preparations obtained by other means.

2.1 Eggs

Shell and membranes were from normally laid and premature hen eggs, and occasionally incubated hen eggs, and eggs of guinea-fowl, duck, turkey, emu and ostrich. By premature eggs is meant either prematurely removed eggs taken from the oviduct post-mortem or prematurely laid eggs (Evers, 1967). Unless otherwise mentioned the species referred to in further discussion of eggshells and membranes will be the domestic fowl *(Gallus gallus)*. Generally eggshells and membranes of laying breeds (White Leghorn and a cross of White Leghorn and Rhode Island Red) from the Institute at Beekbergen have been used. The membrane thickness was determined in 37 eggs laid by four pullets (White Leghorn hen no. 568 and 597; Rhode Island Red hen no. 253 and 502).

Fragments were usually taken from the widest part of the egg, called here, in accordance with Professor Tyler of Reading, the waist, in contrast to the middle of the egg, which he calls the equator. There is some confusion about the meaning of these two terms. Webster's Third New International Dictionary (1961) gives the following definitions, among others: equator – a circle about a body at the place of its width, e.g. equator of an egg; waist – the middle or central part esp. when narrower or less thick than the ends.

2.2 Light microscopy

2.2.1 Ground sections

Pieces of shell 1 × 1 cm were dried at 50° C for 24 h and embedded. The embedding mixture consisted of Vestopal H 85, monostyrene 15, cyclonox 0.5 and cobalt octoate 0.2 g, and was thoroughly mixed and cleared of bubbles before the pieces of shell were embedded in it. After embedding, the mixture was polymerized for 24 to 48 h at 22° C and 50 mm Hg pressure in a desiccator. They were

hardened for a week at room temperature and suitable fragments about 4 mm thick were sawn out with a diamant saw.

Then cross-sections were successively ground and polished with carborundum powders with grain-size of respectively 44 μm, 25–30 μm, 16–20 μm, 13–16 μm and 4 μm. The carborundum powders were mixed with glycerine to a fine paste and placed on a cast-iron plate revolving at a speed of 100–200 revs/min. Polishing was then continued with AB metadi diamond paste which has a grain-size of 3 μm, on a nylon cloth wetted with an AB metadi fluid and placed on a bronze disc. Finally the preparations were polished with the diamond pastes AB alpha and AB gamma micropolish, having a grain-size of 0.3 μm and 0.05 μm respectively, on a silk cloth wetted with demineralised water and likewise clamped to a bronze disc. With the polished side downwards, the piece of shell was affixed to a clean, degreased slide in a mixture of Vestopal H 10 g, cobalt octoate 0.1 g and cyclonox 0.2 g. Care was taken to ensure that no air bubbles remained when the polished fragment was pressed firmly on to the slide. After 24 hours of hardening the other side was ground and polished in the same way. The preparation was ground with carborundum 240 (grain-size 44 μm) to a thickness of about 500 μm, with carborundum 320 (grain-size 25–30 μm) to a thickness of about 300 μm, with carborundum 400 (grain-size 16–20 μm) to a thickness of about 200 μm, with carborundum 500 (grain-size 13–16 μm) to a thickness of about 85 μm, and with carborundum 1000 (grain-size 4 μm) to a thickness of about 20 μm. Polishing was then completed with AB metadi, AB alpha and AB gamma micropolish to a thickness of about 10 μm as described previously. In this way radial and tangential cross-sections were obtained of normal shells of hen and guinea-fowl eggs.

A number of shell pieces, some of which had been treated with sodium hydroxide and sodium sulphide as described below, were mounted in plastic, ground and polished to thin sections by the method described by Tyler (1964), except that diamond pastes were used instead of grinding powders.

In most cases the resultant preparations were studied under a Leitz Ortholux light microscope with polarized and ordinary transmitted light.

2.2.2 Decalcified sections

Some of the preparations were embedded in low-viscosity nitrocellulose after being dried, fixed and washed in absolute alcohol. Subsequently the shell was decalcified in a 5 % ethylenediaminetetra-acetic acid (EDTA) solution containing 6 % formaldehyde at a pH of 7.2 (Simkiss & Tyler, 1957). In many cases the trisodium salt of EDTA was used instead of the disodium salt. When the disodium salt was used it was adjusted to pH 7.2 with sodiumhydroxide. After decalcification the preparations were dehydrated and embedded as follows:

1. 30 min rinsing in 50 % alcohol
2. 30 min rinsing in 70 % alcohol

3. 30 min rinsing in 95% alcohol
4. 2 × 30 min rinsing in absolute alcohol
5. 30 min rinsing in a mixture of 50 parts of absolute alcohol and 50 parts of benzol
6. 2 × 30 min rinsing in benzol
7. 40 min impregnation with benzol paraffin at 70° C
8. 3 × 40 min impregnation with benzol paraffin at 70° C.

(When the preparation was mounted in nitrocellulose, butyl or isobutyl alcohol was used.)

After cooling, sections were cut (thickness: 100 μm) from the resultant blocks on a freezing-microtome. In most cases these sections were stained for 5 min with Masson's trichrome stain. Some sections were stained for one minute with a 0.1 % thionine solution. After dehydration and clearance first with alcohol and then xylol, the sections were then mounted in Canada balsam.

This method produced sections of normal eggshells with which it was possible to study shell-membrane thickness and factors affecting shell strength.

Several preparations, including those treated with sodium hydroxide and sodium sulphide were not embedded in low-viscosity nitrocellulose, but directly in paraffin wax and sectioned on the rotary microtome.

2.2.3 Measurements of thickness of shell membranes

Pieces of shell with the shell membranes still attached were placed in a 30 % acetic acid solution for five minutes at 20° C. The membranes were then removed with tweezers. The inside of the shell was examined with a microscope to check complete separation. The isolated membranes were first washed in distilled water, then acetic acid and finally distilled water again in order to remove residual calcium carbonate. After drying for an hour the thickness was measured with the deformation apparatus without a weight at the cap (Schoorl & Boersma, 1962). A comparison was made between the membrane thicknesses thus obtained and the thickness in ground and decalcified sections.

2.2.4 Treatment of the shell with sodium hydroxide and sodium sulphide

Pieces of shell 15 × 5 mm from the waist with their longest side in line with the egg's axis were placed for different times in 10 % NaOH or 30 % $Na_2S.9H_2O$ at about 95 ° C. Ten pieces of shell taken from each of 20 eggs laid by 4 birds were subjected to the following treatments after the membranes had been removed mechanically:

1. control
2. 9 h in water at 95° C
3. 1 h in 10 % NaOH at 95° C

4. 2 h in 10 % NaOH at 95° C
5. 3 h in 10 % NaOH at 95° C
6. 9 h in 10 % NaOH at 95° C
7. 1 h in 30 % $Na_2S.9H_2O$ at 95° C
8. 2 h in 30 % $Na_2S.9H_2O$ at 95° C
9. 3 h in 30 % $Na_2S.9H_2O$ at 95° C
10. 9 h in 30 % $Na_2S.9H_2O$ at 95° C.

Decalcified sections and plastic embedded ground sections were made of these pieces of shell as described above, and the shell thicknesses also measured. The strips were snapped inwards on the apparatus described by Tyler & Coundon (1965) using the method of Tyler & Thomas (1966). This research was carried out in Reading under Professor Tyler (Simons et al., 1966).

2.3 Electron microscopy

2.3.1 Decalcified sections

After the contents of the egg had been removed the inside of the shell was carefully rinsed with water. This was not done for the first experiment. The shell was decalcified in a 4 % EDTA solution containing 6 % formaldehyde at a pH of 7.2 for about two days at room temperature. The pieces of shell with attached membranes were secured to a cotton thread to reduce damage during handling. Following Tyler (1965 a) cetyl pyridinium chloride was sometimes added to the decalcification fluid to prevent further solution of mucopolysaccharides, but with no visible result. After decalcification the preparations were usually fixed in a 1 %–2 % osmium tetroxide solution for 1–2 h (Mercer & Birbeck, 1966). Sometimes they were fixed for 2 h in a 1 % osmium tetroxide solution to which was added 500 ppm ruthenium red (Luft, 1966) in order to stain the acid mucopolysaccharides present. Control preparations were placed in a 1 % osmium tetroxide solution for 2 h, and fixed in a solution of 1 % osmium tetroxide and 1 % Sorensen buffer. The preparations were then dehydrated with ethanol (Mercer & Birbeck, 1966).

For the first experiments the preparations were embedded in a mixture of methyl- and butylmethacrylate (20 : 80) (Mercer & Birbeck, 1960). Subsequently epon was used instead (Luft, 1961), the specimens being passed twice through propylene oxide for 30 min and once for 30 min through a mixture of 50 parts of propylene oxide and 50 parts of the embedding mixture. Still later specimens were embedded in a mixture of styrene and butylmethacrylate (Kushida, 1961).

A glass knife was used for cutting ultrathin sections on a Porter-Blum or an LKB microtome. Some of these sections were stained with potassium permanganate for one hour, and then put in dilute citric acid for one minute (Lawn, 1960). In some cases the preparations were stained afterwards with lead citrate (Reynolds, 1963) and uranyl acetate (Mercer & Birbeck, 1966), or with lead citrate only.

The materials treated by the method described were eggshells of normal hen eggs, eggshells of the same thickness but varying strength from hen eggs, eggshells of premature eggs, eggshells of hen eggs obtained at high environmental temperatures and high relative humidities from the experiment as described by El-Boushy (1966), and guinea-fowl eggshells. Eggshell membranes were extracted in 10 ml portions of water for respectively 10, 20, 40 and 60 h in an autoclave at a pressure of 1.76 kg/cm² (Balch & Cooke, 1968). We obtained these membranes from Dr D. A. Balch in Reading already treated. Then they were dehydrated, embedded and stained as described above.

The cross-sections obtained were viewed with a Philips EM 100, Siemens Elmiskop I or Philips EM 300 transmission electron microscope.

2.3.2 Replicas

Formvar replicas (Bradley, 1954) were made of the surface of the egg and of small pieces of radially fractured surfaces through the shell. It was possible to obtain larger surfaces of radially fractured surfaces by means of tylose replicas (Pohlmann & Volk, 1959).

The effect of the various methods of washing, i.e. with a jet, by hand, with a cloth, and after addition of Nusan, was examined using the formvar replica technique. The effect of the temperature of the water was determined by placing the eggs for 5 min in water of temperatures of respectively 20°, 30°, 40°, 50°, 60°, 70°, 80°, 90° and 100° C. The effect of boiling in 10 % potassium hydroxide for 5 minutes was also examined.

The resultant preparations were studied with a Philips EM 100, Siemens Elmiskop I or Philips EM 300.

2.3.3 Shell membranes

The inner membrane was separated from the outer first by sawing off a slice of shell from the pointed end of the egg. Then the egg was emptied and the inside of the shell thoroughly rinsed several times. A hole was made through the shell and outer membrane at the blunt end of the egg. By blowing through this hole into the air space the inner membrane was detached. Two membranes representing the inner and outer part of the outer membrane were then removed by tweezers from the inside of the shell. After the remaining pieces of shell had been decalcified for 1–1½ h in the above-mentioned EDTA solution, a fourth membrane was removed from the shell. Preparations of each membrane were fixed for 2 h in a 1 % osmium tetroxide solution to which was added 500 ppm of ruthenium red (Luft, 1966), the other control preparations remained for 2 h in a 1 % osmium tetroxide solution. These membranes were then placed on copper grids and studied with a Philips EM 300.

2.3.4 Scanning electron microscopy

Pieces of shell and shell membrane taken from the waist of the egg were glued with a cellulose acetate glue to a round aluminium plate (diameter 1 cm) and coated with carbon and gold in a vacuum chamber. The photographs were taken at an accelerating voltage of 10 kV. During this examination the structure of the shell membranes and shell were studied with the Stereoscan scanning electron microscope of the Institute of Medical Physics, Münster, West Germany. Radial and tangential sections were ground and polished on one side by the method described in Section 2.2.1. The sections were etched by boiling them for 10 minutes in a 10 % sodium sulphide solution. The inner shell membrane was separated from the outer one by the method described in Section 2.3.3. After 1 h in a 5 % EDTA solution at a pH of 7.2, the true cuticle, a layer of organic material on the outside of the shell, was removed with most of the pore plugs of the shell by spraying a jet of water onto the surface of the egg. Some of the calcite from the surface crystal layer were probably removed as well by this treatment which enabled the columns of the calcified shell to be seen. Besides the above shell fractures and surfaces were studied with the scanning electron microscope.

In a second examination, the specimens were glued to the flat top of a cylindrical copper rod 1 cm in diameter. The preparations were evaporated with gold in a vacuum chamber and examined with a Jeolco JSM 2 scanning electron microscope at an accelerating voltage of 25 kV in the Jeol demonstration centre, Paris. The two types of examination gave complementary data on shell structure. A study was also made of the fracture planes of eggshells and shell membranes of the duck, turkey, guinea-fowl, emu, ostrich as well as fracture planes of shells of incubated eggs.

2.4 X-ray microanalysis

Thin ground sections were made by the method described in Section 2.2.1. These preparations were polished by Drs R. P. E. Poorter to still smoother sections. They were then studied with the electron probe X-ray microanalyser of the Analytical Chemical Laboratory, Utrecht.

3 Structure of shell and membranes

Schoorl & Boersma (1962) found a correlation coefficient of +0.625 between the shell thickness and the breaking strength which indicates that 39 % of the shell strength is contributed by shell thickness. The breaking strength was determined by the weight necessary to crack the egg with a flat ended pin (diameter 3 mm) on the blunt end of the egg. Results of a study of shell structure may be of importance if factors influencing shell strength are discovered.

In general the bird egg contents are enveloped by the shell membranes, the shell and the organic true cuticle. The shell consists of an organic matrix intermingled with calcareous depositions. For more than 100 years light microscopic studies have been made of the structure of the shell. Polarized light studies of the crystalline structure of the shell started about 1900. Electron microscopists as yet have given little attention to the structure of the eggshell.

3.1 Nomenclature

The terminology for the various layers surrounding the egg contents varies. It is generally assumed that the first description about shell membranes and shell was given by Purkinje (1830) who termed the shell membranes *'membranae testae'* (L. *testa* = shell). Baer (1837) noticed that these membranes were given many names: *membrana testae, membrana testacea, membrana putaminis, membrana ovi propria, membrana succingens, membrana ovi liquores amplectens* and *pellicula.*

Landois (1865) observed four layers in the shell from inside to outside: the 'Faserschicht' (fibre layer), the 'Uterindrüsenschicht' (uterus gland layer), the 'Schwammschicht' (spongy layer) and the organic 'Oberhaut' (epidermis). Blasius (1867) described the structure of the shell membranes and the shell. He divided the fibre layer in two: the inner and the outer fibre layer. He disagreed with the term 'Uterindrüsenschicht' which he called 'Kernschicht' (core layer) owing to its organic cores. Von Nathusius' numerous publications between 1868 and 1898, formed an important basis for later studies. He distinguished an inner and an outer membrane. On the inside of the shell a layer of 'Mammillen' (mammillae) was found through the ends of which run membrane fibres (von Nathusius, 1882). He referred to the organic covering of the shell as the 'Oberhäutchen'. Romankewitsch (1934) termed this layer 'Kuticulum' (cuticle). Afterwards many scientists termed the different layers surrounding the egg contents inner and outer mem-

brane, mammillary and spongy layer and cuticle. It was possible to use this nomenclature for the different layers of organic matter in shell and membranes, but studies of the crystalline structure of the shell necessitated other terminology. Therefore Schmidt (1958 b) termed from inside to outside the shell membrane, the 'Lage der Kegel und Basalkalotten' (layer of cones and basal caps) and the 'Palisadenlage' (palisade layer). The covering outside the palisade layer previously 'Auflagerungen' (Schmidt, 1958 a) was designated during the last years 'Deckschicht', sometimes 'Tegmentum' or 'Überzug' (Schmidt, 1964 a). This layer is sometimes partly calcified. If this layer consisted only of organic matter it was called either 'Schleimhäutchen' or 'Cuticula'. Tyler (1965 b) disagreed with these terms for the covering. He showed that in some species a layer with calcified deposits occurred on the outer surface of the organic cuticular covering and suggested terming these deposits as cover ('Ueberzug' by von Nathusius, 1882). The organic layer under this calcified layer was referred to by Tyler (1965 b) as the true cuticle 'Schleimhäutchen' after Schmidt (1958 a) and this true cuticle covered a thin layer with crystals named surface crystal layer.

The nomenclature for the different layers around the egg contents used in this work is as follows: inner membrane, outer membrane, layer of cones and basal caps, palisade layer, surface crystal layer and true cuticle (Fig. 1). Calcified deposits on the organic true cuticle will be termed as cover. They do not normally occur in the hen egg.

Von Wittich (1851) found larger or smaller holes in the shell which ran through the different shell layers and corresponded to the openings in the 'Epidermis' (organic cuticular layer on the shell). Von Hemsbach (1851) referred to them as 'Poren' (pores). This term is still used.

3.2 Literature

3.2.1 Shell membranes

Layers with fibres and meshes The shell membranes may be subdivided in the inner and outer membrane. Baer (1837) termed the two membranes together 'shell membrane' which he divided into two sheets adhering to each other except for the blunt end of the egg where the air space was formed. Sajner (1955) saw three shell membranes. After he had torn two membranes from the shell a third one remained. It is possible to separate this third membrane from the shell after a treatment with an acid or EDTA. The third membrane which is only a portion of the outer membrane firmly adhering to the first calcified deposits, was termed by Sajner 'Körnchenhäutchen' (granulated membrane), because parts of the calcified mammillae were visible in it.

Blasius (1867) showed that more than two concentric layers of fibres can be separated from the membranes. Von Nathusius (1868) even mentioned a great

number of membrane layers of fibres. Hays & Sumbardo (1927) and Romankewitsch (1932) demonstrated a lamellar structure. The former showed that the different lamellae are not quite separated, the fibres of one entering the other. Moran & Hale (1936) found that the inner membrane consisted of one layer and the outer of three layers, each layer having a mesh of fibres. Also Simkiss (1958) reported that the membrane could be dissected in layers. He was unable to see them with the aid of microscopic techniques.

Purkinje (1830) showed that the membranes are fibrous. Von Hemsbach (1851) described the shell membranes as consisting of fibres which crossed each other with traces of larger blood vessels and many pores, giving the membrane a sieve-like appearance. At the same time fibres with small meshes were said to be present in these membranes (von Wittich, 1851). The organic fibres crossed each other in many directions, being connected in a network (Blasius, 1867). Von Nathusius (1868) demonstrated that the membranes consisted of interwoven fibres running in different directions firmly connected by cementing material. These fibres did not form branched systems or anastomoses. Between the flattish fibres were air-filled interstices. Romankewitsch (1932) described the membrane as an interwoven network with anastomosing fibres with such minute meshes that proteins were unable to pass through it.

Inner layer of inner membrane Dark stripes going from the blunt to the pointed end were present on the inside of the membranes of freshly opened eggs (Landois, 1865). According to him these were impregnated with protein. Baer (1837) showed that the inner membrane had a smooth surface inside. As meshes in the inner part of the inner membrane were difficult to find (von Wittich, 1851) a fairly compact layer must exist on the inside. Von Nathusius (1868) showed that the inner margin of the inner membrane was formed by a homogeneous transparent film. On this film or in the innermost layers of fibres he saw granules of about 1 µm. Schmidt (1965 a) showed that the inner membrane of the shell of a swan *(Cygnus olor)* egg was closed on the inside by a thin lamella. Masshoff & Stolpmann (1961) also described this inner membrane as being smooth on the inside.

Fibre diameter Coste (1847), Blasius (1867), von Nathusius (1868), Romankewitsch (1932) and Masshoff & Stolpmann (1961) showed that the outer membrane had thicker fibres than the inner one. The broader fibres, especially in the outer layer, may be formed by more than one fibre (von Nathusius, 1868). This author then stated that the membrane fibres of larger eggs were not thicker than those of smaller eggs.

Blasius (1867) found in kinglet *(Regulus regulus)* eggshell membranes, fibres with a diameter of 0.5–0.6 µm. Von Nathusius (1893) measured in the inner membrane of the hen eggshell, fibres with a diameter of 1.0–1.5 µm and in the outer one fibres with a diameter of 2.0–3.0 µm. Romankewitsch (1932) gave values of 8–12 µm for the thickness of the fibres of the outer membrane and

values of 2–3 μm for those of the inner membrane. These measurements are too high compared with the results of other authors. Moran & Hale (1936) distinguished in the inner part of the outer layer, fibres with a diameter of 0.8 μm and in the outer part, fibres with a diameter of 2.5 μm. Sajner (1955) also found in the granulated membrane (the outer part of the outer membrane) fibres of 2 μm diameter. Wolken (1951) mentioned membrane fibres with a diameter of the order of 1 μm. Fibres with diameters of 0.5–1.0 μm and 0.8–1.0 μm were seen by Simkiss (1958) and Masshoff & Stolpmann (1961) respectively in electron micrographs. These values on the average are low for the fibre diameter in both membranes. The authors probably studied a part of the inner membrane with fibres of smaller diameter, and thought that the fibres were branched.

Diameter of meshes Von Wittich (1851) found spaces between the fibres. Von Nathusius (1868) described them as being filled with air. The number of meshes per cm^2 was invariably greater in the inner membrane than in the outer one (von Wittich, 1851; Hays & Sumbardo, 1927). Wolken (1951) found meshes of 1–2 μm probably in the inner membrane. The largest measured meshes in the outer membrane were 28 μm (Von Wittich, 1851).

Structure of fibres Romankewitsch (1932) distinguished a denser outer layer and a less dense core in the membrane fibres using a silver impregnation method. Masshoff & Stolpmann (1961) saw fibres with cores surrounded by a less electron dense mantle with a thickness of 0.5 μm. Between the core and the mantle they found a 0.3–0.1 μm cleft. After a treatment with gold chloride von Nathusius (1893) saw air channels in many outer shell membrane fibres.

Masshoff & Stolpmann (1961) determined, by a much greater enlargement of the fibre core, fibrils with a diameter of 3–4 nm. They reported that the core consisted of keratin and the fibrils were embedded in a matrix. These findings have yet to be confirmed.

3.2.2 Layer of cones and basal caps

This layer consists of a single layer of primatic bodies anchored in the outer membrane. Von Nathusius (1868) showed that the interior of this inner layer of the shell had numerous protuberances penetrating the membrane of the shell. These he termed 'mammillae'. Together they form the mammillary layer.

Shape and size of mammillae Von Nathusius (1868) suggested that the mammillae are branched inward and fuse outward. The mammillae had an irregular polygonal shape. In a tangential section Szielasko (1913) found them roundish, often passing into an elliptical shape. Landois (1865) gave a diameter of 32–40 μm for the mammillae and a distance of 100–800 μm between their centres. These values differ from those of Simons (1962) who in 70 radial sections measured

distances of 36.5–75.0 µm between the centres of the mammillae. The value of 800 µm must be an error. Even if a pore is present between two mammillae a value not much greater than 100 µm would be expected. Blasius (1867) found in a premature egg, diameters of between 96–144 µm for the mammillae in the shell. These values also seem to be high. The diameters of the mammillae decreased from outside to inside and interstitial spaces were present between them. Measurements of the diameters of the mammillae taken at different heights may account for these differing results. Von Nathusius (1871), who studied eggshells of different species of birds, showed that variations in size of mammillae were smaller than variations in shell thickness. The mammillae of the same family or genus were not necessarily of the same size. Schmidt (1966 b) also noticed that the mammillae may differ in shape and size in the same eggshell and vary in their distance from each other. One-third of the shell thickness was accounted for by the mammillary layer (Burmester, 1940) and this was about 110 µm (Romanoff & Romanoff, 1949). The mammillary layer of ostrich *(Struthio camelus)* eggshells was also approximately one-third of the shell (von Nathusius, 1885).

The part of a mammilla embedded in the outer membrane was termed the basal cap (Tyler, 1965 b). The outer part of the mammillae belonged to the layer of cones. The basal caps had a very irregular surface. Numerous wartlike protuberances of different size and shape were seen in the swan eggshell (Schmidt, 1965 b). The curved basal boundery often had an irregular bulge. The same kind of irregularities in the hen eggshell were found by El-Boushy (1966) and can be seen in Schmidt's paper (1966b). Heyn (1963) noticed on the inside of the shell small randomly-oriented calcite microcrystals. Further outward, the surface of the mammillae was smoother with a polygonal or roundish boundary (Schmidt, 1965 b). The mammillae were fused at the end furthest from the membrane and formed groups (Schmidt, 1966 b). Between the groups of mammillae, pores were formed (Schmidt, 1966 b) which passed through the shell and communicated on the inside with interstitial spaces between the mammillae and open spaces between the membrane fibres (von Nathusius, 1868).

Anchorage in outer shell membrane Purkinje (1830) found that parts of crystals of the inner part of the shell were present in the outer part of the shell membrane. Von Nathusius (1868) showed that the inner ends of the mammillae were inserted in the outer layer of the shell membrane. Romankewitsch (1932) saw in the outer layer of the outer membrane, stellate figures interconnected by membrane fibres running through them. Sajner (1955) saw the figures as granules of about 30 µm diameter in the membrane, which caused a thin layer of membrane to remain on the shell after the membranes had been detached. It is usually stated that the shell membranes are firmly attached to the shell. Masshoff & Stolpmann (1961) suggested that the membrane fibres ran through the whole shell. Schmidt (1965 a) stained the fibres of the shell membranes deep violet brown in ground sections by the gold chloride-hydrazine hydrate method. He showed clearly that the fibres

of the outer membrane only run through the bases of the mammillae. The form of connection between the fibres and shell is still a matter of discussion. Terepka (1963 a, b) saw thick fibrous rings in decalcified sections of the eggshell. These rings, which were the remains of decalcified mammillae, were connected to the fibres of the shell membranes. Sajner (1955) and Tyler & Simkiss (1959 a) also found this kind of ring in the granules on the outer membrane in radial sections of the shells of hatched eggs.

Distribution of organic matter The mammillae, like the entire shell, consisted of an organic collagen-like matrix (Almquist, 1934) and crystalline inorganic material (Schmidt, 1962 a). The matrix is distributed over the cone and the basal cap. Simkiss & Tyler (1957) showed the organic material was more concentrated in a central core of the mammillae. However the cores they described did not correspond in position with the dark cores seen in radial ground sections with a light microscope by Terepka (1963 b). These conclusions agreed with the results of Schmidt (1967 a) who said that the dark cores seen under a light microscope were caused by the gas inclusions which appeared just outside the central core.

Crystalline structure The crystalline structure of the eggshell has been studied in detail over the years. Von Nathusius (1868) noticed the existence of columns in the eggshell. Kelly (1901), Clevisch (1913) and Young (1950), using a polarization microscope observed large crystal individuals in the shell more or less perpendicular to its surface and described them. They studied the palisade layer in particular. Kelly (1901) already reported rhombohedral crystals in the shell. The most important work on the crystalline structure of the eggshell was done by Schmidt. He showed that the bird's eggshell consisted of rhombohedral calcite crystal individuals (Schmidt, 1962 a). According to this author the crystal individuals were not held together by organic material but by cohesion at their lateral boundaries. Masshoff & Stolpmann (1961), who studied the eggshell with a transmission electron microscope, cut it with a diamond knife and saw many small separate submicroscopical crystals. The large crystal individuals were split by cutting, and many small rhombohedral calcite crystals of the same shape as the original were formed (Schmidt, 1962 a). He noted that twin lamellae occurred in some crystals. A part of a crystal individual might have shifted with respect to a given plane in the shell. Both parts then grew symmetrically towards each other and a twin crystal was formed. Sometimes there where many of these shifts in one crystal individual and numerous parallel lamellae were arranged at smaller or greater distances. These lamellae were termed 'twin lamellae'. The occurrence of split and twin lamellae in the shell, and the fact that the shell did not fall apart into small crystals after several days' treatment with a 10 % potassium hydroxide solution at 50° C showed that the large crystal individuals described by Schmidt were present in the shell.

Inclusions Von Nathusius (1868) found opaque layers with inclusions in the cone layer of the eggshell. He described them as roundish grains which may differ in diameter in various bird's eggshells. After acid treatment they disappeared and he therefore assumed that they consisted of lime compounds. Kelly (1901) thought that calcium phosphate was present in these inclusions. After studying the refraction index at different points of the shell Schmidt (1962 a, 1965 b) concluded that small spherical gas inclusions were present and formed concentric growth lines in the cone of the bird eggshell. Near the outside of the cone he also observed a dark layer with many gas inclusions. Similar inclusions were seen by Heyn (1963), in a radially fractured surface of the palisade layer, as pitlike holes containing small irregular crystals. The composition of these inclusions is still uncertain and more research is needed.

Dark triangular columns with intermediate transparent ground substance in radial sections (von Nathusius, 1868; Tyler & Simkiss, 1959 b) and dark triangles surrounded by transparent substance in tangential sections (von Nathusius, 1869), were found in the layer of cones of ostrich eggshells. Schmidt (1958 b) also observed these dark triangles in tangential sections through the cone layer of the hen, duck, goose, swan and seagull eggshell. According to him every triangle was located inside a calcite individual. Schmidt (1964 d) observed that the gas inclusions described were concentrated in the central part of the calcite individuals which in a tangential section showed clouded triangles surrounded by clear calcite. This clear layer near the boundary of the crystal individual grew thinner towards the outside of the cone and finally disappeared (Schmidt, 1967 a).

The inclusions and clefts (splits filled with gas and occurring between crystal individuals) in the shell might have formed special patterns in some bird eggshells (Schmidt, 1964 e, 1966 c, 1967 b, c). They sometimes occurred in the cone layer, but more often in the palisade layer.

When the cones were fused outside the outer membrane, their boundaries gradually become toothed and more difficult to recognize (Schmidt, 1962 b). This is where the palisade layer begins.

3.2.3 Palisade layer

This crystalline layer, interwoven with an organic matrix, begins outside the cone layer and goes to within a short distance of the surface of the shell. It accounts for two-thirds of the thickness of the eggshell (Stewart, 1935; Marshall & Quickshank, 1938; Burmester, 1940 and Romanoff & Romanoff, 1949). It differs in structure from the rest of the shell.

Landois (1865) called it the spongy layer because after the shell had been decalcified, a spongy substance remained. He described this layer as structureless. But von Nathusius (1868) noted that this layer had a stratified structure in the ostrich and swan eggshell. The name palisade layer (sometimes column layer) was

given by Schmidt (1958 b) who showed the presence of crystal columns in the shell.

Organic matrix A few years ago little was known about the structure of the organic matrix in the palisade layer. The presence of an organic layered matrix in the shell was noticed by von Nathusius (1868) who decalcified ground sections of shells with chromic acid, which hardened the residual organic matter. Schmidt (1958 b) also observed after decalcification of the eggshell an organic mass with layers running parallel to the surface. Romanoff & Romanoff (1949) saw three different zones in the palisade layer after protein staining the eggshell. The distribution of matrix seemed to be fairly uniform within each layer. The matrix material was most plentiful in the inner layer and sparse in the outer. After etching the shell with hydrochloric acid Tyler (1956) also demonstrated different zones in the shell. Except for the outer part, the palisade layer seemed to be the most resistant. The outer part and the mammillary layer of the shell were less resistant. These differences in resistance may be explained according to Tyler (1956) by differences in organic matter content. There might be also a possibility that the differences in resistance are caused by the variations in crystalline structure (e.g. a difference in density) in the different layers. Terepka (1963 b) also saw zones of relative EDTA resistance in radial sections of the shell. In this case the outer zone of the palisade layer also decalcified rapidly and he suggested that it had a higher organic matrix content. This would disagree with the results of Romanoff & Romanoff (1949). In decalcified sections Terepka (1963 b) observed a well-ordered fine orientation with a herring-bone appearance in the organic matrix. According to him this matrix was not fibrous and was very finely interspersed throughout the mineralized portions of the shell.

Crystalline structure Kelly (1901) found that the palisade layer consisted of regularly-formed rhombohedral calcite crystals, with their principal axis perpendicular to the surface of the eggshell. At the transition of cone to palisade layer the number of calcite individuals decreased (Schmidt, 1962 b). One or more calcite individual(s) of the cone continued to grow on the surface (Schmidt, 1962 b) and formed the palisade layer consisting of united calcite columns (Schmidt, 1958 b). In tangential sections the columns were often polygonal. He demonstrated these conclusions using polarized light in thin radial and tangential ground sections.

The columns in the eggshells of different species may differ in structure and shape. In radial ground sections of the domestic hen eggshell studied by polarized light, the crystal columns clearly ran perpendicular to the surface from the inside to the outside of the shell. The diameter of these columns measured in electron micrographs was of the order of 50–100 μm (Heyn, 1963).

As described for the layer of cones and basal caps it was generally agreed that the mineralized part of the shell consisted of rhombohedral calcite individuals. When the palisade layer was examined under high magnification it was seen that the columns were not homogeneous but composed of block-like smaller units

10–15 μm wide (Terepka, 1963 a). The author was not certain whether this was the smallest crystal unit in the shell. But within each column all the smaller units were optically oriented in the same direction. In a transverse fracture surface of the palisade layer studied under the transmission electron microscope Heyn (1963) found the crystalline structures with striations in specific directions. In polarized light studies Terepka (1963 a) and Schmidt (1964 f) observed that the crystallographic C-axis of the calcite individuals was maintained in a radial direction. X-ray diffraction studies on the orientation of the crystals were conducted by Cain & Heyn (1964), Favejee et al. (1965) and van der Plas (1966). Cain & Heyn (1964) concluded that the hexagonal C-axis of the calcite crystals in the outer layer of the eggshell were inclined at 28° ± 16° from the normal to the shell surface. Favejee et al. (1965) and van der Plas (1966) showed that the pole on 104 (crystal plane) was roughly parallel to the normal of the shell surface and that the angle between the C-axis and the pole on 104 was about 45°. The C-axis was arranged in a cone with an apex of 90°. The results of the polarized light studies disagreed with the results of X-ray studies. Possibly the difference in the X-ray studies was due to one study being made of the thin surface crystal layer deposited on the palisade layer. It would therefore seem necessary to repeat this X-ray research on a larger number of eggshells with different crystalline structures. It would be interesting to select eggs of different strains and species.

Inclusions The inclusions in the eggshell were first noticed by von Nathusius (1868) as roundish grains with lime compounds. Kelly (1901) saw them arranged in lines parallel to the surface and believed them to contain calcium phosphate deposits. About ten years ago Schmidt described them as air inclusions. Later Schmidt (1964 d, e) called them gas inclusions which he assumed to be filled with air or carbon dioxide. During the last years he has referred to 'globular inclusions' (Schmidt, 1967 a, b). The palisade layer in ground sections appeared quite dark owing to the presence of these inclusions (Schmidt, 1958 b). The author found fewer of them in the outer part of the palisade layer. Heyn (1963), working with a transmission electron microscope, saw them in replicas of the radially fractured surface of the eggshell. He described them as pitlike holes containing small irregular crystals. The nature of these crystals is still uncertain but may be settled by an X-ray microanalyser with a small probe-diameter.

Measurements of the diameter of these holes (inclusions) were reported by von Nathusius (1882). He showed that the diameter may vary from one species to another. He estimated 0.3–0.4 μm in the goose, swan, hen and ostrich eggshell; owing to their small diameter they were difficult to measure. The highest value (3.75 μm) was found in the parrot eggshell. In the author's opinion the diameter was not the same in all layers of the shell.

Dark patterns were seen in sections of the eggshell under the light microscope possibly due to different arrangements of the inclusions, clefts and outgrowing sutures (Schmidt, 1967 b). When a cleft became narrower more to the outer part

of the shell and finally disappeared, there remained a line between the crystal individuals which was called by Schmidt (1967 b) an outgrowing suture. The herring-bone pattern was described for the herring-gull *(Larus argentatus)* eggshell by Schmidt (1964 e) as alternate regular layers not parallel with the shell surface. Some of these layers were said to have many and others few spherical inclusions forming stripes. The stripes were in a section in two directions at about the same angle to the surface of the shell. Terepka (1963 b) also observed such a pattern in decalcified radial sections. When the pattern and the growth lines occurred in the shell, the growth lines were parallel to the surface across the entire shell, whereas the herring-bone pattern was interrupted at the column boundaries. When the globular inclusions were regularly accumulated an arrangement of inclusions called a globular pattern could be formed (Schmidt, 1967 b). The inclusions were sometimes arranged in stripes running obliquely against the shell surface and formed according to the author a stripe pattern. In shells of some species if the clefts, outgrowing sutures or globular inclusions were arranged in rectangular or rhombic areas, block and rhomboidal patterns may be formed. These patterns were described in the swan (Schmidt, 1966 c) and albatross *(Diatomea exulans)* eggshell (Schmidt, 1967 b, c). They easily break off at the boundaries of the columns. This may be due to differences in formation of the calcite individuals in the shell.

3.2.4 Surface crystal layer

Tyler (1965 b) termed the final thin layer of fine vertically oriented crystals above the palisade layer and under the organic cuticle as surface crystal layer. He showed this layer in sections of shells of the Anatidae (Tyler, 1964). In 1862 Nasse observed a layer which was rich in organic matter in the outer part of the calcified shell (Blasius, 1867). Von Nathusius (1868) saw in thin radial sections of the ostrich shell, viewed under polarized light, that its thin outer calcified layer was translucent. Kelly (1901) and Marshall & Cruickshank (1938) saw a vertical layer of crystals in the outer part of the shell. This thin outer layer probably corresponds with the surface crystal layer, studied by Tyler (1965 b). There is still controversy about this layer, because Schmidt (1964 a) described it as the inner part of the 'Deckschicht'. In radial sections of the swan eggshell, viewed by transmitted polarized light, he saw that the part of this layer lying on a column extinguished at the same time as the column. The extinction was not quite so regular. The author observed small crystal individuals in this layer, as described by Tyler (1965 b). Air inclusions were often present in clefts, formed between these calcite individuals perpendicular to the surface.

3.2.5 True cuticle

The whole shell is covered by an organic cuticular substance. It is termed true cuticle (bloom: Szielasko, 1913).

Arrangement in layers The true cuticle was described first by Baudrimont & St. Ange (1847), as a structureless epithelial layer. This layer could easily be lifted from the shell by action of acids on the calcified shell beneath (Dickie, 1848). In a more detailed study, he suggested that this layer consisted of epithelial cells over a basement membrane. The deeper layers should consist of nucleated cells and the more external ones did not necessarily include these nuclei. Romankewitsch (1934) also observed two layers in the organic cuticle. An outer granular layer and an inner transparent structureless one. Schmidt (1964 a) saw two layers in the 'Deckschicht' of which the inner one was calcified. This inner layer is the surface crystal layer as described previously. The outer layer he saw, is the true cuticle. After staining with gold or methyl green von Nathusius (1893) demonstrated in the shell cuticle numerous strongly refractive granules with diameters from 1.0–1.2 μm. In special uncoloured preparations von Nathusius (1887) saw many vesicular holes with a diameter less than 1 μm. No explanation for this difference was given. Von Nathusius (1868) observed vertical and horizontal striations in this layer, which covered the whole egg including the pores.

Hair cracks Von Nathusius (1868) showed that hair cracks were present in the cuticle but not the pores, as von Wittich suggested. Marshall & Cruickshank (1938) considered the true cuticle as a porous apparently structureless substance, which contained 'plaques' as thickenings of the true cuticle over the mouths of the pores.

Attachment to shell According to Romankewitsch (1934) the organic cuticle in the hen and goose egg were strongly attached to the shell but in the duck egg the connection was looser.

Air inclusions may occur in the true cuticle, which would make the exchange of gas easier (Schmidt, 1961). This is important for a developing embryo.

Thickness The thickness of the true cuticle differed very much in the different eggshells (Dickie, 1848). Water-birds had a very thick organic cuticle but with singing birds this layer might be very thin (Landois, 1865). Some workers have given values for the thickness of the true cuticle. Schmidt (1958 a) estimated in *Caccabis gracea* eggshells that the thickness of this cuticle was about 2 per cent of shell thickness. For the hen egg von Nathusius (1893) gave values from 5 to 10 μm, Sajner (1955) between 3 and 5 μm and Simkiss (1961) about 10 μm. The true cuticle seemed thinner at the end of the laying period and was unevenly divided over the shell (Keller, 1940/1941). If a calcite column was lower than its

neighbouring ones, a thicker layer of cuticular material may be deposited on this column (Schmidt, 1962 a). This may explain its uneven thickness. Von Nathusius (1894) studied the variation in the thickness of the true cuticle of spotted eggs. He observed that an outer layer of 7 μm contained no pigment. In a radial section he found through a spot under the uncoloured layer of 7 μm a coloured cuticular layer with a thickness of 12.5 μm. If the radial section was not made through the spot, the true cuticle was 7 μm thick.

3.2.6 Cover

A calcified layer was deposited on the true cuticle in many species (Schmidt, 1958 a). This layer is termed cover. Normally this calcified cover was not present on the hen egg (von Nathusius, 1893; Romankewitsch, 1934 and Schmidt, 1966 b). Sometimes, however, this covering layer was found on the hen, duck and turkey egg (von Nathusius, 1894). The author showed that its inner surface was attached to the layer underneath. Its outer surface made the shell rough. According to Schmidt this layer consisted of calcite grains, which may be variable in size (von Nathusius, 1882). They disappeared after a treatment with chromic acid and may have consisted of unorganized calcium salts (von Nathusius, 1869).

Often the cover occurred on eggshells of broiler-type pullets. These pullets sometimes laid two eggs a day. The first egg frequently had a hard shell with a rough cuticular covering, sometimes over the entire surface of the egg, but usually as a band over a part of it (van Middelkoop & Simons, 1970). The covering was about 40–50 μm thick.

3.2.7 Pores

Although Sajner (1955) said there were no pores in the eggshell, it is generally agreed that they are present. Pores were observed in the middle of the nineteenth century by Baer (1837), Baudrimont & St. Ange (1847), von Hemsbach (1851) and von Wittich (1851). They were found perpendicular to the shell surface through the different layers of the shell from the interstices between the mammillae to the surface and were in direct connection with the interstices between the mammillae (von Nathusius, 1868).

Penetration by true cuticle The pore was sealed up by the true cuticle, which covered the calcified shell according to Marshall & Cruickshank (1938). This organic covering may penetrate as a plug in the outer part of the pore. The largest pores were visible with the naked eye as depressions. According to Romanoff & Romanoff (1949) this pore mouth opened outside in a narrow, branched groove. This groove may be compared with cracks on the surface of the true cuticle. The pores could be seen clearly when the shell was partly decalcified (von Hemsbach, 1851) by which procedure the cuticular substance was loosened from the shell

and after boiling with sodium hydroxide (Landois, 1865) which dissolved the organic material.

Shape Marshall & Cruickshank (1938) claimed that the pores passed right through the shell. The pores were oval (von Nathusius, 1868) and normally funnel-shaped (Simkiss, 1961). The smallest diameter occurred near the base of the palisade layer. In some bird eggshells, including the hen's, the pores were unbranched (Tyler & Simkiss, 1959 b). Schmidt (1958 b) not believing the pores to have their own organic wall, observed in tangential sections of premature eggs triangular or quadrangular shaped pores (Schmidt, 1966 b). He suggested that the calcite columns bound the pores. These results about shape differed from Tyler (1956, 1964) who embedded the shell in plastic and after dissolving the shell with concentrated hydrochloric acid, saw that the plastic moulds of the pores which did not show an angular boundary, were left behind.

Size Von Nathusius (1868) gave for the maximum pore size a largest diameter of 29 μm and a smallest diameter of 22 μm and for the minimum pore size a largest diameter of 11 μm and a smallest diameter of 9 μm for shell pore size. These values were lower than those found in the duck, swan and turkey eggshell. In a soft-shelled hen egg he observed lower values for the pore diameter than in a hard-shelled egg, which may be related to the enlargement of the egg during its shell formation. Haines & Moran (1940) found a pore with a diameter of 13 μm at the top and 6 μm at the bottom, but pores up to 15 μm occurred. These values are very low, compared with Tyler (1956) who measured the plastic moulds of the pores and found in two shells the respective mean values 63 μm and 45 μm at the top and 23 μm and 17 μm at the bottom.

Number Schmidt (1958 b) described different methods to make the pores visible and to count them. Dye solutions that stain the pore plug could be put inside or outside the shell, or the air bubbles pressed through the shell under water at the places where the pores were present could be counted. Tyler (1953) used another method. He marked the pores by immersing the shell in concentrated nitric acid for a short time and then counted them under the light microscope. The author was able to count very small pores, which were enlarged by this treatment. With dye solutions it was difficult to count the small pores as the colours ran.

Therefore the numbers of pores counted by the different methods varied. As quoted by Stewart (1935), Rizzo (1899) found 6000–8000 pores per egg. Romanoff & Romanoff (1949) found about 7500. These authors used dye solutions. Tyler (1953) using the nitric acid method counted 7000–17000 pores per egg.

The pores were not distributed at random over the shell (Tyler, 1953, 1955). At the broad end of the shell were more pores/mm^2 than at the narrow end (Romanoff & Romanoff, 1949; Tyler, 1958 and Schoorl & Mos, 1968).

Pore wall Not much is known about the structure of the pore wall. Lumps of cuticular material were found in the pore channel against the wall (Tyler, 1964). As the true cuticle was deposited, this material may have been drawn inwards. Tyler (1964) noted further, that the organic matrix of the palisade layer has a streamlined effect inwards at the pore channel. This indicated that the growth of the shell around the pore was retarded.

3.3 Results and discussion

3.3.1 Shell membranes

Layers with fibres and meshes One complete transverse section through the two membranes was examined. The inner membrane had a thickness of 22 μm and the outer one 48 μm. The inner membrane was much denser than the outer one. From inside to outside in this membrane first a layer of transversely cut fibres (2–3 fibres thick), then a second layer of longitudinally cut fibres (1–3 fibres thick) and a third layer of again transversely cut fibres (4–10 fibres thick) were observed. In the first layer there were irregular open meshes of about 2 × 3 μm; the second layer was very dense; the third layer was still more open than the first with meshes of about 5 × 7 μm.

At the line of contact between inner and outer membranes over a distance of 53 μm only 11 fibres of the inner membrane were fused by their mantles to those of outer membrane fibres. Therefore the contact seemed rather loose. It may be, however, that a very few fibres of the outer membrane crossed over into the inner membrane, as on the inner side over a length of 23 μm at the frontier between the membranes only 2 thicker fibres were seen among the thinner ones.

The outer membrane in this preparation had wider meshes (about 8–10 μm) and from the side of the inner membrane in an outward direction there were 6 layers of fibres alternately cut longitudinally and transversely and 5–9, 3–5, 1–2, 5–8, 1–4 and 4–6 fibres thick respectively.

The diameter of the meshes may be of importance for the penetration of micro-organisms. Some micro-organisms, for example *Pseudomonas* occurring in the contents of rotten eggs (Board, 1968) and having a diameter of 2 × 1 μm (Haines & Moran, 1940) can penetrate through the meshes of the inner and the outer membrane quite easily. For other micro-organisms with larger diameters it is more difficult.

Inner layer of inner membrane The inner layer of the inner membrane is seen as a smooth surface under the light microscope. In our transmission electron microscopical work it looked at first quite different in various eggs (figs 2, 3 and 4). Simons & Wiertz (1963) described the possible artefacts in this layer caused by a too rapid decalcification of the shell. Figs 2, 3 and 4 follow the sequence of ageing of the EDTA solution, i.e. the order of decreasing rate of decalcification. Later

we used a decalcification fluid 2 % weaker than Simkiss & Tyler (1957) and 1 % weaker than in the preceding work. As some protein of the egg contents may have been attached to the inside of the inner membrane, in later experiments the interior of the shell membranes were rinsed several times with water after emptying the egg. In the first experiments they were only rinsed once or twice. Sections of these membranes then showed a thinner inner layer of the inner membrane (Fig. 5). Therefore in the preparations of figs 2, 3 and possibly Fig. 4 there may have been some egg protein attached to the inside of the inner membrane. The origin of this layer in Fig. 4 is doubtful because it is of approximately the same granular structure as the mantle of the membrane fibres. Its outer zone (0.1 μm wide) fused with the mantles of the adjoining fibres and was about the same thickness as the similar layer in Fig. 2 and the whole inner layer of the inner membrane in Fig. 5. In Fig. 4 this zone shows a centre of greater electron density of about 0.03 μm width which can be found in some places in the other pictures of this layer. Inwards from the outer zone the layer shows an interrupted row of small openings (Fig. 4). A similar though more discontinuous row lies in the centre of the inner layer of the inner membrane. The structure inward of this row is looser than outward of it except the innermost border which in certain places is even denser than the layer's outer half. The inner contour in this picture is approximately a straight line, which is in contrast with the inside of the inner membrane in other pictures (figs 2, 3, 5 and 6). Granules can be seen of up to 1 μm in short rows mainly in one direction, in a tangential preparation with the scanning electron microscope (Fig. 6). Granules of about 1 μm on the inner layer of the inner membrane were seen many years ago by von Nathusius (1868). The organic deposits probably protein at the inside of the inner layer in figs 2 and 3 also have a diameter of about 1 μm. Possibly in the preparations studied by von Nathusius (1868) and in those examined with the scanning electron microscope some egg protein was still present on the inside of the membranes, although after emptying the eggs for the latter examinations were rinsed several times with water.

Attention should be drawn to the 0.03 μm wide canal passing through the entire inner layer of the inner membrane in Fig. 3. Its diameter is so small that micro-organisms could not penetrate it. Such a canal was only seen once during the study of many shell membrane preparations.

Course of fibres The fibres are attached to the inner layer of the inner membrane. 16 membrane fibres were attached over a length of 20 μm. In the outer membrane and sometimes in the inner one also composite bundles of fibres were seen. In the outer membrane, bundles of up to 4 fibres were found (Fig. 7). The 4-fibre bundle can be traced for more than 1 mm. The fibres in the membranes were oriented slightly. The thicker fibres ran almost parallel in systems forming diamond meshes. A predominant direction was indicated to some extent.

Thickness of fibres The average fibre diameter in the inner membrane was less than in the outer one. The maximum fibre diameter in the inner membrane was 1.5 μm with an average of about 0.9 μm. Most of the fibres of the outer membrane had a diameter of around 1.3 μm but sometimes over 3 μm especially in the outer part of the outer membrane. The average fibre diameter as reported by Sajner (1955) and Moran & Hale (1936) for the outer part of the outer membrane and by Wolken (1951) for both membranes are comparable with the values given above. Measurements in electron micrographs obtained by Simkiss (1958) and Masshoff & Stolpmann (1961) of between 0.5 and 1.0 μm are too low. The authors probably measured only the fibre diameter of the fibres of the inner membrane. Von Nathusius (1893) and Romankewitsch (1932) gave higher values than described here. They were possibly unable to measure the diameter of the smaller fibres under the light microscope and obtained therefore only values of the larger fibres or bundles of fibres.

We found membrane fibres of the duck and turkey egg with about the same diameter as those of the hen egg.

Structure of fibres A fibre consists of a core with a mantle. The core contained an electron-denser material than the mantle (figs 4 and 5). Using a staining method Romankewitsch (1932) distinguished under the light microscope a denser outer layer (mantle) and a less dense core. The densities in his and our experiments could not be compared.

Holes sometimes occurred especially in thick fibres (Fig. 8). In some longitudinally cut fibres there were oblong holes which indicated that the holes in the core of the fibre may follow a longitudinal direction. Maximum diameter of the holes was 0.3 μm in membrane fibres of normal eggs. In a membrane fibre of a premature egg about 80 holes were found, some with a diameter of 1 μm (Fig. 9). In this fibre the holes lay more or less in rows parallel to the surface of the core. In many outer shell membrane fibres, air channels with a diameter of 0.7 μm were seen by von Nathusius (1893). He was, however, unable to see the smaller holes. In some wide fibres he occasionally identified three of them close to each other.

The clefts between core and mantle as mentioned by Masshoff & Stolpmann (1961) were seen in many sections (figs 4, 5 and 9) although they were sometimes not visible at the places around the cores. After membranes marked with a staple were kept in a 4 % formaldehyde solution for a long time, clefts could be seen as electron-dense spaces in a premature eggshell membrane (Fig. 10). Some copper or iron which went into solution during this time, was deposited in the clefts. The thickness of the clefts was less than mentioned by Masshoff & Stolpmann (1961). Although difficult to measure, the maximum cleft thickness found, was about 0.1 μm.

The thickness of the mantle varied in the different parts of the membranes between 0.1–1.0 μm. It was thinner in the inner than in the outer membrane

fibres. Examining with the transmission electron microscope a layer with fibres – by tearing off the membrane – it was possible to see membranes (Fig. 11) and threads (Fig. 12) which both attached fibres to each other. These contacts were mostly near places of fusion of mantles of different fibres. They especially occurred in the outer membrane. There were many budlike protuberances on the surface of the fibres of the inner and outer membrane (figs. 7, 11, 12 and 13). Measurements for the diameters of these protuberances went up to 1.6 μm and their length was variable. At one place there were 12 protuberances of about 0.5 μm over a distance of 8 μm. Spirally arranged details of surface structure could be seen crossing the whole outer surface of the fibre (Fig. 13). Many small protuberances were seen on these spirals. The direction of the fibres which fused with the others was parallel with the spiral surface structure of the latter.

3.3.2 Layer of cones and basal caps

The outer boundary of the outer membrane was not delimited but its fibres entered the calcified part of the shell. The basal caps being the bases of the calcified shell were anchored in the outer membrane.

Shape and surface of mammilla Just beneath the base of the mammilla the outer membrane contained many irregular calcified deposits through which fibres pass that penetrated the mammillary base in tufts and ensured a firm connection between the mammillary layer and the membranes (figs 14 and 15). These calcified deposits were seen in radial sections as protrusions of the mammillae. Except where the fibres entered the mammilla there seemed to be no contact between it and the calcified deposits. The basal boundary of the caps looked quite irregular with the presence of these deposits. Small crystals as Heyn (1963) mentioned were not seen on the inside of the shell. The surface of the sides of the mammillae and between the deposits at their bases was smooth, rounded off and covered with a thin layer of organic material (figs 14, 15, 16, 17 and 18). The deposits were also enclosed by a thin dense organic layer possibly the same as that present on the surface of the mammillae (figs 14 and 19). The polygonal surface of the crystal individuals lying underneath this organic layer in the mammillae was still recognizable at its outer surface. Many holes with a diameter of about 0.3 μm occurred in the organic surface layer. This layer, also seen in decalcified radial sections, was 0.1–0.5 μm thick. The interstitial spaces between the mammillae were bounded by an organic layer. These layers of neighbouring mammillae were fused outwards if there was no pore between them and formed a cleft between the mammillae (figs 14, 15, 16 and 18). The fused organic layer penetrated the shell for more than 70 μm. It was not expected from the literature that a cleft was rounded outwards as seen in Fig. 18. This cleft could be a part of a pore near its inward end, although the distance between the walls was small (0.2–0.5 μm). Sometimes two neighbouring mammillae were in contact by a

calcified bridge (Fig. 20). This contact was bounded by the same kind of organic layer as the greater part of the surface of the mammillae. The distance between them was about 8 μm.

Anchorage of shell in outer shell membrane The contact between membrane and shell was made by the irregular calcified deposits at the extreme base of the mammilla penetrating into the meshes of the fibre network (figs 14 and 19). The thin organic layer which enclosed the deposits seemed to continue the outer lining of the fibre mantle (Fig. 19). The interior of these deposits studied in decalcified sections was of a loose, granular nature, partly arranged in strands, which gradually merged into the denser organic mammillary matrix. This organic matrix in which two directions characteristic of the calcite crystal were seen, enclosed small open spaces varying in diameter. During decalcification, calcified material was probably dissolved from these now open spaces.

At the place of contact between the fibre and basal cap the appearance of the mantle changed abruptly (figs 21 and 22). The homogeneous fine granular structure turned into much coarser, granular strands branching out fanlike with a finer network in between (Fig. 21). The thickness of the mantle increased gradually to 1 μm. In the strands were many small holes probably formed during decalcification. The outer boundary of the strands was not sharply outlined, its branchings being fused with the mammillary matrix. Nevertheless the mantle stood out sharply against the matrix background because of its much greater electron-density.

Further into the mammillary base the fibre core showed more vacuolisation especially along its outer margin (figs 21 and 22). Then the core branched into electron-dense, fibrillar material and probably also membraneous. As the distance from the core increased the branching became finer. The arrangement of this organic network clearly showed that in calcified shells the membraneous matter was deposited around crystalline material in which some fibrils may be seen. Outwards from the core the crystals became smaller as the branching became finer. In decalcified sections these anastomosing strands showed a loose network which occurred in a belt up to 3 μm wide. This may be the original fibre mantle. At the periphery of the belt the strands merged into a much denser mammillary matrix. Just outside this belt the mammillary matrix was seen as an even more densely arranged organic material (Fig. 22).

The membrane fibres were measured in the shell up to a depth of about 20 μm. Masshoff & Stolpmann (1961) illustrated the entrance of the membrane fibres into the base of a mammilla and suggested that these fibres pass throughout the calcified part of the shell. This, however, was not the case. The fibres were only present in the inner part of the mammillary base, where they were anchored in its organic matrix and the rest of the calcified shell was without membrane fibres. Next to the anchorage just described were a few membrane fibres, some with vacuoles (Fig. 23). They were surrounded by coarse granular branching strands. There were large cavities in the mammillary base about 10 μm above the anchoring

site of the membrane fibres. These were irregularly branched, about 9 μm maximum diameter and with walls of uneven thickness (figs 23 and 24). The walls had about the same electron-density as the fibre cores and they may correspond with the vacuolated fibre cores. Strands of mantle-like material were seen around it in Fig. 23. These cavities might be the EM equivalent of Sajner's rings as described and illustrated by Tyler & Simkiss (1959 a). In tangential sections some holes were seen together in a ring which may correspond with the fibrous rings seen on the outer membrane after decalcification (Terepka, 1963 a, b). Terepka observed these rings connected with fibres which supported the idea that cavity walls were of the same origin as fibre material.

Organic matrix All different calcified layers of the shell were interwoven with an organic matrix which could be studied easily after decalcification. The side of the mammillae which did not contain membrane fibres was covered by an organic layer which varied in thickness. This layer was connected in some places with the outer lining of the fibre mantle as was described for the surrounding organic layer of the irregular deposits at the inside of the mammillary bases. The organic matrix of the basal cap and the inner part of the cone consisted of a very fine fibrous network (figs 18, 21, 22, 23, 24, 25 and 26). This matrix was most dense between the fibres of the outer membrane and just outside it in the central part of the mammilla (mammillary core). Simkiss & Tyler (1957) among others observed that the organic material was more concentrated in the central core of the mammillae. The maximum diameter of the fibrils of the mammillary matrix was about 0.008 μm. The larger of the meshes formed by decalcification have a diameter of about 0.1 μm. Many fibrils were definitely oriented in two directions probably originating from their position at the crystal faces of small crystallites.

Smaller or larger areas within this organic matrix were surrounded by a denser organic layer (figs 22 and 23). This layer corresponds with what was called 'loculus' by Schmidt (1965 c), enveloping a single crystal individual (See further Chap. 6).

In the axis of a mammilla a 7 μm long fan-shaped arrangement was seen (Fig. 25). At approximately a right angle with this axis the mammilla was entirely or partially transversed by membraneous lamellae in the matrix (figs 21 and 25).

Holes with a maximum diameter of 0.8 μm and with a wall thickness of maximal 0.13 μm occurred in the meshwork of the matrix as seen in decalcified sections (figs 24, 25, 26 and 27). They were mostly vesicular but sometimes had a more angular boundary (figs 26, 27 and 29). However they will be defined in decalcified preparations as vesicular holes. Heyn (1963) saw them in the palisade layer as pitlike holes in which tiny crystals could be found. Fig. 28 shows these pitlike holes in a fracture surface studied by scanning electron microscope. A definite conclusion cannot be made about the form of the tiny crystals in Fig. 28, but vague diamond and hexagonal contours point to rhombohedra. The greatest diameter of the pitlike holes in Fig. 28 is 0.8 μm. It was not clear in the decalcified

radial sections whether these holes form growth lines as reported by Schmidt (1962 a, 1965 b). This might be due to the greater magnification we used. Outside the membraneous lamellae in Fig. 27 the presence of growth lines was indicated. This photograph also shows the boundaries of the crystal individuals. The vesicular holes in decalcified sections were more concentrated in the central part of the crystal individuals especially in the inner part of the cone layer. To the outside of this layer the holes were more numerous and also near the boundaries of the crystal individuals (Fig. 27). This agrees with Schmidt (1964 d). These concentrations of holes might show up in tangential ground sections as the dark triangles, first observed by von Nathusius (1868). In the decalcified guinea-fowl eggshell the holes described did not occur in the very dense organic core or in the outer part of the core where the organic matrix was less dense (Fig. 29).

The outer part of the cone layer of the hen eggshell showed a completely different structure. Towards the outer layer the reticular character of the matrix gradually loosened and the fibrils broke up into loosely arranged strands ot granules. The outer layer was sharply defined, being much more compact. It consisted of numerous vesicular holes up to 0.5 μm diameter intermingled with coarse granular masses (Fig. 30). The outer part of the cone layer in the guinea-fowl eggshell differed from its inner part and from the outer part of the cone layer of the hen eggshell. It contained many of these holes, the walls of which were either attached to or lay in the course of the fibrils at right angles to the axis of the cone. They have a diameter of about 0.6 μm (Fig. 31). The structure of the organic matrix in this layer looked like that of the palisade layer in the hen eggshell but had more vesicular holes and was less dense in structure than the matrix in the palisade layer of the guinea-fowl eggshell. Von Nathusius also pointed out the fine horizontal striations in the inner layer of radial sections of guinea-fowl eggshells.

Crystalline structure The calcified shell is composed of calcite crystal individuals. A crystal individual is defined as a united part of the shell extinguishing at the same time, when seen in ground sections viewed by polarized light. These crystal individuals radiated in all directions from the centre of the mammillary core. Some of the outwardly directed ones from one mammilla possibly reached the outer surface. Within the crystal individual, smaller crystalline units occurred which will be called crystallites from now on. Their crystal faces may be partly connected, run parallel, and the crystallites have therefore within the crystal individual the same direction of extinction when placed between crossed Nicols (Chap. 6).

Crystalline structure of the shell has been studied in radially fractured surfaces mainly with the scanning electron microscope (figs 16, 17, 28, 32 and 33). A fracture through the centre of the mammilla showed crystal individuals with maximum widths of 15 and 30 μm radiating from the centre of the mammilla. At the periphery of the crystal individuals the crystallites seemed to be oriented

nearly perpendicular to their surface. The same orientation occurred at the surface of transverse fractures through columns (cf. the radial peripheral striations of crystal aggregates seen by Heyn, 1963, Fig. 6). This may explain why after the crystal individuals break apart along their organic envelope in the emu eggshell striations mainly parallel to its surface are seen (Fig. 34). Unfortunately no information on the history of the egg was available. The shell was green and the egg had a very uneven surface. The crystal individuals in this shell had a width of between 15 and 30 μm. Figs 16 and 28 show a radially fractured surface with a zone 10 μm wide and rich in organic material starting at the line of contact between two mammillae. After this zone the quantity of organic material between the mammillae decreases and lamella-like crystallites are seen. In Fig. 17 a zone of about 15 μm rich in organic material can be seen in a fractured surface near the side in the outer region of a mammilla. At this place a crystal individual may be broken out. In this photograph there are many striations oriented more or less perpendicular to the axis of the crystal individuals and probably representing a boundary between two crystal individuals.

3.3.3 Palisade layer

The palisade layer begins outside the layer of cones. The change from cone layer to palisade layer was more gradual (Fig. 30) than expected from the previous results of Simons & Wiertz (1963). In the upper part of the photograph especially to the left there are some fibrils which are part of the palisade layer.

Organic matrix The appearance of the decalcified palisade layer in radial sections was dominated by fibrils with a maximum length of 10 μm and thickness of about 0.01 μm parallel to the surface of the shell (Fig. 35) and by vesicular holes approximately 0.4 μm wide whose walls were attached to the fibrils or lying in their axis. These walls had a thickness of up to 0.06 μm. The fibrils had very thin branches, short in relation to their length, anastomosing with the walls of the holes or with branches of neighbouring fibrils and more or less perpendicular to the axis of the fibril. It was impossible to tell from the cross sections of these branches whether a small amount of granular material was also in the palisade layer. The herring-bone appearance of the organic matrix observed by Terepka (1963 b) was not seen in the electron micrographs. Possibly the magnification we used was too high and limited our interpretation of the arrangement of vesicular holes.

Three layers with a different distribution of organic material seen by Romanoff & Romanoff (1949) in the palisade layer were not visible in the investigated ultrathin sections. The matrix structure of the outer part of the shell, including the organic matrix of the surface crystal layer, was more compact than the matrix elsewhere in the palisade layer (Fig. 36). This more compact layer probably corresponds with the layer of the shell less resistant to etching with hydrochloric

acid as reported by Tyler (1956).

In the palisade layer the appearance of the matrix in tangential sections (Fig. 37) differed from that in radial sections. In tangential sections the organic material appeared to be unevenly distributed over the shell. Some parts had a compact structure and others a loosely-arranged one. To what extent this is caused by the method of preparation is not clear. The tangential sections contained fewer and shorter fibrils than the radial ones. Fibrils of up to 5 μm long were seen. Within a certain area they follow a certain predominant direction with granular branchings more or less perpendicular to them. The predominant direction of the fibrils in the lower part of Fig. 37 differs from that of the upper part because they each may belong to different crystal individuals.

The decalcified palisade layer of the guinea-fowl eggshell was characterized by a very fine division of its organic matrix far denser than in the hen eggshell. Holes of the same diameter as described in the palisade layer of the hen eggshell were also found in the shell of the guinea-fowl but they were not so numerous.

Crystalline structure The crystalline structure of the palisade layer was especially studied in fractured surfaces with the transmission electron microscope after using the replica technique and with the help of the scanning electron microscope. Polarized light studies in ground sections were also carried out. After studying a shell fracture with the scanning electron microscope it was possible to fit them together with the results obtained with the transmission electron microscope. In the palisade layer the plane of fractures deviated from the strictly radial direction characteristic of the cone layer. Transverse breaks of about 50 μm diameter occurred here and there through the columns (figs 39 and 40) so that in these places the shell fracture was in a tangential plane parallel to the surface of the shell. Organic material (matted fibrils with attached vesicular holes), which partly enclosed small crystallites with a longest side of about 0.5 μm, was seen in this plane (Fig. 40). Also Heyn (1963) found, in radially fractured surfaces, rather flat areas which were rich in organic material with small crystallites in the matrix. He called this structure softer and less pronounced crystalline. Although he spoke about a transverse fracture through the shell it was probably a tangential plane through a column. In radial sections of decalcified shells layers of organic material were found parallel to the shell surface at relative distances of about 2 μm. These corresponded with the distances between the tops of the rhombic crystallites visible at the front side of the broken column in Fig. 39 which indicated several of the potential fracture planes. A more detailed photograph of such an area was obtained by replica technique (Fig. 41). The horizontal direction seemed parallel to the surface, the crystallites being at right angles to it. The organic matrix and small crystallites were not visible in this picture.

A radial rather flat break through the calcified column showed calcified material in thin parallel layers with holes inside and some organic matrix between (figs 42

and 43). The homogeneous block-like units with a width of 10–15 μm seen by Terepka (1963 a) were not seen in these preparations. Most of the palisade layer in the hen, duck, turkey and ostrich eggshells showed this flat break. Sometimes, however, transverse breaks through a whole column occurred as previously described. The radial fracture of one guinea-fowl eggshell showed this flat break over the whole palisade layer. In a detailed picture of a replica possibly representing this flat fracture pattern in the hen eggshell (Fig. 44) the small crystallites with their holes and fibrillar matrix could be clearly seen. The fibrils had a main direction parallel to the surface and branched at about right angles to it. They were probably located at the crystal faces of the small crystallites with dimensions of about 0.5 μm.

After removing the true cuticle with EDTA the denuded surface of the shell showed in some places angular boundaries of columns of the palisade layer (Fig. 45). The columns had diameters of sometimes over 80 μm. Heyn (1963) found diameters of the order of 50–100 μm.

Holes and inclusions In the organic matrix of the palisade layer decalcified sections were studied with the transmission electron microscope (figs 35, 36 and 37) and vesicular holes with diameters of about 0.4 μm were seen. Holes of about the same diameter but less numerous were also found in the guinea-fowl eggshell (Fig. 38). Size of the diameters were similar to those of 0.3–0.4 μm reported by von Nathusius (1882). In the scanning electron microscope work and in the photographs obtained by replica technique the holes were shown to be pitlike with a maximum diameter of 0.5 μm in which often tiny crystals can be seen (figs 40, 41, 43 and 44). Sometimes minute crystals lay close to the holes, probably having fallen out of the holes and come to rest there. A definite conclusion about their form could not be made but vague diamond and hexagonal contours pointed to rhombohedra as for the layer of cones and basal caps. Figs 4 and 5 of Heyn (1963) gave the same impression although he called them irregular. Kelly (1901) believed that they contained calcium phosphate. Schmidt (1964 d, e) saw them as dark points under the light microscope and referred to them as gas inclusions. Some air was present possibly between the tiny crystals and the hole walls. The content of the holes is still controversial. However the problem may be solved using an X-ray microanalyser with a small probe diameter.

Patterns due to hole arrangements, outgrowing sutures and clefts (Schmidt, 1967 b) were not encountered in this work.

In the palisade layer larger holes also occurred (Fig. 46 one of 3.5 μm). In sections of shells from climatically stressed hens we found holes with diameters of 3.1, 5.8 and 9.0 μm. In ground sections through these shells much larger cavities were present (El-Boushy et al., 1968). The walls were of uneven thickness, in certain places up to 0.6 μm. The wall substance of these holes looked similar to that of the small vesicular ones (Fig. 47). Walls of the latter were seen attached to the wall of a large hole and in some places the small and the large holes had

coalesced. In the outer part of the palisade layer of an emu eggshell many similar holes occurred (figs 48 and 49).

Schmidt (1968) found triangular holes with the point directed outwards in the inner part of the palisade layer of the guinea-fowl eggshell. We found them in the inner as well as in the outer part of the palisade layer. In our decalcified sections and also in the ground sections they were more numerous in the inner part. They had sides up to about 3 μm (Fig. 50). In one case a round hole was found with a diameter of 1.6 μm (Fig. 50). This hole had a wall of up to 0.06 μm in thickness which was much thicker than the wall of the triangular holes.

All these large holes except the triangular hole of the guinea-fowl eggshell look more or less like the cavities described in the section on the layer of cones and basal caps. However they did not have such an irregularly branched form.

3.3.4 Surface crystal layer

The surface crystal layer is deposited on top of the palisade layer in varying thickness. In ground sections of the shell examined in polarized light the thickness varied between 3 and 8 μm. In sections of guinea-fowl eggshells the surface crystal layer had an average thickness of 5 μm. In preparations of eggshells of the Anatidae (Tyler, 1964) a value of about 10 μm was measured for this surface crystal layer.

The scanning electron micrographs showed that this layer consisted of small calcite crystals with their greatest dimension approximately at right-angles to the egg surface (figs 51 and 52). Not so many pitlike holes were present in this layer as in the palisade layer. This may be the reason why von Nathusius (1868) saw a very thin transparent outer calcified layer in ground sections of ostrich eggshells. This outer calcified zone was attached to the palisade layer. The surface of an old egg showed that the boundaries of the columns went further into the surface crystal layer. Schmidt (1964 a) saw, in radial sections of the swan eggshell under polarized light, the parts of this layer lying on a column extinguished at the same time as the column. We also found this in the hen and guinea-fowl eggshells.

In decalcified radial sections a zone of 1.5–2 μm has been observed predominantly fibrillar (figs 53 and 54). The long fibrils (or fibrillar membranes?) were thicker (0.1 μm) than in the palisade layer and abounded in fine branchings perpendicular to their axis. The layer was much thinner here than was observed in the scanning electron microscope perhaps caused by shrinkage during decalcification. From the figs 53 and 54 this surface crystal layer in relation to other parts of the palisade layer seemed rich in organic matter. This had already been reported by Blasius (1867). On top of the surface crystal layer a thin membrane formed an irregular surface making contact with the true cuticle.

3.3.5 True cuticle

The organic true cuticle in decalcified sections was completely vesicular. In its lower half irregular open spaces of up to 0.5 × 2.8 μm in size occurred between the vesicles (Fig. 54, white egg). In a few months old brown egg (Fig. 53) its structure was much denser. Schmidt (1961) suggested that air inclusions occurred in the true cuticle. The vesicles were spherical or ovoid with diameters up to a little over 1 μm while neighbouring ones may have fused. They were probably seen by von Nathusius (1887) as refractive granules with diameters of 1.0–2.0 μm. The vesicles were empty or partly filled with granular material along the inner periphery sometimes branching off in thin anastomosing strands towards the interior. A few vesicles had a central aggregation of coarser granules. These aggregations may have been seen by Dickie (1848). He suggested that nuclei were present in the vesicular substance. The brown egg true cuticle showed more and coarser granules than the white one.

In Fig. 54 the outer quarter of the true cuticle was more compact than the rest with vesicles of about 0.2 μm. The vesicles in Fig. 53 had about the same size. Also Romankewitsch (1934) mentioned the existence of two layers in the organic cuticle.

The cuticular surface showed many star-shaped crack systems (Fig. 55). The big crack systems indicated the surfaces of the oval pore plaques. In the cracks cuticular vesicles were seen of up to about 1 μm (Fig. 56).

The thickness of the true cuticle varied in different eggs and in different places of the shell of one egg. It varied in the ultrathin sections of the hen egg between 0.5 and 12.8 μm. Von Nathusius (1893) gave values from 5 to 10 μm, Sajner (1955) between 3 and 5 μm and Simkiss (1961) about 10 μm.

3.3.6 Cover

The calcified covering occurred sometimes on eggshells of broiler-type pullets, and was about 45 μm in thickness. It has not yet been studied with the transmission electron microscope and scanning electron microscope.

The surface of the true cuticle of a boiled egg (Fig. 57) had many rhombohedric crystals of 2 μm diameter. Tyler (1964) found calcium carbonate crystals in the outer cuticular layer of Anatidae eggs. Heyn (1963) described small cuticular bumps from underlying crystals. From his photograph their dimensions were about 0.2 μm. This was the first time we had seen crystals in the true cuticle of a normal hen egg.

3.3.7 Pores

The big crack systems on the surface in the true cuticle indicated the surface of the pore plaques (Fig. 55). Romanoff & Romanoff (1949) saw them as narrow branched grooves. The diameter of the surface of the plaques amounts to over 100 μm.

In a tangential section through the decalcified shell the pore plug, which normally filled the upper part of a pore with cuticular material, was seen. Its diameter was about 11 μm. Part of this plug can be seen in Fig. 37 at the right side below the middle. It consists of the same vesicles with the same diameter (about 0.2 μm) and structure as of the true cuticle in Fig. 53 and the outer part of the true cuticle in Fig. 54. A membraneous wall of organic material to which the fibres of the palisade layer were attached enveloped the sides of the pore plug.

The denuded surface of the shell, visible after removal of the true cuticle, showed the pore holes in the shell (Fig. 45). At least part of the surface crystal layer had disappeared.

The pores were oval with diameters amounting to more than 40 μm (figs 55 and 58). Together with the true cuticle most of these plugs were removed. Fig. 59 shows a pore in which part of this plug is still present. The wall of the pores was covered with a layer of organic material. Fibrils could be seen parallel to the surface of the shell in the pore wall (Fig. 60) at a distance of about 2 μm apart. They could be the edges of the matted layers of fibrils seen in radial sections through the palisade layer (figs 35 and 36), which have the same relative distance.

4 Membrane thickness

The economical importance of shell strength has stimulated research. As shell membranes may influence shell strength, their thickness is considered in this chapter. Values for membrane thickness vary widely and so the methods of measurement were critically compared.

4.1 Literature

Two main methods are used to determine the thickness of the isolated membranes:

1. using some sort of calliper
2. using a microscope with an ocular micrometer.

Hays & Sumbardo (1927) used the first method and found a membrane thickness of between 56 and 75 μm of which the inner membrane varied from 13 to 17 μm. Moran & Hale (1936) obtained values of between 70 and 84 μm, the inner membrane varying from 40–48 μm and the outer one from 30–36 μm. This was the only published result found with the inner membrane thicker than the outer. Balch & Tyler (1964) measured 48.5–57.4 μm for dry and 97.2–123.0 μm for wetted membranes. Romankewitsch (1932) used the second method, obtaining values from 60–125 μm and von Nathusius (1893) a value of 74 μm. Other authors, who did not clearly describe their experimental procedure, have given the following values: Wolken (1951), 60–70 μm; Romanoff & Romanoff (1949), for Brahma, Leghorn and Bantam eggs 92 (22+70), 65 (15+50) and 50 (10+40) μm, respectively. In brackets is given the thickness for the inner and the outer membrane, respectively.

Blasius (1867) noticed that the shell membranes of the eggs of larger birds were usually thicker than those of smaller birds. Asmundson et al. (1943) established a relation between thickness and egg size. The actual thickness of the membranes decreases with decreasing egg size (Romanoff & Romanoff, 1949). Characteristic diferences in membrane thickness within the clutches were observed by Tyler & Geake (1961). Subsequently Balch & Tyler (1964) found that the membranes were not uniformly thick over the whole surface. The pattern of variation for a given characteristic was identical in different eggs from the same birds. Different birds could have different patterns.

4.2 Results and discussion

Within an egg there was a large variation in membrane thickness in a longitudinal direction (Tyler, 1961). Around the latitudes there was far less variation. Therefore the samples were taken from the waist where the variation in membrane thickness was small. The membrane thickness was measured in pieces of isolated membranes, in decalcified sections and in ground sections. The values for the membrane thickness in ground and decalcified sections were the average of independent determinations by two persons each based on duplicate measurements in 1–3 ground sections or 1–15 (mostly 5) decalcified sections. The values for the isolated membranes received from Prof. Dr Ir P. Schoorl from the Institute at Beekbergen were the average of 3–4 measurements. The results of the measurements are presented in Table 1.

The thickness of the shell membranes measured in decalcified and ground sections was on the average 63.4 μm. Hays & Sumbardo (1927) and Romanoff & Romanoff (1949) found an average thickness for both membranes of 65 μm of which the inner one was 15 μm and the outer one 50 μm. In an electron micrograph a thickness for both membranes of 70 μm was measured to which the inner membrane contributed 22 μm. Within the clutch no characteristic modifications were observed. Tyler & Geake (1961), however, expressing membrane thickness in mg/cm² found in two egg clutches, that second eggs had thinner membranes than first eggs. For three egg clutches the second egg had the thickest membrane. For four and five egg clutches they obtained variable results from season to season. The mean membrane thickness – according to the authors – appeared to increase gradually with clutch size up to four eggs per clutch and fell again with five eggs. The differences between these results and our findings might be due to the method of measurement. The weight/cm² within the clutch was possibly not highly correlated with the membrane thickness. The thickness of the membrane fibres could influence the weight/cm² but it is not known if their thickness varied within the clutch. On the other hand it is understandable from the structure of the membranes that it is very difficult to measure their thickness.

Despite the quite different ways of preparation used, the average difference between membrane thickness in decalcified and ground sections was only 5.5 μm, which amounts to 7.2–10.5 % of the measured values. Mostly the ground sections gave a lower value, the embedding in hot molten resin probably causing some shrinkage.

The thickness in the isolated membranes was always much lower than those taken in sections, the average difference from the values in decalcified and ground sections being 25.3 and 19.5 μm, respectively. In their method description the workers pointed out that microscopic inspection of the interior of the calcified shell after isolation of the membranes took place. It was found that a few fibres of the outer membrane always adhered to the shell. The membrane fibres were present in the mammillae up to a depth of 20 μm. Thus the average differences

Table 1. Measurements of membrane thickness (in μm).

Hen	Date of laying	a Isolated membranes	b In decalcified sections	c In ground sections	Difference b–a	Difference c–a	Difference b–c
253	16/4	38.5	71.0	55.5	32.5	17.0	11.5
	17/4	37.5	70.5	54.0	33.0	16.5	16.5
	19/4	40.5	69.0	60.0	28.5	19.5	9.0
	20/4	40.5	65.0	55.5	24.5	15.0	9.5
	21/4	–	67.0	66.5	–	–	0.5
	22/4	40.0	64.0	68.0	24.0	28.0	– 4.0
	24/4	39.5	67.5	70.0	28.0	30.5	– 2.5
	25/4	37.5	67.0	63.0	29.5	25.5	4.0
	27/4	43.5	57.5	65.0	14.0	21.5	– 7.5
502	16/4	47.5	73.5	72.0	26.0	24.5	1.5
	18/4	42.0	63.0	56.0	21.0	14.0	7.0
	20/4	36.5	64.0	65.0	27.5	28.5	– 1.0
	22/4	45.5	67.0	74.0	21.5	28.5	– 7.0
	23/4	52.5	65.0	64.0	12.5	11.5	1.0
	24/4	43.5	59.0	53.5	15.5	10.0	5.5
	26/4	41.5	57.5	58.0	16.0	16.5	– 0.5
	27/4	42.5	56.5	59.0	14.0	16.5	– 2.5
568	16/4	36.5	70.5	59.0	34.0	22.5	11.5
	17/4	37.5	74.5	64.5	37.0	27.0	10.0
	18/4	41.5	66.5	57.5	25.0	16.0	9.0
	19/4	42.5	63.5	53.5	21.0	11.0	10.0
	20/4	41.5	65.5	63.0	24.0	21.5	2.5
	21/4	–	65.5	60.0	–	–	5.5
	22/4	38.5	60.0	52.5	21.5	14.0	7.5
	23/4	40.0	72.5	62.0	32.5	22.0	10.5
	24/4	43.5	73.5	54.5	30.0	11.0	19.0
	26/4	41.5	62.5	55.5	21.0	14.0	7.0
	27/4	38.5	69.0	57.5	30.5	19.0	11.5
597	16/4	41.5	75.5	62.0	34.0	20.5	13.5
	17/4	42.0	76.0	63.0	34.0	21.0	13.0
	18/4	36.5	70.0	59.0	33.5	22.5	11.0
	20/4	41.5	65.5	–	24.0	–	–
	21/4	–	61.0	62.0	–	–	– 1.0
	22/4	41.5	61.0	58.5	19.5	17.0	2.5
	23/4	37.5	53.5	59.0	16.0	21.5	– 5.5
	26/4	43.5	69.0	61.0	25.5	17.5	8.0
	27/4	37.5	68.0	59.0	30.5	21.5	9.0
Average differences					25.3	19.5	5.5
Standard deviation					± 6.8	± 5.5	± 6.8

from isolated membranes in thickness of decalcified and ground sections (25.3 and 19.5 μm, respectively) were of the expected size and similar to the depth to which the membrane fibres occurred in the shell. Sajner (1955) also described a 'granulated membrane' retained in the calcified shell after tearing off the membranes and only isolated by special procedure. At the 16th day of incubation this 'granulated membrane' would separate from the rest of the calcified shell, its calcium carbonate then becoming available to the developing skeleton of the embryo. For the hen egg he mentioned a value of 25 μm for the thickness of this 'granulated membrane'. A discrete layer of the outer shell membrane to which the mammillae were attached was also mentioned by Robinson & King (1963).

Thus actual thickness of dry eggshell membranes can only be measured in sections, and without correction, values for membrane thickness taken from isolated membranes should not be compared with those taken in sections.

5 Shell breakage and the influence of various structural factors on it

Weak-shelled eggs broken during transportation cause a loss of eggs and soil neighbouring ones. The practical methods used to measure shell strength have disadvantages making it difficult to find the shell factors that contribute to strength. Research on the influence of shell structure on strength was therefore desirable. Fractures through the shell were also studied to see how it breaks.

5.1 Literature

5.1.1 Per cent breakage

Opinions vary as to the percentage of breakage. Cray (1953) concluded that in the United States 8.8 % of eggs were cracked on the way from the farm to the wholesaler, i.e. 2.8 % on the farm, 2.6 % at the time of grading, 2.5 % in the grading station and 0.9 % on the way from the grading station to the wholesaler. The total annual loss caused by breakage in the United States was estimated at some $ 60 million. In 1965 the average number of layers during the year in the United States was 302 million producing 65,692 million eggs (Anonymous, 1969 a). Gleaves (1967) estimated the loss due to shell breakage on US poultry farms in that year at $ 50 million starting from a breakage of about 3 % on the farms.

Leach & Knowles (1969) found in the United Kingdom a breakage of 4.22 % and 3.67 % in pullet eggs laid in batteries and on the floor, respectively. The loss was considerably greater in eggs laid by older birds. From June 1967 up to and including May 1968 the United Kingdom had on an average 75 million laying hens which laid 15,590 million eggs during that year (Anonymous, 1969 b). The estimated loss due to egg breakage in the United Kingdom was £ 3 million per annum (Taylor, 1966).

In Czechoslovakia 5 %–13 % breakage occurred on poultry farms (Anonymous, 1967). Breakage was highest on battery farms. According to the author it could be reduced to 2 %–3 %. In addition to breakage on the farm a considerable amount of shell damage occurred in transit and during handling.

According to the Animal Nutrition Bureau the breakage in France amounts to 7 %, of which 5 % is due to the birds themselves. But Mongin (1966) pointed out that 10 % of the eggs produced in France do not reach the consumer owing to breakage.

Eggs from three entries were tested fortnightly for hair-cracks and cracks at the

Netherlands Random Sample Testing Station from February to August 1968 (Schoorl, 1968). In battery eggs there was an average of 5.1 % hair-cracked and 4.5 % cracked eggs, the figures for birds kept on the floor being 4.4 % and 1.5 % respectively. Towards the end of the laying period 12 % of the battery eggs were hair-cracked and 5.6 % cracked, the percentages for eggs laid by birds kept on the floor being 8.4 % and 1.2 %. Compared with the values known from other countries these percentages are rather high.

5.1.2 Measuring shell strength

Many methods have been developed for measuring eggshell strength, but all have their deficiencies. One of the most widely-used routine methods of determining shell quality, favoured by breeders of laying stock, is the measurement of the specific gravity of the whole egg (van Tijen, 1963). This specific gravity is influenced by evaporation of water through the shell during storage, which is not uniform for all eggs owing to differences in shell porosity. It is therefore very important that the eggs are tested as soon as possible after laying. Another method often used is the deformation under load. The change in shape of the shell is measured when the egg is subjected to a load of 500 g (Schoorl & Boersma, 1962). Eggs found broken or cracked after transportation were not mainly those found previously with high deformation measurements (Shrimpton & Hahn, 1968). Shell thickness is often used as a parameter of shell strength. However it is a rough parameter because sometimes thinner shells are stronger than thicker ones caused probably by differences in structure.

5.1.3 Influence of shell membranes and structural shell factors

There is some doubt about the influence of shell membranes on shell strength. Bokx (1962) found no relationship between thickness of shell membranes measured in isolated membranes and breaking strength even when shell thickness was eliminated. Snapping pieces of shell outwards Tyler & Thomas (1966) showed the strengthening effect of shell membranes. Influence of membranes on shell strength was not noticeable when the shell was snapped inwards.

A regular distribution of the mammillae was very important as it contributed to greater strength (Robinson & King, 1970).

Guinea-fowl eggshells were stronger and thicker than eggshells of the domestic hen. They were stronger even when allowance was made for their greater thickness. Petersen & Tyler (1967) saw no crystal columns in the palisade layer of the guinea-fowl eggshell and suggested that this layer was built up of small polyhedral crystals. This suggestion was not accepted by Schmidt (1968) who showed in very thin sections between crossed Nicols that the guinea-fowl eggshell exhibited an ordinary palisade layer with columns in which he discerned an inner and outer palisade layer. In the inner palisade layer the various columns had irregular lateral

boundaries and were interpenetrating. In the outer palisade layer there was less interpenetration of prisms, their boundaries being smooth and vertical. Schmidt (1968) suggested that this interpenetration in the inner palisade layer possibly caused the greater strength of the guinea-fowl eggshell compared with that of the hen eggshell as found by Petersen & Tyler (1967). No layer of organic matter was present between the united crystal individuals in the palisade layer, as is the case between those in the cones (Schmidt, 1965 c). The lateral boundary, however, of neighbouring columns had a wavy appearance under low magnification, but seemed smooth under higher magnification (Schmidt, 1962 a).

Tyler & Thomas (1966) found that shells were strengthened by the presence of the true cuticle when snapped inwards.

Many scientists have looked for a relation between organic matter of the shell and shell strength. Tyler & Geake (1958) demonstrated that the shell thickness increased with a decreasing percentage of shell nitrogen. They attributed this to the presence of a higher percentage of organic matter in the mammillary core than in the rest of the shell. The shell thickness is a frequently used parameter for shell strength. The percentage of nitrogen in the membrane-free shell was about 1.7 times higher in the guinea-fowl than in the hen eggshell (Petersen & Tyler, 1967). The authors suggested that this extra nitrogen contributed to the greater strength of this shell. Hen eggshells with special structural flaws gave high values for shell strength (Tyler, 1969). These flaws contained more protein, more fat and the same amount of acid mucopolysaccharide as the rest of the normal shell. Diamantstein (1966) noted that the percentage of acid mucopolysaccharides in the calcified shell matrix was highly correlated with the breaking strength ($r = 0.74$). It was very difficult to interpret these results. An increase in a given organic substance or the organic matter as a whole may be accompanied by a finer distribution of the organic matrix, giving the shell a different calcification and possibly greater strength. More research is required in this field.

5.2 Results and discussion

Many structural shell factors may influence shell strength. Some of them were examined with the light microscope, transmission electron microcope and scanning electron microscope and the results are described here.

5.2.1 Influence of shell membranes

Landois (1865) and Schmidt (1957, 1966 a) were able to pull fibre layers of the membranes in a spiral from the egg. The strength of the shell membranes varied in different directions. This may have contributed to the controversy about the effect of membranes on shell strength. The directional difference in strength in the membranes may be explained by the indication for the predominant direction of the fibres in the shell previously described.

5.2.2 Influence of mammillae and columns

The bases of the mammillae of the cone layer in the guinea-fowl eggshell penetrated into the outer shell membrane. There were more mammillae per unit of surface area in the guinea-fowl eggshell than in the hen eggshell (figs 61 and 62) and they looked more regularly distributed in the guinea-fowl. Hence the centres of the mammillae were closer to each other and the mammillae grew together at a lesser depth than in the hen eggshell. As a result the spaces between the mammillae were small in the guinea-fowl eggshell. There were a large number of irregular calcified deposits at the foot of the mammillae through which the fibres penetrating the mammillary base passed in tufts. These factors together may contribute to the greater strength found in the inner part of the shell (Petersen & Tyler, 1967). Robinson & King (1970) also attributed the greater strength of the hen eggshell to a more regular distribution of the mammillae. After removing the membranes by boiling for 10 minutes in 2.5 % sodium hydroxide tangential preparations of the shell were examined. Thus the number of mammillae/mm^2 in many hen eggs was counted on the inside of the shells. Within the egg there was a large variation in the number of mammillae/mm^2. On average for 20 eggs, the point, waist and blunt end of the hen eggshell had 157.5, 236.3 and 193.8 mammillae/mm^2, respectively. The average of these values for point, waist and blunt end of two guinea-fowl eggshells was 275 mammillae/mm^2. Many of these were even compound mammillae (two or more mammillae fused at an early stage, which may be seen as one mammilla, Chap. 6). So guinea-fowl eggshells had more mammillae/mm^2 than the hen eggshells as was expected from observations on radial ground sections (figs 61 and 62). The average diameter of the mammillae at the waist varied little between eggs from one hen but varied more between eggs from different hens. This factor could possibly be used in selection. The mammillae of ten thick and ten thin eggshells of one breed were measured but their average diameters were not different. Taking shells with the same thickness and a different deformation, measured by the method of Schoorl & Boersma (1962), the eggs with the highest deformation clearly had the fewest mammillae/mm^2. The diameter of the mammillae affects shell strength: narrow mammillae fuse at a lower level than wider ones; the wider mammillae impair shell strength because fractures are formed most easily between the mammillae.

Also Schmidt (1968) showed that the guinea-fowl eggshell contained mammillae which rapidly fused in their growing period. This probably resulted in the formation of columns with a smaller diameter than in the hen eggshell. The small diameter of the columns of the guinea-fowl eggshell may also contribute to their greater strength. Van der Plas (1966) pointed out that when the diameter of column is lower relative to length shells may be stronger. The ratio of diameter of column : length of column should be at least 1 : 4. The relation between average diameter of the columns and shell strength was examined in sections with polarized light. The surface area of about 40 crystal columns was measured

in tangential ground sections of 6 eggshells with about the same shell thickness and a different deformation measured by the method of Schoorl & Boersma (1962). The average area of the columns in the three shells with the highest deformation (average: 23 μm) was 3033 μm^2 and the average area in the ones with lowest deformation (average: 20 μm) was 2747 μm^2. It is indicated from these measurements that as the width of the column decreases the deformation becomes lower and the shell stronger. Research with a larger number of eggs would be useful.

5.2.3 Influence of distribution of matrix

The many pictures, obtained with the transmission electron microscope of the structure of the decalcified palisade layer in eggs of different strength, suggested that the compactness of the organic matrix of this layer is related positively to shell strength. One eggshell having a high deformation relative to thickness and being quite weak showed fairly wide vesicular holes in the palisade layer. The number of holes did not seem to be increased. The average mammillary diameter as seen under the light microscope with a larger field of vision, was extremely high which may have influenced shell strength unfavourably. Pieces of two eggshells of approximately the same thickness but very different breaking strengths were obtained from Mr N. R. King from the Food Research Institute in Norwich, England. Robinson & King (1970) investigating pieces of these same shells by light microscope saw an irregular distribution of the mammillae in the weaker shell. Decalcified radial sections through the palisade layer showed little difference in matrix compactness and the average diameter of the vesicular holes but quite a difference in their number per surface unit. The number of vesicular holes in the palisade layer of the strong shell looked quite normal; on the contrary in the palisade layer of the weaker shell we counted about a 60 % more holes. The fibrils were rather loosely arranged in the palisade layer of the weaker shell. Its structure looked more like that of the outer part of the cone layer of a normal eggshell than that of a normal palisade layer.

The palisade layer of the guinea-fowl eggshell in decalcified sections (Fig. 38) was characterized by a very fine division of its organic matrix, again far denser than in the hen eggshell (Fig. 35). Much of this organic material was situated at the boundaries of small rhombic crystallite deposits whose dimensions were estimated to be about 0.04 μm. This is about 1/10th the size of the small rhombic crystallites in the hen eggshell. Vesicles similar to the vesicular holes of the same diameter as described in the palisade layer of the hen eggshell were also found in the shell of the guinea-fowl egg, but were far less numerous. These results again indicate that the division of the organic matrix and the number of vesicular holes contribute to shell strength and agree with the findings in the hen eggshell.

5.2.4 Relation porosity and shell quality

In eggs with the same shell thickness and varying deformation there seems to be a positive relationship between deformation and porosity (weight loss during a certain time). The porosity becomes higher as the deformation increases. This may possibly be associated with the presence of a thinner true cuticle assuming that the true cuticle especially influences the evaporation. Shell compactness and number of pores may also vary in strong and weak shells of the same thickness and affect rate of evaporation of water from the egg contents. This relation has not yet been examined but there is a need for research.

5.2.5 Effect of sodium hydroxide and sodium sulphide

This experiment was carried out in the Laboratory of Physiology and Biochemistry of Professor Tyler in Reading.

One piece from each of 20 eggs was treated identically. There were 10 treatments as described in Chapter 2. The membranes were removed as cleanly as possible from all the 200 pieces of shell by scraping with a scalpel before treatment. One strip from each shell served as control. After the treatment with either water, sodium hydroxide solution or sodium sulphide solution snapping strength, shell thickness and histological sections of these shells were studied to examine the effect of these treatments on shell strength and find the cause of the changes. The means of twenty eggs are given in Table 2.

Table 2. Changes in shell as shown after heating with water, sodium hydroxide and sodium sulphide.

	Treatment in h at 95° C	Strength (g)	Thickness (μm)	Ground sections
Control	0	76.6	313	normal columns
Water	9	68.7	312	normal columns
NaOH	1	53.0	289	small needle crystals [1]
	2	45.9	300	small needle crystals [1]
	3	45.9	307	small needle crystals [1]
	9	44.8	310	small needle crystals [1]
Na_2S	1	70.6	310	normal columns
	2	70.0	310	normal columns
	3	61.3	311	normal columns
	9	49.0	310	normal columns

1. The small needle-like crystals were present in the outside layers of the shell and in the mammillary knobs.

After heating the shell in hot water for 9 h the snapping strength was significantly lowered, the shell becoming weaker. This reduction in strength may partly be caused by removing watersoluble compounds from the true cuticle. Tyler & Thomas (1966) showed that the presence of the true cuticle contributed to the shell strength when the shell was snapped inwards. Also watersoluble compounds from the shell matrix may dissolve during this treatment. However the effect of treatment with water is small compared with the treatment with sodium hydroxide and sodium sulphide solutions.

After a treatment with sodium hydroxide solution shell strength decreased rapidly at first and then more slowly. Sodium sulphide caused a steady diminishing of strength with time. After treatment for 9 h the shells were still a bit stronger than after the sodium hydroxide solution treatment.

The values obtained for shell thickness after these treatments indicated that the membranes had been efficiently stripped from the shell as the differences in shell thickness (membranes removed) between water treatment and control was very small. Heating the shell in water or in sodium sulphide solution did not change its thickness. The hydroxide solution, however, caused a decrease in thickness after one hour followed by a gradual increase to about the original value after 9 h. After 1 h treatment in sodium hydroxide sections showed the inside of the calcified shell (inner part of the mammillary layer) already damaged, possibly causing the shell to become thinner. The increase in thickness may be explained by the formation of small needle-like crystals in the outer and inner part of the shell. By splitting up parts of the large crystal individuals in small needles small spaces may be formed between them and thickness may increase.

It was seen in decalcified sections that sodium hydroxide or sodium sulphide removed the true cuticle and the fragments of membrane. Some disintegration of the shell matrix occurred after a 1 or 2 h treatment with sodium hydroxide. A 1, 2 or 3 h treatment with sulphide did not seem to cause any change in the matrix. After decalcification of these shells, a 3 and 9 h treatment with hydroxide and a 9 h treatment with sulphide resulted in disintegrated residues from which no sections could be prepared. Normally the organic matrix of the shell was partly interwoven with the crystallites and partly enveloped crystal individuals in the cone layer (Schmidt, 1965 c) and crystallites. Shell fractures tended to follow the organic layers. Understandably the shell is weakened by removing this organic matrix.

The ground sections of these shells were studied with polarized light between crossed Nicols. The results are given in Table 2. Treatments with sodium hydroxide caused a change in the structure of the calcite individuals in the outer part of the shell and in the mammillae. Small needle-like crystals occurred in the outer part of the columns and in the inner part of the mammillae. An expansion of the organic matrix in sodium hydroxide possibly split up the crystal individuals. The amount of the shell which gradually disintegrated increased with the time of heating in hydroxide. Shell disintegration did not occur in sodium sulphide solution. Also Heyn (1963) reported that sodium sulphide does not affect the

calcite of the shell even after a long treatment. The splitting up of the crystal individuals may also weaken the shell slightly. This may explain why even after a 9 h treatment with sodium sulphide the shell was still a bit stronger than after a 9 h treatment with hydroxide.

This experiment suggests that the disintegration of the organic matrix in particular, and also the splitting up of the crystal individuals weaken the shell. This might again indicate that the organic matrix has an important influence on shell strength.

5.2.6 Shell fractures

The scanning electron microscope was most useful for studying fractures through the shell. It was very interesting to see that fractures over the greater part of their length followed the concentrations of organic matter. The only exception we saw is shown in Fig. 32 (left mammilla) and Fig. 33. It means that the strength of the organic matter is an important factor in the breaking strength of the shell. This agrees with the positive relation between compactness of organic matrix in the palisade layer and shell strength, and the relation between removing or disintegration of the organic matrix and snapping strength.

As described in Chap. 3 the organic coverings of neighbouring mammillary bases fused between the mammillae and sometimes penetrated the shell for more than 70 μm (Fig. 63). Fractures followed these 'lines of least resistance' (figs 14, 15, 16, 17 and 63). At many places entire crystal individuals were broken out of a mammilla, the fracture following again the thin layer of organic material between the crystal individuals (Fig. 63).

In the palisade layer the plane of fractures sometimes deviated from the strictly radial direction characteristic for the cone layer. In some places there were transverse breaks through columns of about 50 μm diameter (figs 39, 40 and 63) so that here the fracture was in a tangential plane parallel to the surface of the shell. But here again the fracture followed thin layers of organic material (matted fibrils with attached vesicles). These lay at relative distances of about 2 μm throughout the palisade layer as observed in decalcified sections with the transmission electron microscope. The tops of the rhombic crystallites visible at the front side of the broken column in Fig. 39 indicated several of these potentially fractured planes. These layers of organic material can be seen far more clearly in Fig. 64. These tangential layers of fibrils were missing in the cone layer and a greater amount of organic material was found between the crystal individuals of the cone layer, than between the columns. Both these facts may account for the strictly radial course of the fractures through the cone layer.

Replicas of the fracture of the palisade layer in radial fractures also showed the radially (Fig. 41) and the tangentially fractured planes (Fig. 44), respectively. The latter was rich in organic material. Its structure was described in Chap. 3. The same type of photographs was also taken by Heyn (1963; figs 4 and 5).

According to Heyn his figs 4 and 5 showed a 'compact crystalline' and a 'softer less pronounced crystalline' region respectively of the radially fractured surface. The 'softer less pronounced crystalline' region corresponded with the structure in a tangential plane through a column. The 'compact crystalline' structure gave a view in a radial plane.

Summarizing, structural shell factors thought to influence shell strength are the shell membranes, the distribution and average diameter of the mammillae, the average diameter of the columns, the distribution of the organic matrix, the largeness and number/mm^2 of the vesicular holes, the crystalline structure within the columns and the presence of the true cuticle.

6 Formation of shell and membranes

Practical studies on feed composition and environmental conditions of hens have so far produced few results of use for poultry farmers in improving shell strength. If good criteria were found by collaboration between physiologists, biochemists and geneticists, shell quality could ultimately be improved by breeding. Formation of the shell and membranes was therefore studied as a basic understanding of formation is important in the discovery of such criteria.

6.1 Literature

6.1.1 Shell membranes

Formation of shell membranes The shell membranes were deposited around the egg contents in the isthmus. The isthmus is the part of the oviduct which precedes the shell gland (also called uterus). It had a length of about 10 cm and the egg stayed there on average for 74 minutes (Warren & Scott, 1935). Von Wittich (1851) recorded that membrane fibres were residues of smooth muscle fibres originating in the oviduct. Blasius (1867) on the other hand believed these fibres were formed by coagulation of proteins. According to Richardson (1935) granular materials were secreted by glands of the isthmus. This author reported that these granules coalesced to form fibres. Romankewitsch (1932) suggested that the granules swelled and formed a colloidal mass. Taylor (1965) doubted that the inner membrane was deposited by secretions in the isthmus. He wondered if the inner membrane could be formed afterwards from the egg contents.

Chemical composition Many 19th century scientists looked at the chemical composition of the membranes. As quoted by Blasius (1867), Purkinje mentioned in 1825 that many organic structures such as vessels, membranes, cells and fibres were formed by proteins coagulating. Baer (1837) noticed that the shell membranes consisted of 'animal substance'. According to Landois (1882) the membranes were a feltlike mass of fibrinous or albuminous fibres. This idea was based on the possible precipitation of albumen in water. Von Nathusius (1882), however, noted that the membrane consisted of fibres composed of elastin, i.e. the same substance as the elastic connective tissue found in animals, but not fibrin or albumin. Calvery (1933) found that the protein of eggshell membranes had the amino acid composition of a typical keratin. Other chemical analyses by Baker &

Balch (1962), histochemical studies by Moran & Hale (1936) and Simkiss (1958) showed that the shell membranes mainly consisted of keratin. Wolken (1951) concluded from X-ray diffraction studies that the shell membranes were mostly α-keratin. Moran & Hale (1936) found some mucin in the membrane fibres and Simkiss (1958) established a positive reaction for sugars bound in the membrane fibres. Baker & Balch (1962) noted that the membranes were mainly keratin with a minor amount of polysaccharide containing hexosamine, galactose and probably mannose. A fibre consisted of a core and a mantle. Masshoff & Stolpmann (1961) reported that cores of the membrane fibres consisted of keratin and their mantle contained mucopolysaccharide.

Formation of air space Baudrimont & St. Ange (1847) noted that an air space was formed between the two shell membranes. According to Baer (1837) and Romankewitsch (1932) these air spaces were usually found at the blunt end between the membranes. Romankewitsch (1932) stated that this air space *(Folliculus aëris)* was formed at the boundary between the outer shell membrane with larger fibres and the inner membrane with smaller fibres. He said this was the point of least resistance and the fibres of the inner membrane were torn from the fibres of the outer one. Microscopic examination revealed fibres which had been torn loose.

Course of fibres Landois (1865) described the membrane fibres as being formed in a spiral from the oviduct muscles. He showed this helical arrangement by pulling fibre layers from the membrane of a boiled egg. Schmidt (1957, 1966 a) also found that the membrane fibres were in a spiral around the axis of the egg.

6.1.2 Shell

According to Baer (1837), a liquid (calcareous milk) in diluted protein came from the uterus glands and was responsible for calcareous crystals being deposited in the shell membrane. Von Hemsbach (1851) thought that this shell was formed by loosening and calcification of the mucous membrane of the uterus. Some years later Landois (1865) stated that the glands of the mucous membrane of the uterus were deposited on the eggshell membrane and formed the inner part of the shell. Blasius (1867) doubted whether there was a cellular substance in this layer. The formation of the mammillary layer took place partly in the shell gland and partly in the isthmus (Taylor, 1962). The rest of the shell was formed in the shell gland.

Composition of organic matter and its role in calcification The mammillary layer contained about two-thirds of the shells organic matter (Carter, 1969). The organic material of the various layers of the shell differed chemically (Simkiss, 1968). In the mammillary layer of eggshells of ratite birds there was less protein and fat and more carbohydrates than in the palisade layer (Tyler & Simkiss,

1959 b).

Blasius (1867) saw in a tangential section of the shell, at the base of the mammilla, an organic core which he believed was of cellular material. Simkiss & Tyler (1957) clearly showed that the organic material of this mammillary layer was more concentrated in a central core of each mammilla. Simkiss (1968) thought it consisted of small masses of organic matter embedded in the true shell and resting on the outer layer of the shell membranes. These masses of organic matter have been shown histochemically to contain protein, polysaccharide and reducing groups (Simkiss, 1958). The reducing groups may be sulphydryls and/or phenols (Simkiss, 1968). They were not present elsewhere in the shell matrix. The calcium ions were thought to be bound to the organic cores by sulphonic acid groups on the mucopolysaccharide-protein material of the core (Taylor, 1970). The core may be deposited before the calcification of the shell begins (Simkiss & Tyler, 1957). In this way the organic core may play an important part in the initial calcification. Taylor (1970) suggested that the bound oriented calcium ions deposited here acted as seeds or nuclei for the growth of the crystallites of the shell. The membrane fibres which passed the cores may become attached to them, possibly through the development of the disulphide links (Simkiss, 1961).

Carbonic anhydrase may be an important aid to shell calcification. There is some disagreement about its presence in the mammillary cores. Robinson & King (1963) stated that they localized carbonic anhydrase in the mammillary core, but Diamantstein et al. (1964) were unable to confirm this. They said that an unspecific non-enzymic reaction may be involved.

Carbonic anhydrase has been found in blood cells and in cells of some tissues (Mitchell, 1946) and may be an obligate intracellular enzyme. The shell gland contained more of it than other oviducal tissues (Common, 1941). Blasius (1867) thought there was cellular material in the mammillary core and Purkinje (1830), von Wittich (1851) and von Nathusius (1882) indicated its presence in shell membranes, whose fibres partly passed through the mammillary core. These references are old and not too much value should be attached to them but there is a possibility that animal tissue occurs in the mammillary bases which should be taken into account.

The matrix of the remainder of the shell consisted of a protein acid mucopolysaccharide complex (Simkiss & Tyler, 1957). Histochemical tests showed that the cone and basal cap with the exception of the core contained chondroitin sulphate and a protein complex different from that of the palisade layer (Simkiss, 1968).

Simkiss & Tyler (1958) stained decalcified sections of eggshells in a series of solutions of 0.01 % toluidine blue with different concentrations of calcium ions. The pieces stained metachromatically with toluidine blue solution, but the staining decreased when more calcium ions were added. Other metal ions behaved like calcium. The authors concluded that calcium ions could expel toluidine blue from the shell matrix. They suggested that an acid mucopolysaccharide acted in this way. A chelating agent in the shell might be of great importance to calcification by

binding calcium. If this chelating process occurred, calcium carbonate in the shell will be deposited in the organic material already present, as in bone. This theory is quite different from that of Schmidt (1962 a) who believed that organic and inorganic materials were deposited at the same time.

Development of layer of cones and basal caps The following description of the development of mammillae is taken mainly from Schmidt and his terminology is used. Crystallization centres (formation centres) were formed on the outer shell membrane, probably by secretions from each glandular stoma of the uterus glands (Schmidt, 1958 b, 1960). The first secretion partly penetrated the shell membrane (Schmidt, 1966 b). Giersberg (1922) described the mammillae as having an organic core formed by secretions from the uterus glands sticking to membrane fibres. According to Schmidt (1966 b) a drop of secretion fluid crystallized in this centre near the shell membrane forming a small spherite. A spherite consisted of a collection of crystal individuals extending in a certain direction. The material deposited just around the formation centre was called primary spherite and was described in detail in tangential and radial sections by Schmidt (1964 b, c, respectively). The primary spherite was deposited very rapidly and used up the entire supply of calcium ions (Schmidt, 1965 b). Schmidt (1965 c) observed a dark spherical primary grain in the centre of the primary spherite of nandu *(Rhea americana)* and duck *(Anas domestica)* eggshells. He (Schmidt, 1964 b, c) pointed out the dark centre of the primary spherite was due to the presence of air, and had a ring of small radial crystals (needles: Schmidt, 1962 b) around it. According to him there were no spherical gas inclusions as further outwards in the shell. The dark centres were formed by air penetrating between the calcite elements of the primary spherites after drying and shrinkage of parts of the shell and the shell membrane fibres. The presence of the air made it difficult to study this part of the shell with a light microscope. Some of the small radial crystals of the primary spherite enlarged when new calcification material was supplied by the uterus glands (Schmidt, 1965 b), forming the secondary spherite over the rest of the shell (Schmidt, 1962 a).

The location of the primary spherite approximately corresponded to that of the organic core as described by Simkiss & Tyler (1957) and Terepka (1963 a, b). Unlike Schmidt, Simkiss (1968) suggested that outside the organic core the first calcite crystals of the forming shell were deposited. Schmidt (1965 d) observed the organic mass in the calcite of the eggshell of a black-headed gull *(Larus ridibundus)*. According to Schmidt (1965 c) the organic part of the calcification fluid may partly remain between the calcite crystal individuals after being crystallized. He described the loculose organic structure in the basal caps and cones of nandu and duck eggshells, each loculus enveloping a single crystal individual.

Schmidt (1962 a, 1965 b) distinguished the eisospherite growing from the crystallization centre into the outer shell membrane from the exospherite growing

outward. Both consisted of calcite individuals. The eisospherites (basal caps of the mammillae) were anchored in the outer membrane. As the crystal individuals developed in the eisospherite, their growth was impeded, according to Schmidt (1965 b) by the resistance of the outer membrane fibres and the reduced flow of calcification material. After the cone has formed this flow of material from the uterus glands should have more difficulty reaching the eisospherites via open spaces between the cones. The eisospherite developed in a much shorter time than the exospherite, and the eisospherites were deposited before the cones fused outside the outer membrane (Schmidt, 1965 b). According to this author who examined the swan eggshell, the crystal individuals of the eisospherite did not have a regular boundary due to their growing in the outer membrane. The spherical gas inclusions and the growth lines which were present in the exospherites, have not been found in the eisospherites. The eisospherites had a structure parallel to the surface derived from the membrane fibres running through it. Air inclusions were particularly common in the vicinity of these fibres when the fibres dry up after emptying the egg. The boundary at the base of the mammilla was not round but slightly flattened. The eisospherites were usually wider than the bases of the cones in the swan eggshell. The theory of the formation of basal caps is not founded on research and it inadequately explains why the fibres penetrate the mammillary base in tufts.

Particularly in some species two or more spherites may be found in one mammilla, which is then termed a 'polyspheritical mammilla' (Schmidt, 1965 b). The calcite spherites of such a mammilla may be termed 'testispherites' (Schmidt, 1968). This phenomenon occurred in the eggshells of all birds studied by Schmidt. He showed for example that they were very numerous in guinea-fowl *(Numida meleagris)* eggshells. The testispherites may be formed when the uterus glands were arranged in groups, the distance between the glands in a group being small and less than that between the groups.

In the swan eggshell Schmidt (1965 b) sometimes found the crystallization centre well outside the outer membrane. He mentioned that in this case the crystal individuals, oriented towards the outer membrane, had the properties of the exospherite up to the contact point with the outer shell membrane. The part of the eisospherites penetrating the outer membrane had the usual properties of the eisospherite.

Some crystal needles of a primary spherite should grow further outwards and together formed the exospherite (Schmidt, 1962 b). The exospherites were divided by Schmidt (1962 a) in a basal layer with cones having flat contours and a distal layer with columns (palisade layer). In the cone (inner part of the exospherite) a number of calcite individuals grew outwards from the crystallization centre and widened. Schmidt (1967 a) described the part of the calcite individual in the cone layer as a wedge. These wedges, according to Schmidt (1965 b) were surrounded by the organic loculus, as previously described for the single crystal individual of the basal cap. He examined these wedges in nandu and duck eggshells and found

their boundary smoother than that of the columns.

When the exospherites fused outside the outer membrane their boundaries became more irregular and were more difficult to see (Schmidt, 1962 b). The palisade layer with their columns began at this point.

Deposition of columns Columns (crystal individuals in the palisade layer) grew at about the same rate over the entire shell (Schmidt, 1962 a) resulting in growth lines which according to the author contained numerous air inclusions. The growth lines of a column ran further into the neighbouring ones. Schmidt thought that certain columns sometimes grew more rapidly than neighbouring ones causing in some places an elevation in the growth lines of columns. How the crystal individuals terminated near the surface of the shell was said to affect the smoothness or roughness of the surface (Schmidt, 1958 b). If columns or groups of columns ended at different heights the shell could be rough. It may be concluded that the rate of growth for the different crystal individuals is about the same but their growing period may differ and their calcification may stop at different stages.

Surface crystal layer This thin layer of small crystals is deposited on top of the palisade layer with the crystals oriented in a direction dependent on that of the crystal faces in the columns. According to Schmidt (1964 a), after the columns have formed, the amount of calcium in the secretion fluid has local differences. Therefore calcite crystals did not form at every position around the egg. This might be why Tyler (1966) did not find this layer in the eggshells of the Falconiformes.

Presence of inorganic materials About 95 % of the eggshell consisted of inorganic matter made up of calcium salts, small amounts of phosphate and magnesium, and traces of other minerals (Romanoff & Romanoff, 1949). A hen eggshell had about 0.02 g magnesium and 0.02 g phosphorus (Romanoff & Romanoff, 1949). This was about 1/3 % of the shell weight. Terepka (1963 a) detected a small amount of phosphate in the cone layer but most of the phosphate and magnesium seemed to occur in the outer part of the shell (Itoh & Hatano, 1964). Brooks & Hale (1955 a) also showed that in the palisade layer the ratio magnesium : calcium decreased inwards and Smith et al. (1954) found that most of the phosphates were deposited in the outer part of shell.

6.1.3 Pigments of shell and true cuticle

Eggshells of different species of birds may have different pigments. The ground colour of the eggshell may be partly in the organic cuticle and partly in the deep calcified layers (Dickie, 1848 and Romankewitsch, 1934). According to Landois (1865) these pigments were 'cholephyrin' (bilirubin) or biliverdin. Blasius (1867) suggested they consisted of bile pigments which came into contact with the eggs

via the faeces in the cloaca. The colours were limited to either the red or green part of the spectrum (Romanoff & Romanoff, 1949).

The reddish-brown pigment of the domestic hen eggshell has been termed ooporphyrin (Fischer & Kögl, 1923, 1924 and Fischer & Müller, 1925). Furreg (1931), Völker (1940) and Fox & Vevers (1960) confirmed that ooporphyrin (protoporphyrin) occurred in the pigmented areas of the shells of bird eggs. Völker (1940) showed that ooporphyrin was even present in white eggshells and caused red fluorescence in ultra-violet light although it may disappear in daylight. According to Fox & Vevers (1960) the amount of porphyrin was very variable and not proportional to the amount of brown, supposed melanin, pigmentation. I was unable to find any other references to confirm this suggestion. Schmidt (1958 b) reported that the brown pigment was based on porphyrin grains and deposited in the organic cuticle in particular. Accumulation of these grains caused spots. Ooporphyrin seemed to form from blood haemoglobin (Romanoff & Romanoff, 1949).

6.1.4 True cuticle

When the true shell is formed, an organic cuticular substance is deposited over the surface crystal layer. This organic layer is termed true cuticle (bloom: Szielasko, 1913). The true cuticle is believed to protect the egg contents against micro-organisms and evaporation of water. The cuticle sealed the pore mouth reducing the permeability of the shell (Fromm, 1963). According to this author relative humidity, abrasion of shell, egg washing, ambient temperature and temperature changes should – after oviposition – influence the permeability. Permeability increased the rate of carbon dioxide loss, which then caused a rise in pH of the albumen. Thus the amount of thick egg white and internal egg quality decreased.

Chemical composition Von Nathusius (1887) thought the true cuticle of the hen, swan, duck and goose eggshell had the chemical composition of elastin. Stewart (1935) considered the protein of the true cuticle to be keratin. For many years it was generally accepted that this layer consisted of mucin as found by Moran & Hale (1936). Schmidt (1961) thought keratin was part of the true cuticle. Some 90 % of the true cuticle was protein, distinguished from shell membrane and matrix protein by Baker & Balch (1962). This protein contained disulphide groups, sulphydryl and/or phenol groups together with some polysaccharide and possible some lipid material (Simkiss, 1968). The protein contained a high content of tyrosine, glycine, lysine and cystine. Hexosamine was present together with galactose, mannose and fucose (Baker & Balch, 1962). Acid mucopolysaccharide, which is essential for the deposition of calcium carbonate, seemed to be absent in the true cuticle (Simkiss & Tyler, 1957). Landois (1865) noted, that the organic cuticular layer in the duck eggshell was impregnated with fat drops, which give the egg a glossy outward appearance. Romankewitsch (1934)

found varying amounts of fat in the true cuticle of the hen eggshell and it may be present in large amounts in the cuticular substance of some other species (Simkiss, 1961).

Baker & Balch (1962) found 3.49 % inorganic material in the true cuticle. Brooks & Hale (1955 b) found some potassium chloride in the true cuticle. The potassium chloride was dissolved by washing and this fact was sometimes used to indicate whether eggs had been washed.

6.1.5 Cover

Tyler (1964) studied the cover in the Anatidae eggshells with microscopical and histochemical techniques. It was a powder, consisting of calcium carbonate and organic matter. This layer seemed also to cover the pore mouth so that the pore could not often be seen. The powder could easily be scraped off. With histological techniques Tyler (1969) showed in Podicipitiformes and Pelecaniformes (except for the Phaethontidae) eggshells, that this cover consisted of organic matter in which many small calcite crystals were embedded.

Von Nathusius (1869) considered this cover to originate from drops of thick calcification liquid, which had run together, but not coalesced.

6.1.6 Pores

As the mammillae were formed, there was already some grouping of calcite spherites. Within these groups, the spherites fused and between them some open spaces or pores remained (Schmidt, 1966 b). The inward movement of fluid during the shell formation might keep these pores open (Tyler, 1956). Crystal growth may be prevented by this inflow of material. This theory of how pores are formed is not perfect. It does not explain the shape of the pore, the structure of its wall and the distribution of the pores over the shell as described in Chap. 3.

6.1.7 Porosity

Many workers showed that the true cuticle was permeable to gases and water vapour and important in the incubation process. These substances may be transported by cracks of the true cuticle or by the true cuticle itself. Marshall & Cruickshank (1938) found that the true cuticle may transport water. They dyed the interior of the egg and saw stained pores with the true cuticle stained immediately around the exits. According to these authors, the effective evaporating area was not the pore surfaces but the stained area. The ratio of the two areas is about 1 : 13. The authors suggested that removing the true cuticle should retard evaporation.

The amount of water evaporation is often represented as porosity. Porosity can be measured by calculating the loss of weight per day per surface unit under

standard conditions of humidity and temperature. This loss of weight is the amount of water evaporated. Black & Tyler (1944) found that the first egg of a clutch had a porosity coefficient less than that of any of the remaining eggs of the clutch. A lower hatchability of the first egg of the clutch (Mérat & Lacassagne, 1961) might be connected with this lower porosity.

6.2 Results and discussion

Various aspects of eggshell formation were studied with photographs obtained from the different eggshell and membrane preparations. Some premature eggs were used in a part of this study.

6.2.1 Shell membranes

Comparison of structure in premature and normal eggs A radial section of the shell membranes of a premature egg removed from the isthmus 20½ h before estimated laying time showed the complete inner membrane (Fig. 65). For many years the presence of the inner membrane in isthmus eggs had often been a point of discussion. Von Nathusius (1893) found an increase in fibre number in premature eggshell membranes on which the shell calcification had started. This author did not expect branching of fibres and suggested a growth from the inside formed from the granules he saw in the inner layer of the inner membrane (Chap. 3). He said no new fibres could be formed on the outside because the mammillae had been deposited. A further reason for accepting this suggestion was that the shell membranes were about 50 µm thick in the premature egg but 74 µm in a normally laid egg (von Nathusius, 1893). A thickness of 22 and 48 µm was found for the inner and outer membrane, respectively in a section through the shell membranes of a normally laid egg. We measured for the inner membrane of a premature egg a thickness of about 18 µm. The value for the inner and outer membrane in one section varied from 59–63 µm. Although we did not find the low value of von Nathusius the thickness of the inner membrane and outer membrane seemed to be less in the premature eggs than in the normal ones. The formation of the outer membrane was possibly incomplete.

The inner layer of the inner membrane of a prematurely removed isthmus egg (Fig. 66) did not differ much in structure from that of normally laid eggs. The zone of greater electron-density seemed to be thicker in the premature shell membranes (probably about 0.05 µm). This zone had about the same electron-density as the fibre core and might therefore consist of keratin as Masshoff & Stolpmann (1961) reported in the core.

The fibres of shell membranes of this premature egg (Fig. 66) were found to have cores of the same diameter as those found in normally laid eggs (Fig. 4) which contradicts the results of von Nathusius (1893), who reported that fibres thickened when shell membranes developed. The fibre mantle in these premature

eggshell membranes was thicker with a less regular distribution of its granular substance and with a more irregular boundary than in the normally laid egg. The outer part of the mantle sometimes looked less electron dense than the inner part (Fig. 66). Especially in the outer part of the outer membrane of these premature eggs many fibres were cemented to each other by the mantle substance (Fig. 67). If the fibre arrangement became more loose, thread-like or membraneous connections occurred between the fibres. Thus the meshes formed between the fibres were smaller than in normally laid eggs. The granular substance of the mantle was more loosely arranged.

Development In accordance with the structural facts described the following method of membrane development is suggested: When the egg enters the isthmus, fibrillar and granular materials secreted by the glands of the isthmus are deposited on the outer surface of the thick egg white. At first the more electron-dense fibrillar material probably forms a thin layer which surrounds the contents. Afterwards the secretions of these fibrillar materials in a short time combine to form the fibre core which is deposited on the outside of the egg (Fig. 68). The granular material probably penetrates between these cores and surrounds them, thus forming the mantles. From the inside to the outside of the premature eggshell membrane the loosely arranged granular materials in the meshes between the fibres occur progressively more often. The section of the outer part of the outer membrane from the prematurely removed isthmus egg shows accumulations of fibres (Fig. 67) with rounded-off cores. The mantle substance of the fibres is present between these cores. Plumping fluid is added to the egg white, during the first five hours the egg is in the shell gland, when it is enveloped with a soft membraneous covering (Burmester, 1940). During this process the egg becomes larger and the fibres are frequently pulled apart. As a result more and larger meshes arise in the shell membranes. The zone of greater electron-density in the inner layer of the inner membrane probably becomes thinner during this process. Thus it is easily understood that the meshes in premature shell membranes taken from the isthmus are small or in some places not even present. Shell membranes from prematurely laid eggs obtained 12, 8 and 4 h respectively before estimated laying time did not show any difference from those of normally laid eggs. It is therefore supposed that these structural transformations take place during the plumping period. The meshes in the inner membrane of the isthmus eggs are smaller than those in the inner part of the outer membrane. This may be caused by the fibres being more numerous in the inner membrane. The meshes in the outer part of the outer shell membrane are smaller than those in the inner membrane, possibly due to fibre material being deposited there just before removing the egg. When the fibres are pulled apart during the plumping process they may remain attached by thin membranes or threads (Fig. 66). These membranes and threads also occur in the normal shell membranes (figs 11 and 12) but less often than in the shell membranes of premature eggs. These attach-

ments may break off during egg growth and this could explain why there are sometimes protuberances on fibres of shell membranes.

Holes occurring in the large membrane fibres of the isthmus egg contained some granular organic material. This material of about the same electron-density as the mantle substance, was enclosed in the core and a hole might have been formed by shrinkage or the drying up of this material.

It is unlikely that fibres stretch when plumping fluid is added because the diameter of fibres in shell membranes of normally laid and premature eggs is about the same. More fibres which originally constituted a part of bundles are pulled apart during the plumping period. Under the light microscope it was very difficult to distinguish bundles with fibres from single fibres and this may account for some authors, one of which was von Nathusius (1893), reporting that the number of membrane fibres increased during shell formation. When fewer fibres are connected with each other, the meshes between them grow, their arrangement becomes looser and it follows that the membranes become thicker. The shell membranes from premature isthmus eggs are compact and very electron dense and it was therefore not possible to study tangential preparations of them with the transmission electron microscope not even at their fracture boundary. In tangential preparations of normal shell membranes with a more loose fibre arrangement this was possible (figs 11 and 12).

Chemical composition According to Balch & Cooke (1968) each shell membrane was mainly of protein which owing to its high cystine content and insolubility has often been described as keratin. The shell membranes also contained, according to these authors, carbohydrate (less than 4 %). A protein polysaccharide complex was extracted from both membranes by autoclaving. We obtained some of these autoclaved shell membranes from Balch & Cooke and found on studying them with the transmission electron microscope that in the outer shell membrane the mantle of the fibre became progressively thinner and its structure more granular and less compact. Its thickness varied between 0.05 and 0.4 μm which was less than half of the control (not autoclaved). The core also appeared to be affected because more holes were present after autoclaving than before. In most of the holes there was still some granular material irregularly distributed. Although the micrographs suggested that more mantle than core substance was dissolved, some mantle material was still present after 60 h of autoclaving. The thickness of the fibre mantle from the inner shell membrane also showed little change even after the above mentioned time of autoclaving, but its structure had become more granular and less compact. There was some evidence that the fibre cores were affected, but this was, however, less marked than for the outer shell membrane fibres. The inner layer of the inner membrane was certainly affected by autoclaving. The electron micrographs showed that autoclaving, whilst dissolving as much dry matter from the inner as from the outer membrane (Balch & Cooke, 1968) had a less destructive effect on the mantle of the inner membrane fibres. This may be

caused by the higher percentage of mantle substance probably present in the inner membrane. This higher percentage of mantle substance could be related to its smaller fibres which are more numerous and to the presence of its inner layer to which some mantle material was attached. It was implied from this experiment that the main part of the protein polysaccharide complex is in the mantle. In an effort to establish this, pieces of shell membranes were fixed for two hours in an osmium tetroxide solution to which some ruthenium red was added in order to stain the acid mucopolysaccharide. In many of these sections the mantle looked more electron dense than the core (Fig. 69) which was the reverse of that seen in former sections. The larger electron-density was especially concentrated at the points of contact between two fibres, and in the outer part of the mantle. More ruthenium red had probably penetrated these parts than further in the mantle.

This experiment again indicated that the protein mucopolysaccharide complex may be especially deposited in the mantle. Balch & Cooke (1968) reported that the mantle was richer in carbohydrate and hydroxyproline than the corresponding membrane, but contained much less cystine. Collagens were according to Mahler & Cordes (1966) the only proteins known to contain significant amounts of hydroxyproline. Block & Weiss (1956) and Grau (1969), however, indicated the presence of hydroxyproline in collagen and elastin. The percentage hydroxyproline in elastin and in the egg membranes were of the same order. Using the 'Taenzer-Unna' orcein and the 'Maskar' safranelin staining method for elastin, both described by Romeis (1968) we succeeded in staining the mantle of the membrane fibres positively for the presence of elastin. Collagen, however, could not be shown in these fibres histochemically using the 'Cason', the 'Maskar' safranelin and the 'Heidenhain's' azan staining method (Romeis, 1968). According to Partridge (1962) elastin contained little, if any cystine. Elastin is therefore probably present especially in the sheath or mantle, while most of the core may be keratin.

Formation of air space The air space present between the membranes is formed mostly at the blunt end. As there are more pores per mm^2 at the blunt than at the narrow end, the air space may be formed here as a result of the larger number of pores.

6.2.2 Shell

Hypothesis about growth of shell At approximately 20 h before laying the egg enters the shell gland, the deposition of the mammillae having just been started whilst the egg was still in the isthmus. During the beginning of this period of calcification the mammillae became gradually wider and longer. A watery secreted solution is still added to the egg contents during this period (plumping period) increasing the egg size (Burmester, 1940). Thus there is formed a larger distance between the base of the mammillae and the intermammillary spaces increased.

During the plumping period there were open spaces between the mammillae by which the watery solution was transported to the egg contents. About 15 h before laying the mammillae will fuse (Evers, 1967) to form a continuous calcified shell. If the egg stayed in the shell gland for 20 h by calculation the mammillary layer will take about 5 h to form. During the last 15 h the palisade and surface crystal layer as well as the true cuticle are deposited successively.

The length of the mammillae of the hen eggshell varied from 94 to 144 μm while the thickness of the eggshell varied from 320–410 μm (Simons, 1962). The length of these mammillae may depend upon the rate of calcification (weight of shell deposited in a certain time) because as Bradfield (1951) suggested the mammillary layer was formed in a period of slow calcification while the palisade layer was formed in a period of more rapid calcification. According to this author during the last 5 h the egg was in the shell gland there was also a slow calcification. This may be when the surface crystal layer and the true cuticle are deposited. It would mean that the whole palisade layer is formed in about 10 h. The causes of these phases of slow and rapid calcification are not known.

Primary shell deposition The first crystal seeds of the eggshell were secreted in the isthmus (Taylor, 1962). A radial decalcified section of such a crystal seed can be seen in Fig. 21. As soon as the first drop of secretion fluid for the shell deposition and containing abundant mucopolysaccharide as well as some calcium carbonate (Schmidt, 1966 b), made contact with the membrane fibres, their mantle changed abruptly in structure and the secreted liquid crystallized (Fig. 22). The mantle substance and shell matrix contained a protein polysaccharide complex, as described. Simkiss & Tyler (1958) showed that the shell matrix had a calcium binding capacity. Its acid mucopolysaccharide acted in this way. This chelating capacity was greater in the cone layer than in the outer part of the shell (Tyler & Simkiss, 1959 b), which was probably related to the beginning of the eggshell calcification. It was suggested by Simkiss & Tyler (1958) that mucoitin sulphuric acid acted as chelating agent in the eggshell matrix. A calcium binding capacity of the fibre mantle has not yet been described. From the change of this mantle structure at the contact points with the mammillae (figs 19, 21 and 22) and its chemical composition a chelating capacity due to the presence of mucopolysaccharide or possibly elastin might be expected. The granular fibre mantle became, at first contact, much coarser and branched in a network in which crystals with a varying size up to 0.3 μm were deposited. All the crystals lying in the same direction have the shape of calcite. Also further outwards the whole shell matrix was calcified which did not agree with the descriptions of Simkiss (1968) who suggested the organic core was covered by the first deposit of calcite crystals.

About 7 μm outwards from the centre of the mammillae membrane-like lamellae were formed possibly by the pushing forward of some organic material during the crystallization process of the first drop of secretion fluid (Fig. 21). The rim of organic material may have formed the boundary of this first drop. Its diameter

was similar to that of the primary spherite seen by Schmidt (1964 c). Further outwards in figs 21 and 27 rims were also seen not parallel with the first ones formed possibly from succeeding drops. The crystal individuals grew, according to Schmidt (1962 a) from the crystallization centre inwards and outwards. They may also push some organic material forwards, some of which remained at their boundaries in the layer of cones and basal caps (figs 22, 26, 27, 29, 34 and 70). There was also a higher concentration of organic matter at the boundaries of the crystallites, within crystal individuals than within the crystallites themselves (figs 22 and 29). Schmidt (1965 c) also observed layers of organic matter between the crystal individuals in the basal caps and cones of nandu and duck eggshells. He called it a loculose structure, each loculus enveloping a single crystal individual. He suggested that the expanding crystals were formed in a self-purification process, the organic secretions remaining in the interstices between the individuals.

The first drops of calcification fluid will penetrate a small distance into the outer shell membrane between the membrane fibres. At this stage of formation, there were accumulations of membrane fibres especially of the outer part of the outer membrane with small open spaces between them in some places (figs 65 and 67). As the egg size increased during the plumping period the shell membranes were stretched which may increase the number of open spaces between the fibres. Some of the fibres were partly embedded in the calcified part of the shell. As the secretion continued it penetrated the outer shell membrane between two already present central parts of neighbouring mammillae and was deposited on the inside or side of these calcified parts. The fluid might crystallize along some membrane fibres lying near each other. This way of calcification may explain why the bundles of fibres penetrate the bases of the mammillae via the calcified deposits. Calcification fluid may easily flow from the mammillae along the fibre bundles and by the chelating properties of their mantles, crystallization may rapidly take place and the deposits will be formed along the outmost part of the fibre.

In the mammillae, there were irregular cavities with walls of about the same electron density as the fibre cores, about 10 μm above the anchoring site of the membrane fibres as described in Chap. 3 (figs 23 and 24). The formation of these walls possibly consisting of mainly core material might occur as the secretion of membrane material decreased or the secretion of calcification fluid increased. It is interesting whether this wall material was secreted in the isthmus or in the isthmo-uterine junction, the part of the oviduct where according to Simkiss (1967) the first secretions of calcification fluid probably occurred. In this isthmo-uterine junction secretion cells with unusual multiple granules have been seen by Johnston et al. (1963). In the isthmus the secretory granules were more uniformly dense in structure. Just at the outside of a premature isthmus egg still without calcified material around it, these cavities were seen. They might be formed at the end of the period in which the membrane fibres have been deposited.

Formation of holes and dark triangles During the formation of the layer of cones the shell deposition was more advanced in the central part of the crystal individuals than at their sides (figs 70 and 71). The appearance of vesicular holes was associated with the earlier deposition in the central part as seen in decalcified radial sections (figs 27 and 71). In fractured and premature eggshell surfaces these holes were seen as pitlike holes with tiny crystals inside (figs 28, 72, 73 and 74). Due to these holes von Nathusius (1868), Schmidt (1958 b) and Tyler & Simkiss (1959 b) saw in some places in tangential ground sections through the cone layer dark triangles surrounded by a transparent substance. Each triangle corresponded with a group of holes of a crystal individual. The transparent layer around these cloudy triangles became thinner towards the outside of the cone and finally disappeared (Schmidt, 1967 a). The following model is suggested as to its formation in the layer of cones. When the inside of the shell gland moved over the surface of the shell, crystallization possibly took place under a higher pressure or with less calcification fluid in the central part of the crystal individuals than near their sides. As a result more holes – with small crystals and perhaps gas (probably carbon dioxide) inside – might be formed in the central part of the crystal individuals than near their sides. Also meeting obstacles might influence the number of holes. These holes were not formed in the eisopherites (figs 21 and 22) where there were many membrane fibres, along which the calcification fluid might flow during formation. The crystallization may take place here with more calcification liquid or under a lower pressure than in the central part of the crystal individual in the cone layer. Schmidt (1965 b) reported that gas inclusions did not occur in this area.

The so-called gas inclusions by Schmidt (1967 a) occurred just outside the primary spherite and were present in the exospherite. Schmidt (1962 a) reported that these inclusions were formed in growth lines which lay in concentric curves outside this primary spherite in the inner part of the shell. Further outwards in the shell according to the author, these curves diminished. From our pictures we could not see clearly that the pitlike holes occurred lying in these growth lines. Many pitlike holes were formed especially in the outer part of the layer of cones but also in the palisade layer (Fig. 40). The definite shape of the crystals inside was not established. It was known that about 37 % of the shell consisted of calcium. Potassium, sodium, magnesium, phosphorus, iron, copper, manganese, sulphur and zinc occurred in small amounts in the shell. Where exactly these minerals were deposited in the eggshell was not known. They may be in part waste products deposited in the crystals of the holes. The crystals may shrink during crystallization by which some gas may fill in the holes around them. Schmidt (1965 b) saw these holes as dark points under the light microscope and thought that the entire holes were filled with gas. Initially Schmidt (1958 b) reported that the water containing organic matrix shrunk after drying and the empty spaces would be filled with air. The same author (1964 e) suggested that these holes were filled with air but carbon dioxide might also be present. In the

latter case he probably expected that carbon dioxide, released during shell calcification, would penetrate the holes. The X-ray microanalyser was used to examine the type of mineral occurring in the pitlike holes but without success as the probe diameter of the analyser was too large (about 5 μm). It would be useful to repeat this experiment with an X-ray microanalyser of probe diameter smaller than 1 μm.

Relation between organic and inorganic material Initially Schmidt (1962 a) thought organic and inorganic material was deposited simultaneously. This idea was based on the presence of organic as well as inorganic matter in the calcification fluid. Some years later he showed that in the layer of cones and basal caps of the bird eggshell some organic matter was pushed forward in the growing crystal individuals. Simkiss & Tyler (1958) suggested that the deposition of the organic matter in the shell precedes the inorganic. They supposed that acid mucopolysaccharides were in the shell matrix and may play an important part when the eggshell was deposited. This idea if proved would lead to more conformity between shell and bone formation.

The shell of an egg laid about 18 h before estimated oviposition showed on the outside, a compact organic covering which was up to 1 μm thick and which may correspond in structure with the organic layer present at the sides of the mammillae (Fig. 71). The mammillae in this case were not fused. A shell of a 4 h prematurely laid egg showed that the top of the developing palisade layer was also covered by a layer of organic material in some places $\frac{1}{3}$ μm thick (Fig. 75). These organic layers at the outside of premature eggs might have taken part in the crystallization process if this process had continued with taking up inorganic materials and forming the organic matrix. The idea that organic matter was primarily deposited before crystallization of inorganic material in the avian eggshell takes place was therefore supported. The mean rate of deposition of the palisade layer was about 19 μm/h (Evers, 1967). By calculation the small calcite crystallites were formed in the palisade layer about one minute after the organic matter was deposited.

Replicas of the outside of an estimated 16–17 h premature egg were studied with the transmission electron microscope (figs 70 and 72). The mammillae in this shell were not yet fused. Their outer surface varied from a flat to irregular structure. Small crystallites were seen in some places in layers running parallel with the outer surface of its mammilla. These layers were about 0.04 μm thick. The width and length of these crystallites were of the same order. This size corresponded with the meshes of the decalcified organic matrix in this part of the shell. Within one crystal individual the small crystallites were oriented in the same direction. Its direction varied in the different individuals (Fig. 70). The organic matrix which may partly envelop these crystallites could be seen particularly in the flatter areas. Many variations in structure occurred in different places of the replicas and this might be due to different calcification phases in

the different cones or crystal individuals.

Replicas of the shells of 4 h prematurely laid eggs showed a cross-section through a whole column with a diameter of about 15 μm (Fig. 73). There was more relief in this surface than in that of the 18 h premature eggs. This may be due to the larger crystallites in this layer. Many of these small crystallites together formed a column in which the orientation of the crystallites was about the same. However differences in this orientation between the columns did occur. The size of these small crystallites in the palisade layer was very difficult to measure. It was clear that their size was much larger than those of the cone layer and there were many crystallites with size of about 0.3 μm (figs 73 and 74) but they varied in size. Their size was such that they fitted in the structure of the organic matrix of the palisade layer (figs 35 and 75). Organic matter was seen which may surround these crystallites and partly penetrate them, which corresponded with the theory of how the eggshell was formed as described above.

The reasons for the differences in calcification of the cone and the palisade layer have not yet been investigated. One reason may be the difference in chemical composition of organic material between the two layers in the shell discussed below.

Chemical composition of organic matrix The organic matrix of the mammillary core consisted of a very compact network which probably took part in the shell calcification as previously explained. This organic material contained acid mucopolysaccharides (Simkiss & Tyler, 1957) to which a calcium binding capacity is ascribed. Tyler & Simkiss (1959 b) showed that the organic matrix of the palisade layer of ratite bird eggshells was different from that of the cone layer. They suggested that there were two protein-acid mucopolysaccharide complexes in the eggshell, one occurring in the cone layer and the other in the palisade layer. The first had the better chelating capacity. This may possibly be the same in the hen eggshell. Histochemical tests showed differences in polysaccharides between the cone and the palisade layer (Simkiss, 1968). We tried to stain the acid mucopolysaccharides after fixation of decalcified pieces of shells in an osmium tetroxide solution to which ruthenium red was added. No difference in electron density of the matrix was found between the stained preparations and controls in the layer of cones and basal caps as well as in the palisade layer. So the presence of acid mucopolysaccharides was not established.

Baker & Balch (1962) did not find hydroxyproline in the shell membranes, shell matrix and true cuticle. Some years later, Balch & Cooke (1968), using a different method, detected hydroxyproline in the membrane fibres and probably in their mantle. It is thought that collagen and elastin were the only proteins which contain hydroxyproline (see chemical composition of shell membranes). Almquist (1934) suggested that the shell matrix was collagenous. The gradual change in structure from membrane fibre mantle to shell matrix at the place of their contact suggests that they could be similar in composition. It would therefore be

interesting to repeat the research for the presence of hydroxyproline in the shell matrix.

Potassium, magnesium and phosphate in the shell An examination of a radial ground section through the shell with the X-ray microanalyser showed that the amount of potassium and magnesium in the shell increased from inside to outside. Most of the magnesium and phosphate occurred in the outer part of the shell (Itoh & Hatano, 1964). The higher phosphate content in the outer part of the shell was probably related to the 75 % increase in blood serum phosphate which occurred when the egg was in the shell gland (Feinberg et al., 1937). According to Black & Tyler (1944) and Common & Hale (1941) bird bones had two types of calcium reserve. The more readily mobilized reserve had a relatively high, and the less readily mobilized a relatively low ratio calcium : phosphorus (Black & Tyler, 1944). The former was probably used more at the beginning of shell formation and the latter more at the end. This may explain why at the end of shell formation more phosphate was found in the blood, one part being deposited in the shell as calcium phosphate (Romanoff & Romanoff, 1949) and another part excreted by the kidneys (Mongin & Lacassagne, 1967). This increase in blood phosphate may help stop shell formation when the shell is completed. An increase of magnesium in the blood during eggshell formation could be explained similarly to that of phosphate. About 70 % of the body's magnesium occurred in the bones with calcium and phosphate compounds. Magnesium may be dissolved like calcium during decalcification of a part of the bones. By this possible increase of blood magnesium during shell formation, the deposition of this element in the shell may be increased. It is according to Romanoff & Romanoff (1949) deposited as magnesium carbonate. Furthermore during eggshell formation pH of the blood decreased slightly (Mongin & Lacassagne, 1964). The potassium excretion in the urine decreased as pH in the blood plasma was reduced (Christensen, 1965). In addition an elevated serum potassium level is expected which may lead to more being deposited in the shell, but where and in which combination is unknown.

Surface crystal layer As far as we know the surface crystal layer of the hen eggshell has not yet been studied. This layer might be deposited slowly, because Bradfield (1951) found at the end of shell formation, a diminishing rate of calcification. As there were structural differences between the palisade and surface crystal layers, differences in chemical composition were also expected. Leonard (1970) showed concentrations of magnesium especially in the outermost layer of the shell. The author did not give the width of this zone. It would be of interest to examine if beside more organic matter there is more magnesium and perhaps more phosphate or potassium in the surface crystal layer than in the outer part of the palisade layer. This outer part should have more magnesium, phosphate and potassium than the inner part of the shell. If so these waste products may prevent further calcification of the shell.

6.2.3 Pigments of shell and true cuticle

Place of deposition Most hen eggs are white, cream or various shades of brown; only the Araucana bird of South America lays blue-green eggs. The colour of the egg was defined by the presence of pigments in the outer part of the shell and in the true cuticle. This colour greatly depended on where the pigment was deposited in the shell and true cuticle (Romanoff & Romanoff, 1949). Each hen laid eggs which were about the same colour. When the true cuticle was removed from the eggshell by keeping a brown egg for one hour in a 5 % EDTA solution, which probably dissolved part of the surface crystal layer, brown pigment was sometimes still seen in the shell. Schmidt (1956) also noted that this pigment was occasionally deposited in the outer zone of the palisade layer (in which he included the surface crystal layer) of the hen eggshell. The blue-green pigmentation of the Araucana eggshell was distributed over the entire thickness of the shell. Furthermore von Nathusius (1894) showed (Chap. 3) in pigmented eggshells that the outer layer of the cuticle may be uncoloured in contrast with its inner layer.

Fluorescence and deposition of ooporphyrin Red fluorescence studies showed that white as well as brown eggs fluoresced. Deeper layers of the shell showed less fluorescence than the outside which according to Völker (1940) was due to the brown pigmented ooporphyrin. Porphyrin may have an affinity for calcium since it also accumulated in molusc eggshells and bones (Fox & Vevers, 1960). An oviduct of a brown and one of a white egg laying bird showed a red fluorescence of their shell glands, which may be related to ooporphyrin being in the shell glands particularly in the last hours of its stay there. 50–74 % of the pigment was secreted during the last five hours the egg remained in the shell gland (Warren & Conrad, 1942). These results did not agree with those of Klosse & Almquist (1937) who found porphyrin secreted in the isthmus and the shell gland. In the egg white of brown eggs, more brown meat spots occurred than in white ones. Red fluorescence studies showed that there might be ooporphyrin in the brown meat spots. It is not known if the ooporphyrin may be transported from the shell gland to the magnum. If this is possible it would explain how Klosse & Almquist (1937) found it in the isthmus.

Deposition of other pigments Eggs did not come into contact with faeces so pigmentation could not take place from bile pigments of the faeces as suggested by Blasius (1867).

Brown meat spots that did not contain ooporphyrin, were also found in the egg white of brown eggs. We looked for a relation between the occurrence of pigment in meat spots and egg colour. As Fox & Vevers (1960) suggested there was melanin pigmentation in the eggshell. We tried to show melanin histochemically in decalcified radial sections of the shell and in the brown meat spots. Using the 'Masson Fontana' method and the ferrous iron technique both described by

Pearse (1961) we did not detect melanin in white and brown eggshells. Therefore melanin is not present in the eggshell or is removed during decalcification.

When the shell is formed with the true cuticle still more pigment is deposited sometimes on the shell as spots or stripes. Accumulations of these pigments were seen particularly on the wrinkled ring in the second laid eggs of broiler-type pullets which laid two eggs a day (van Middelkoop & Simons, 1970). These pigments often have a more reddish colour instead of a brown one. According to Giersberg (1922) these reddish pigments were formed directly from haemoglobin. It could be that the hen was unable to form so much pigment in so short a time thus causing this abnormality, or that this pigment looked reddish because it was not surrounded by cuticular material or perhaps less than in normal eggs.

6.2.4 True cuticle

Blasius (1867) and Ferdinandow (1931) suggested that the organic cuticle was secreted partly in the uterus, vagina and cloaca respectively. This was wrong because we saw from microscopic studies of premature shell gland eggs obtained post-mortem that the true cuticle was secreted in the shell gland. This true cuticle consisted of vesicles which were secreted according to McCallion (1953) as a granular substance. We found a granular material in the vesicles of the true cuticle which possibly corresponded with it.

According to Keller (1940/1941) weight of true cuticle was 0.06 % of egg weight. We found lower values of about 0.02 % of egg weight in white shells. Keller probably measured the cuticle weight in brown eggshells, which can have a thicker true cuticle as described before.

6.2.5 Cover

The calcified covering, which often occurred on eggshells of broiler-type pullets was deposited in the shell gland on the eggshell as a result of a delayed oviposition (van Middelkoop & Simons, 1970). The abnormality was due to two eggs being near each other in the shell gland. The same phenomenon occurred less often with eggs of laying-type pullets. It was also more difficult to observe on white eggs. The calcification will start again on the first egg when the second one starts to calcify. Therefore it may be expected that the chemical composition of this additional calcified layer on the surface is approximately the same as that of the primary shell deposition in the mammillary layer. Compared with the palisade layer, a high amount of organic material with much acid mucopolysaccharide, which is essential for the calcification and present in the primary shell deposition, will therefore probably occur in this cover. In Sphenisciformes eggshells Tyler (1965 b) demonstrated that the covering was indeed richer in protein compared with the palisade and cone layer. Acid mucopolysaccharides were present in large amounts in this covering.

6.2.6 Pores

If the egg was immersed in a suspension with *Pseudomonas* and *Saccharomyces ellipsoideus*, these micro-organisms passed the pores when the temperature of immersion was lower than that of the egg contents (Haines & Moran, 1940). Understandably *Pseudomonas* with a size of $2 \times 1\,\mu m$ passed very easily through the shell pore but this was more difficult for *Saccharomyces ellipsoideus* with a diameter of 8–10 μm assuming the average minimum opening is about 17 μm as Tyler (1956) measured in one shell.

6.2.7 Porosity

Haines & Moran (1940) showed that porosity varied widely between eggs of different hens. The different factors such as size of pores, number of pores per surface unit, thickness of true cuticle and shell thickness, may cause these differences.

Little is known about the relation between size of pores and porosity, but it is likely that there is a positive relation between them.

Haines & Moran (1940) could not correlate the number of pores counted by several staining methods and porosity. Almquist & Holst (1931) also using a dye solution, found a positive linear relation between porosity and the number of pores. The reason for the difference in results is not known.

Marshall & Cruickshank (1938) demonstrated that brown eggs were less porous than white eggs. Several eggs, which had very rough shells, were also quite porous. Both these facts are probably due to the thickness of the true cuticle. Brown eggs have a thicker true cuticle than white eggs (von Nathusius, 1894). The columns not ending at the same height may cause rough shells (Schmidt, 1958 b). If a true cuticle was deposited on such shells, an organic cuticular layer with an uniform thickness over the whole shell cannot be expected. On the columns which grow furthest outwards there will probably be a thin cuticular layer. A thin true cuticle may be more porous than a thick one.

Thick shells seem to have a lower porosity than thin ones (Marshall & Cruickshank, 1938). Nothing is known about differences in number of pores, size of pores and thickness of true cuticle between thick and thin shelled eggs. The influence of shell thickness can probably be reduced to one or more of the above mentioned factors. This is an interesting point for further research.

During the same time percentage weight loss was larger in small than in large eggs (Marshall & Cruickshank, 1938). This was caused by the relative larger surface of small eggs. Per surface unit the rate of moisture loss was the same (Mueller & Scott, 1940).

7 Destruction of shell during incubation

The incubation period of the hen lasts about 21 days. During this period the developing embryo withdraws and utilizes calcium from the shell to form its skeleton. This shell decalcification has not yet been studied in detail probably because without the use of a scanning electron microscope it would have been extremely difficult if not impossible to see the ultrastructure.

7.1 Literature

As quoted by Sajner (1955) it has been mentioned already by Purkinje in 1825 that during the incubation period some minerals (especially calcium) are withdrawn from the shell. These minerals are removed at the end of the incubation period by the blood vessels of the chorio-allantois, located near the inner shell membrane and are particularly used for forming the embryo skeleton (Stresemann, 1927/1934). Numerous microvilli developed in the chorion cells, where they made contact with the inner shell membrane (Leeson & Leeson, 1963) and there were indications that the embryos may obtain 68-80 per cent of their calcium from the eggshell (Simkiss, 1967). The amount of calcium absorbed was about 100 mg or 5 % of the eggshell. Stresemann (1927/1934) further noted that this shell decalcification started about the 12th day of incubation and took place rapidly from the 16th day on. The membranes with parts of the mammillae came off the entire shell, except where there was an air space, by about the 15th or 16th day (Tyler & Simkiss, 1959 a). The outer shell membrane remained attached to the shell in the region of the air space because the chorio-allantois did not come close to the shell here (Simkiss, 1967).

7.1.1 Structure of shell of hatched egg

Sajner (1955) and Schmidt (1965 e, 1967 d) mentioned how holes are formed in the mammillary bases by which the shell membranes with parts of the mammillae may be separated from the rest of the shell during incubation. Schwarz & Fehse (1957) saw them as oval or roundish angular holes at the inside of a shell of a hatched egg.

The spheritical structure of the shell of a hatched egg was described by Schmidt (1965 e, 1967 d). He especially studied shell membranes with calcified remainders of the mammillae on them. He thought the eisospherites almost disappeared

during incubation and the cone rests with irregular organized crystal fragments were left on the shell membranes as the decalcification progressed. The author saw holes with walls in these rests. These walls might correspond with the organic septa between the wedges in the cones. He thought that these septa consisted of two parallel lamellae.

7.1.2 Resorption

Schmidt (1965 e) supposed, that decalcification began in the crystallization centre of every spherite and proceeded radially. He suggested, that the decalcification fluid came by the membrane fibres in the crystallization centre. This author contradicted Tyler & Simkiss (1959 a) and Simkiss (1967) as he believed carbonic acid caused resorption. Partial decalcification of a normal eggshell with an EDTA solution had a similar structural effect on the eggshell as normally seen in shells of hatched eggs (Tyler & Simkiss, 1959 a and Terepka, 1963 b).

7.2 Results and discussion

The change in structure of shell membranes and shell during the incubation period together with shell decalcification have been studied in shells of hatched eggs.

7.2.1 Shell membranes with shell fragments on it

The outside of shell membranes with fragments of mammillae which came off the shell during the incubation period gave a crumbling appearance (Fig. 76). At some places in this picture membrane fibres of the outer part of the outer membrane which are anchored in the mammillary tops, are visible. Part of the remaining shell may have disappeared because part of the calcite dissolved at these anchorage places, or there may be an intermammillary space. The structure of shell membrane fibres in these hatched eggs looks similar to not incubated eggs. There were even protuberances on membrane fibres as mentioned in Chapter 3 for not incubated eggshell membranes. Understandably therefore von Nathusius (1868) stated that the shell membranes were not changed during the incubation period. This is not quite correct because there seems to be more calcium in the membranes at the end of the incubation period (Tyler & Simkiss, 1959 a). This is caused by transport of calcium inwards through the membranes. Whether or not there is a change in structure of shell membrane fibres during the incubation period, was not investigated.

In some places the irregular boundaries of the crumbling mammillae lying on the membranes were still visible (Fig. 76). In the middle of a mammilla, a shallow hole with a diameter of about 16 μm was seen and at some places around it there were smaller holes. The accumulations of organic matter between the calcite

individuals in the layer of cones and basal caps as described for normal shells (loculi of Schmidt, 1965 c) could be seen very clearly in this mammilla. However the accumulations did not consist of two lamellae as Schmidt (1965 e) mentioned. The mammillary core was not observed in this part of the shell although Tyler & Simkiss (1959 a) reported that these parts of the mammillae included the cores.

7.2.2 Outer part of mammillary layer

The outer part of the mammilla still connected to the outer part of the shell was also affected at the end of the incubation period. It looked very porous (Fig. 77). Its demarcation line showed an irregular boundary. Here the mammillae, more clearly as in the separated inner part, had a large central hole with an almost angular boundary as Schwarz & Fehse (1957) mentioned. This is probably where the organic core was deposited (Fig. 78). Before the inner part was freed from the outer part of the shell there was one large hole in the mammilla. There was a ring of smaller holes with irregular boundaries around this large central hole (Fig. 78) probably corresponding with the fibrous rings seen by Terepka (1963 a, b). The break in the mammilla also took place through the small holes which were therefore noticed in both separated shell parts. The small holes may correspond with the cavities seen in decalcified sections (figs 23 and 24) and be the equivalent of Sajner's rings as described and illustrated by Tyler & Simkiss (1959 a) although these authors only observed them in the calcified fragments on the membranes. The shell was eroded from inside of the mammillae in the holes (Fig. 78) and also between the mammillae (Fig. 79). Schmidt (1965 e) supposed that the decalcification fluid reached the crystalline material in the shell via the crystallization centre of the spherite.

7.2.3 Relation of shell structure to its destruction

The calcite individuals in the shell of an egg seemed to be progressively destroyed during incubation by passage of material along the organic matrix (Fig. 79). At several places in the mammilla, the erosion differed probably due to variation in matrix structure. Transport of decalcification fluid may take place firstly by the accumulations of organic matter between the calcite individuals. These accumulations of organic material may be left after hatching. Also Schmidt (1965 e) saw that parts of these so-called 'loculi' remained. From the accumulations of organic matter between crystal individuals, the decalcification fluid may penetrate the individuals along the organic matter between their crystallites (Fig. 79). Schmidt (1965 e) expected that the decalcification followed the concentric growth lines. There were no growth lines in the layer with eisospherites through which the membrane fibres passed. This may be why irregular crystal fragments occurred in this region after hatching.

An organic matrix was not seen in the holes described. The small holes which

formed a ring may correspond with the cavities described in normal shells in which an organic matrix was also not seen and which had walls probably containing fibre core material. The large central hole on the other hand originally contained an organic matrix among which the mammillary core was probably present. From some more-lateral crystal individuals only the surrounding organic matter was visible. The organic matrix from this central hole and some more-lateral crystal individuals may be dissolved or disturbed. It is as yet not known if parts of this mammillary matrix which disappeared were dissolved. If this did happen parts of the fibre mantle, being nearly the same chemical composition as the matrix, might also dissolve. Disappearance of fibre material was not noticed in these pictures which showed only the surface of the fibres. A radial section of the fibre would show this.

The organic matrix in the mammillae was partly disturbed if decalcification took place with EDTA without addition of formaldehyde and this is possibly also the case during incubation. From experiments with EDTA, it appeared that decalcification in a radial ground section of the shell took place most easily, above the region of the organic core where the hole was formed (Terepka, 1963 b). This was where the organic matrix was easily disturbed and it might explain why part of the mammillae stayed on the membrane after hatching. The organic core, however, was not observed among mammillary fragments on shell membranes after hatching. Its decalcification, taking place from the inside and being different from that of the radial ground section, might explain this.

There is some controversy about the way membranes, with mammillary bases, are loosened from the shell. Sajner (1955) suggested that during the incubation period some vacuoles (Sajner's rings) form in the mammillae. These burst and caused the membranes with mammillary bases to separate from the rest of the shell. Schmidt (1965 e, 1967 d) described large holes, formed by decalcification which were in the tops of the mammillae after hatching. He attributed the detachment of the shell membranes to this decalcification. He further suggested that the calcospherites around the holes were weakened, which resulted in the cone breaking under mechanical pressure or by drying of the shell membranes. Tyler & Simkiss (1959 a) thinking that Sajner's rings did not take part in this break off, suggested that the loosening of the membranes with mammillary tops from the rest of the shell did not arise from an indiscriminate solution of calcium carbonate because a great deal of calcium carbonate still remained on the membrane. They suggested that other factors are involved in this break. The porous mammillae with their large holes (Fig. 78) were certainly weakened by the decalcification in the mammillae during the incubation period. However the organic material in the mammillae may take part in the demarcation because fractures over the greater part of their length followed the organic matter. The membraneous lamellae of the matrix in the mammilla (figs 21 and 25) may possibly be seen as preformed lines along which the base of the mammilla separated from the rest of the calcified shell.

7.2.4 Decalcification fluid

Little is known about the mechanism of this decalcification. Schmidt (1965 e) stated, that the calcite resorption of the eggshell was produced from the embryo by the delivery of carbonic acid. Buckner et al. (1924/1925) suggested that the minerals were dissolved as bicarbonates. For many years this theory was accepted. According to Simkiss (1967) there was little doubt, that a high concentration of carbon dioxide did exist within the egg during the latter part of incubation but it was not established if it was sufficient for partial decalcification of the shell. Possibly carbon dioxide was hydrated with carbonic anhydrase, which may be in the mammillary core as described. From these facts it was not proved that carbonic acid attacks the shell and therefore Simkiss (1967) suggested that citric acid may partly decalcify the shell at the end of the incubation period. Citric acid was also produced during this period in the embryo and it was able to form complexes with calcium ions. These two theories have not been investigated thoroughly and need further research. Tyler & Simkiss (1959 a) suggested that enzymes may take part in removing organic matter with the decalcification of the mammillae. It is quite clear from our work and that of Schmidt (1965 e) that some organic matrix in the holes, arisen in the mammillae during the incubation period, had disappeared. The organic matrix may be destroyed. If the second theory (decalcification by citric acid) was right, more of the organic matrix would be destroyed by the carbon dioxide produced than if the first theory (decalcification by carbonic acid) was correct. After shells and membranes were extracted with water, followed by evaporation of this water, we tried to show citric acid in the residue with a paper chromatographic method (Löffler & Reichl, 1953). We found more citric acid in shells with membranes of hatched eggs than in those not incubated. This result supports the second theory. Until now it is not known if some organic matter other than in the mammillary layer of the shell has disappeared during the incubation period. A disappearance would indicate that enzymes might be involved in the shell destruction during hatching.

7.2.5 Outer shell layers

Nothing is known about a change in structure of the palisade layer, surface crystal layer and the true cuticle during the incubation period. The radially fractured surface of a shell of a hatched egg is less irregular than in normal eggshells (Fig. 80). The pitlike holes with their tiny crystals inside were seen probably unchanged. It was not clear from this picture if some decalcification took place over the whole shell, the organic matrix transporting the decalcification fluid, or if perhaps there was a change in structure of the organic matrix material of the palisade layer and the surface crystal layer during the incubation period. Both these possible transformations could make the shell weaker and are possibly important for the embryo to emerge from the egg. However there was certainly

some organic matrix in the palisade layer of the shell after hatching. Radial ultrathin sections through decalcified shells of hatched eggs might show whether or not there is a change in organic material during this incubation period.

According to Almquist & Holst (1931) the number of pores increased as the incubation proceeded. The pores were important for the exchange of gases during this period. More pores will probably be opened by shrinkage of the true cuticle as will be described in the following chapter. Some pores in a normal eggshell ended blindly before reaching the surface of the shell (Romanoff & Romanoff, 1949). In the latter part of the incubation period during decalcification some of these blind pores may be opened in parts of the shell. We observed that during the incubation period the interstices between the mammillae were attacked (Fig. 79) which contributed to this idea.

7.2.6 Shell at blunt end

The air space will occur mostly at the blunt end. During the incubation period there will be no tangential break through the mammillae at the place where the air space was formed. This may be related to the position of the embryo in the egg. In the latter part of the incubation period, its head was at the broad end of the egg, where the exchange of gases could take place more easily.

8 Effect of different factors on the true cuticle of laid eggs

The true cuticle, the outermost organic layer attached to the calcified shell, takes part in protecting the egg contents against penetration of micro-organisms by closing the pore holes (Romanoff & Romanoff, 1949).

Soiled eggs need cleaning before reaching the consumer. There is some doubt about the effect of cleaning on the true cuticle. Studies about the effect of cleaning techniques (e.g. washing) and some related factors on the true cuticle are therefore of economical importance.

8.1 Literature

Von Nathusius (1868) and Schmidt (1961) showed that mostly there were many cracks in the true cuticle of dry shells. These cracks sometimes ran through the true cuticle to the calcified layer underneath (Schmidt, 1961). Romankewitsch (1934) showed regularly distributed depressions on the egg surface.

The first pictures of the surface ultrastructure were published by Heyn (1963), but were of very poor quality. In electron micrographs of the surfaces of the eggshell he saw irregular bumps which he thought were due to small crystals underneath the true cuticle.

Furreg (1931) suggested that the true cuticle was destroyed after exposure to light although the protein remained. The structure of the surface may also be changed after this treatment.

The true cuticle could easily be damaged when it was in a liquid or sticky state just after oviposition (Denison, 1963).

Although the true cuticle had resistance (Simkiss, 1968), it may be destroyed by rubbing (Schmidt, 1961) or by boiling with sodium hydroxide solution (Tyler & Simkiss, 1958). Boiling an egg in water might also damage the true cuticle according to von Nathusius (1893). Lippincott & Card (1939), Romanoff & Romanoff (1949) and Tukker (1957) described the effect of cleaning techniques which may remove the true cuticle. If this occurs cleaning techniques may cause further spoilage. Bacterial spoilage of the egg contents was increased by washing regardless of original condition (Lorenz & Starr, 1952). Washed eggs also lost weight more rapidly than unwashed ones during storage (Briant & Sharp, 1934) and the porosity was thus increased. Lately some authors have described the true cuticle as more resistant (Brooks & Hale, 1955 b; Tyler & Simkiss, 1958 and Schmidt, 1961).

8.2 Results and discussion

The surface of the hen egg normally looks rather smooth and glossy. Many different opinions on the structure of the true cuticle have been published. It has been described as: granular, homogeneous, porous, layered with air inclusions and vesicular. These different descriptions may result from various factors influencing the cuticular structure. The cuticular surface was studied with the scanning electron microscope and in replicas after different treatments with the transmission electron microcope.

8.2.1 Surface structure of fresh eggs

Photographs of the cuticular surface (Fig. 81) confirmed its vesicular structure with airspaces between the vesicles already seen in radial sections of decalcified eggshells. It contained many star-shaped crack systems (Fig. 55). Cracks were also seen by von Nathusius (1868) and Schmidt (1961). The large crack systems represented surfaces of oval pore plaques (Fig. 55). Diameters of the surfaces of these plaques amount to over 100 μm. These oval areas with clearly defined edges were also seen in replicas (Fig. 82). Their surfaces looked more compact and flatter than the surrounding cuticular surface. They were built up of smaller vesicles with hardly any intervesicular airspaces. The smaller crack systems elsewhere on the cuticular surface (Fig. 55) may be caused by the true cuticle shrinking directly after oviposition. The presence of shell underneath the cuticular layer or a thinner cuticular layer itself might have contributed to smaller cracks forming here than in the pores. Heyn (1963) mentioned that there were streaks of cross markings running across the outer surface. He said they followed the egg surface in a spiral and were probably caused by the rotation of the egg when passing through the oviduct. We could not see these streaks.

Brown and white eggshells had the same surface structure. This agreed with von Nathusius (1894) who found that pigment distribution was limited to the basal layer of the true cuticle and the outer part of the calcareous shell.

8.2.2 Effect of time on cuticular surface

Replicas from different pieces of one eggshell showed that as time passed the cuticular surface became flatter and more compact due to the vesicles shrinking and the intervesicular airspaces diminishing. This change was evident 11 days after laying (Fig. 83) and extreme in another egg which was some months old (Fig. 84). Scanning electron microscope work showed that the smaller crack systems outside the pore surface gradually disappeared. This was possibly due to the true cuticle becoming thinner by the vesicles shrinking (Fig. 85). The cracks in the pore plaques on the other hand increased with this shrinkage. In radial sections through the shell of one egg a homogeneous true cuticle was found consisting of small vesicles

(Fig. 53). In another egg we saw a two-layered organic cuticle with small vesicles in the outer layer and larger ones in the inner (Fig. 54). The change may start at the surface and proceed inwards. The age of the two eggs, however, was not known.

The true cuticle of a guinea-fowl eggshell several years old was much changed. The cuticular material had shrunk to such an extent in some places that it looked condensed and spongy (figs 86 and 87). No vesicles seemed to be left.

8.2.3 Effect of washing on cuticular surface

For many years the general opinion was that the true cuticle was mainly mucin. This probably accounts for the idea that 'washing the egg removes the cuticle' (Romanoff & Romanoff, 1949). However, in practice many soiled eggs are washed. Therefore the effect of cleaning on surface structure was studied in some detail. Figs 88, 89 and 90 show that washing, by a stream of water, by hand or with a cloth, produces no apparent change in the structure of the cuticular surface judged by no structural components having disappeared.

In egg washing the temperature of the water should be higher than that of the egg contents (Haines & Moran, 1940 and Brant & Starr, 1940). Then no washing water will be added to the egg contents. Therefore the effect on the true cuticle of keeping the egg for 5 min in water at different temperatures was examined. Water of 40° C and above changed the surface structure (figs 91, 92, 93 and 94). The surface looked flatter and part of the true cuticle seemed to have disappeared. The whole surface was not affected uniformly; there was a patchy distribution of small changed areas among unchanged surroundings. The effect on the true cuticle increased with temperature but even boiling for 5 min did not remove the cuticular structure completely (Fig. 94).

Washing an egg in water with a detergent (Nusan) changed the cuticular surface structure in the same way as did water of high temperatures (Fig. 95). The effect was much more marked, however, and the changed patches were larger.

These findings may be useful for practical egg cleaning. The cuticular structure proved to be much more resistant than was expected. It was not even destroyed completely by boiling for 5 min in 10 % w/v potassium hydroxide. This agreed with Brooks & Hale (1955 b), Tyler & Simkiss (1958) and Schmidt (1961) who also attributed a greater stability to the true cuticle than had previously been supposed.

8.2.4 Shrinkage of true cuticle in old eggs

Simkiss (1958) showed proteins with many disulphide bonds and free sulphydryl groups in the true cuticle. Schmidt (1961) thought keratin was part of this true cuticle. If even small quantities of keratin were present as a structural component enclosing the mucin, the true cuticle could be a fairly stable structure. Speculating

further the possible oxidation of SH- into S-S-groups in this keratin might be related to the structural component of the true cuticle shrinking. This would account for the change of cuticular structure with time although drying out might also have an effect. The cuticular rests on a guinea-fowl eggshell several years old (figs 86 and 87) consisted of structural components with about the same electron density as that of the fibre core. This could support the previous suggestions.

The initiation of the proposed change in structure due to oxidation might also be connected with the change in cuticular fluorescence associated with the photosensitive cuticular ooporphyrin pigment. This also proceeds with time at the surface of the true cuticle (Grini, 1939 and Schoorl, 1965). Furreg (1931) supposed that the disappearance of fluorescence upon exposure to light indicated the destruction of the cuticular layer, although protein remained on the surface after this treatment. In this case the egg surface had a blue fluorescence. The author proved that blue light especially destroyed red fluorescence. This destruction diminished in green, yellow and red light respectively. In the dark, the red fluorescence did not diminish.

8.2.5 Damage of true cuticle

It was demonstrated by Denison (1963) and Tyler & Standen (1969) that the true cuticle may easily be damaged during short time after oviposition. We also confirmed that the true cuticle could be disturbed mechanically shortly after laying. This disturbance is especially produced in batteries by the wire floor or by the claws or beak of the hen. Where damaged translucent streaks will develop on the eggshells. From 4 groups of about 3500 eggs, laid by White Leghorn and Rhode Island Red hens respectively eggs with these streaks were determined from the freshly laid eggs (Oosterwoud, 1970). In batteries about 18 and 25 % of eggs with translucent streaks and consequently with a damaged true cuticle, were found for White Leghorns and Rhode Island Reds respectively. On nests about 6 % of these eggs were found for both breeds.

Eggs with translucent areas are known as mottled eggs. Translucent areas in eggs have been studied by Tyler & Geake (1964). The authors showed that these areas contained more water and were weaker than their surroundings.

Freshly laid eggs showed less mottling than those which were some weeks old. This may be caused by the true cuticle shrinking during this time. The thickness of the true cuticle is not the same over the whole surface of the egg. After shrinking, spots may occur with a very thin true cuticle where a translucent area may be formed.

Summary

The structure of the shell and shell membranes which is diagrammatically represented in Fig. 1 was studied with a light microscope, a transmission electron microscope and a scanning electron microscope. Eggshells and shell membranes particularly of hen eggs laid by laying breeds and those of premature hen eggs were investigated. Incubated hen eggs and eggs of some other species were examined incidentally. The results obtained gave us a better insight into the variability of the thickness of the shell membranes, the structural factors in the shell which may influence shell strength, the formation of shell and shell membranes, the destruction of the shell during the incubation period and the change of the surface of the true cuticle caused by different factors.

The shell membrane thickness of hen eggs was measured by three different methods which were critically compared. Dried isolated membranes were not as thick as membranes in radial decalcified sections and ground sections. This was probably due to the impossibility of separating the whole of the membranes from the calcified shell. Therefore the actual thickness of dry eggshell membranes can only be measured in radial sections.

Structural shell factors which may influence shell strength were studied in eggshells of the hen and guinea-fowl. Guinea-fowl eggshells were examined because they were much stronger than eggshells of the domestic hen even when allowance was made for their greater thickness. From these examinations shell strength may be influenced by shell membranes, the distribution of mammillae over the shell, the average diameter of these mammillae, the average diameter of columns, the distribution of organic matrix, the largeness and number/mm^3 of vesicular holes in the matrix, the crystalline structure within columns and the presence of the true cuticle. Fractures in the shell showed a strictly radial course in the cone layer but deviations to the tangential plane may occur in some places in the palisade layer. In both cases the fracture mostly followed layers of organic material.

Premature eggs were taken from the isthmus (part of the oviduct, which precedes the shell gland) about 20½ h before estimated laying time. The shell membranes of these eggs were compared with normal ones to learn more about its formation. The thickness of the inner as well as the outer shell membrane of these isthmus eggs was thinner than those of normally laid eggs. The inner shell membrane was deposited completely in the isthmus and was not formed, either partly or entirely, later from the egg contents. The cores of the membrane fibres

were deposited before their mantle. Egg shell membranes of 12, 8 and 4 h premature eggs looked identical to those of normally laid eggs. It seems very probable that during the plumping process (first five hours of shell formation when a watery solution was added to the white of the egg) when the egg size increased, the fibres were pulled apart more often, producing more and larger meshes in the shell membranes. The many connections (membranes and threads) which were between the different fibres of the isthmus eggs, may break off during this process but the diameter of the fibres did not change.

The way the different layers of the shell were deposited (from inside to outside respectively: the layer of cones with mammillae, the palisade layer, the surface crystal layer, the true cuticle and in some species the occurring cover) has been described. In the calcified shell there were higher concentrations of organic matter between the crystallites than in them. Also premature eggshells were covered by a thin homogeneous layer of organic material. This seems to be connected with the part the organic matter took in the shell calcification. The deposition of the organic matter precedes that of inorganic material. During shell formation a part of the organic matter took up inorganic material while another part was probably pushed forwards by the growing crystallites. In this manner a layer of organic matter may remain between two crystallites growing towards each other.

Many pitlike holes with tiny crystals inside were observed in the layer of cones and the palisade layer. The composition of these crystals and why they are formed is not yet known.

The amount of potassium and magnesium in the shell increased from inside to outside. It is not clear how much of these minerals is present in the surface crystal layer.

During the incubation period the developing embryo dissolved calcium from the shell for the formation of its skeleton. In each mammilla of the shell a large central hole formed probably originating from the crystallization centre. In the latter part of the incubation period the shell membranes together with parts of the mammillae came off the shell, the plane of separation running across the large holes and the Sajner's rings. Decalcification of the inner part of the shell during the incubation period together with their structural changes have been described. It is clear that decalcification of the shell proceeds along the concentrations of organic material between the crystal individuals and the crystallites. The course of the shell decalcification was influenced by the structure of the organic matrix in the mammilla.

The surface of a fresh hen egg normally showed a vesicular structure of the true cuticle with many star-shaped crack systems. The big crack systems represented the surface of the pore plaques. The surface relief became flatter and more compact by the vesicles of the true cuticle shrinking. The cracks in these pore plaques on the other hand increased due to this shrinkage.

Water with a temperature lower than 40°C did not dissolve the true cuticle.

Water of a higher temperature than 40° C changed the surface of the true cuticle. Washing in water with detergent (Nusan) produced much greater change of the surface and probably dissolved part of the true cuticle. The true cuticle was much more resistant than generally believed. Even after the egg had been boiled in 10 % potassium hydroxide for 5 minutes the cuticular structure had not completely disappeared.

Damage of the true cuticle a short time after laying which produced translucent streaks on the egg, has been described. These streaks were found more often in eggs laid in batteries than on nests.

References

Almquist, H. J., 1934. Proteins of the egg shell. Poult. Sci. 13 : 375.

Almquist, H. J. & W. F. Holst, 1931. Variability of shell porosity in hen's egg. Hilgardia 6 : 61–72.

Anonymous, 1967. Die Eischale und ihre Eigenschaften. Dt. Geflügelwirtsch. 19 : 1003.

Anonymous, 1969 a. Poult. and Egg Situation, June 8.

Anonymous, 1969 b. Meat and Dairy Produce Bull. 22 : 389.

Asmundson, V.S., G. A. Baker & J. T. Emlen, 1943. Certain relations between the parts of birds' eggs. Auk 60 : 34–44.

Baer, K. E., 1837. Über Entwicklungsgeschichte der Tiere. Part. 2. Gebrüder Bornträger, Königsberg.

Baker, J. R. & D. A. Balch, 1962. A study of the organic material of hen's-egg shell. Biochem. J. 82 : 352–361.

Balch, D. A. & R. A. Cooke, 1968. A study of the composition of hen's egg shell membranes. Lecture held at the Symposium on the physiology of egg shell and albumen formation. Grignon, France.

Balch, D. A. & C. Tyler, 1964. Variation in some shell membrane characteristics over different parts of the same shell. Br. Poult. Sci. 5 : 201–215.

Baudrimont, A. & M. St. Ange, 1847. Recherches sur les phénomènes chimique de l'évolution embryonnaire des oiseaux et des bactraciens. Annls Chim. Phys., Series III 21 : 195–295.

Black, D. J. G. & C. Tyler, 1944. Fluctuations in the porosity of egg-shells. Nature, Lond. 153 : 682–683.

Blasius, R., 1867. Ueber die Bildung, Structur und systematische Bedeutung der Eischale der Vögel. Z. wiss. Zool. 17 : 480–524.

Block, R. J. & K. W. Weiss, 1956. Amino acid handbook. Thomas, Springfield. Illinois.

Board, R. G., 1968. Microbiology of the egg: a review. In: Carter, T. C. (Ed.): Egg quality: A study of the hen's egg. Oliver and Boyd, Edinburgh 4 : 133–162.

Bokx, J. A. A., 1962. Invloed van de schaalvliezen op de breukweerstand van eieren. Department of Poultry Husbandry, Agricultural University, Wageningen.

Bradfield, J. R. G., 1951. Radiographic studies on the formation of the hen's egg shell. J. exp. Biol. 28 : 125–140.

Bradley, D. E., 1954. A high-resolution evaporated-carbon replica technique for the electron microscope. J. Inst. Metals 83 : 35–38.

Brant, A. W. & P. B. Starr, 1962. Some physical factors related to egg spoilage. Poult. Sci. 41 : 1468–1473.

Brooks, J. & H. P. Hale, 1955 a. Strength of the shell of the hen's egg. Nature, Lond. 175 : 848–849.

Brooks, J. & H. P. Hale, 1955 b. The detection of washed eggs. J. Sci. Fd Agric. 6 : 37–47.

Bryant, R. L. & P. F. Sharp, 1934. Effect of washing on the keeping quality of hen's eggs. J. agric. Res. 48 : 67–89.

Buckner, G. D., J. H. Martin & A. M. Peter, 1924/1925. Chemical studies of the oviduct of the hen. Am. J. Physiol. 71 : 349–354.

Burmester, B. R., 1940. A study of the physical and chemical changes of the egg during its passage through the isthmus and uterus of the hen's oviduct. J. exp. Zool. 84 : 445–500.
Cain, C. J. & A. N. J. Heyn, 1964. X-ray diffraction studies of the crystalline structure of the avian egg shell. Biophys. J. 4 : 23–39.
Calvery, H. O., 1933. Some analyses of egg-shell keratin. J. biol. Chem. 100 : 183–186.
Carter, T. C., 1969. The hen's egg: relationship between shell thickness and the amount of organic matter in the shell. Br. Poult. Sci. 10 : 165–174.
Christensen, H. N. 1965. Body fluids and the acid-base balance. Saunders, Philadelphia and London.
Clevisch, A., 1913. Beiträge zur Struktur und Physiologie der Vogeleischalen. Phil. Diss., Bonn, W. Germany (Cited by Schmidt, 1962 a).
Common, R. H., 1941. The carbonic anhydrase activity of the hen's oviduct. J. agric. Sci., Camb. 31 : 412–414.
Common, R. H. & R. W. Hale, 1941. Observations on the mineral metabolism of pullets III. J. agric. Sci., Camb. 31 : 415–437.
Coste, J., 1847. Histoire générale et particulière du développement des corps organisés. Masson, Paris 1 : 295.
Cray, R. E., 1953. Cracked eggs costing industry too much money. Poult. Process. and Mktg 59 : 10 and 22.
Denison, J. W., 1963. The effect of mechanical disturbance of the egg cuticle on shell mottling. Poult. Sci. 40 : 771–772.
Diamantstein, T., 1966. Über die lokale Rolle der Mucopolysaccharide bei der Eischalenverkalkung und Eischalenstabilität. Arch. Geflügelk. 30 : 421–430.
Diamantstein, T., K. Bronsch & F. Schluns, 1964. Carbonic anhydrase in the mammillae of the hen's egg shell. Nature, Lond. 203 : 88–89.
Dickie, G., 1848. On the structure of the shell of the egg in birds, and the nature and seat of the colour. Ann. Mag. nat.Hist. 2 : 169–176.
El-Boushy, A. R., 1966. Egg shell quality and microstructure effected by vitamin C, other feed additives and high environmental temperatures. Communications Agricultural University, Wageningen 66–7.
El-Boushy, A. R., P. C. M. Simons & G. Wiertz, 1968. Structure and ultra-structure of the hen's egg shell as influenced by environmental temperature humidity and vitamin C additions. Poult. Sci. 47 : 456–467.
Evers, A., 1967. De vorming en structuur van de schaal van het kippeëi bestudeerd bij prematuur gelegde eieren. Laboratory of Animal Physiology, Agricultural University, Wageningen.
Favejee, J. Ch. L., L. van der Plas, R. Schoorl & P. Floor, 1965. X-ray diffraction of the crystalline structure of the avian egg shell: some critical remarks. Biophys. J. 5 : 359–361.
Feinberg, J. G., J. S. Hughes & H. M. Scott, 1937. Fluctuations of calcium and inorganic phosphorus in the blood of the laying hen during the cycle of one egg. Poult. Sci. 16 : 132–134.
Ferdinandow, V. V., 1931. Handbuch der Vogelzucht und Geflügelindustrie (russ.) I. Teil (Cited by Romankewitsch, 1934).
Fischer, H. & F. Kögl, 1923. Zur Kenntnis der natürlichen Porphyrine. IV Über das Ooporphyrin. Hoppe-Seyler's Z. physiol. Chem. 131 : 241–261.
Fischer, H. & F. Kögl, 1924. Zur Kenntnis der natürlichen Porphyrine. IX Über Ooporphyrin aus Kiebitzeierschalen und seine Beziehungen zum Bluttfarbstoff. Hoppe-Seyler's Z. physiol. Chem. 138 : 262–275.
Fischer, H. & R. Müller, 1925. Zur Kenntnis der natürlichen Porphyrine. XIII Mitteilung. Überführung von Hämoporphyrin in Mesoporphyrin und dessen Abbau zum Ätioporphyrin. Hoppe-Seyler's Z. physiol. Chem. 142 : 120–140.
Fox, H. M. & G. Vevers, 1960. The nature of animal colours. Sidgwick and Jackson, London.
Fromm, D., 1963. Permeability of the hen's egg shell. Poult. Sci. 42 : 1271.

Furreg, E., 1931. Über die Kutikula des Vogeleies und einem Nachweis ihres Bildungsortes. Biol. Zbl. 51 : 162–173.
Giersberg, H., 1922. Untersuchungen über Physiologie und Histologie des Eileiters der Reptilen und Vögel nebst einem Beitrag zur Fasergenese. Z. wiss. Zool. 120 : 1–97.
Gleaves, E. W., 1967. Handling practises can cut egg breakage. Poult. Trib. 73 : April 14 and 36.
Grau, R., 1969. Fleisch und Fleischwaren. 2nd. ed. Parey, Berlin and Hamburg.
Grini, O., 1939. Luminescence test for eggs. Wld's Poult. Congr.: 496–498.
Haines, R. B. & T. Moran, 1940. Porosity of, and bacterial invasion through, the shell of the hen's egg. J. Hyg., Camb. 40 : 453–461.
Hays, F. A. & A. H. Sumbardo, 1927. Physical characters of eggs in relation to hatchability. Poult. Sci. 6 : 196–200.
Hemsbach, H. M. von, 1851. Die Bildung der für partielle Furchung bestimmter Eier der Vögel im Vergleich mit dem Graafschen Follikel und der Decidua des Menschen. Z. wiss. Zool. 3 : 420–434.
Heyn, A. N. J., 1963. The crystalline structure of calcium carbonate in the avian egg shell. An electron microscope study. J. Ultrastruct. Res. 8 : 176–188.
Itoh, H. & T. Hatano, 1964. Variation of magnesium and phosphorus deposition rates during egg shell formation. Poult. Sci. 43 : 77–80.
Johnston, H. S., R. N. C. Aitken & G. M. Wyburn, 1963. The fine structure of the uterus of the domestic fowl. J. Anat. 97 : 333–344.
Keller, H., 1940/1941. Über Nachweis und Darstellung des Eioberhäutchens bei Hühner- und Enteneiern. Z. Fleisch- u. Milchhyg. 51 : 43–48.
Kelly, A., 1901. Beiträge zur mineralogischen Kenntnis der Kalkausscheidungen im Tierreich. Jena. Z. Med. Naturw. 35 : 429–494.
Klosse, A. A. & H. J. Almquist, 1937. The pigment of egg shell membranes. Poult. Sci. 16 : 173–174.
Kushida, H., 1961. A styrene-methacrylate resin embedding method for ultrathin sectioning. J. Electron. Microsc., Chiba Cy. 10 : 16–19.
Landois, H., 1865. Die Eierschalen der Vögel in histologischer und genetischer Beziehung. Z. wiss. Zool. 15 : 1–31.
Landois, H., 1882. Bericht über die (VI.) Jahresversammlung. J. Orn., Lpz. 30 : 8–11.
Lawn, A. M., 1960. The use of potassium permanganate as an electron-dense stain for sections of tissue embedded in epoxy resin. J. biophys. biochem. Cytol. 7 : 197–198.
Leech, F. B. & N. R. Knowles, 1969. An investigation on commercial farms of factors thought to contribute to egg cracking. Br. Poult. Sci. 10 : 139–147.
Leeson, T. S. & C. R. Leeson, 1963. The chorio-allantois of the chick. Light and electron microscopic observations at various times of incubation. J. Anat. 97 : 585–595.
Leonard, E. M., 1970. The molar ratio of calcium to magnesium in the shell of the hen's egg during formation. Proc. physiol. Soc., Camb. 24 P–25 P.
Lippincott, W. A. & L. E. Card, 1939. Poultry production, 6th ed. Kimpton, London.
Löffler, J. E. & E. R. Reichl, 1953. Eine Methode zum Nachweis und zur Bestimmung organischer Säuren in Papierchromatogrammen. Mikrochim. Acta, Heft 1–2 : 78–88.
Lorenz, F. W. & P. B. Starr, 1952. Spoilage of washed eggs. 1. Effect of sprayed versus static water under different washing temperatures. Poult. Sci. 31 : 204–214.
Luft, J. H., 1961. Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol. 9 : 409–414.
Luft, J. H., 1966. Fine structure of capillary and endocapillary layer as revealed by ruthenium red. Fedn Proc. Fedn Am. Socs exp. Biol. 25 : 1773–1783.
Mahler, H. R. & E. H. Cordes, 1966. Biological Chemistry. Harper and Row, New York, Evanston and London, and Weatherhill, Tokyo.
Marshall, W. & D. B. Cruickshank, 1938. The function of the cuticle in relation to the porosity of eggs. J. agric. Sci. Camb. 28 : 24–42.

Masshoff, W. & H. J. Stolpmann, 1961. Licht- und elektronenmikroskopische Untersuchungen an der Schalenhaut und Kalkschale des Hühnereies. Z. Zellforsch. mikrosk. Anat. 55 : 818–832.
McCallion, D. J., 1953. A cytological and cytochemical study of the shell gland of the domestic hen. Can. J. Zool. 31 : 577–589.
Mérat, Ph. & L. Lacassagne, 1961. Faux d'éclosion et position de l'oeuf dans la série chez la poule. Annls Biol. anim. Biochim. Biophys. 1 : 101–104.
Mercer, E. H. & M. S. C. Birbeck, 1960. Electron microscopy. A handbook for biologists. 1st ed. Blackwell, Oxford.
Mercer, E. H. & M. S. C. Birbeck, 1966. Electron microscopy. A handbook for biologists. 2nd ed. Blackwell, Oxford.
Middelkoop, J. H. van & P. C. M. Simons, 1970. Shell abnormalities due to the presence of two eggs in the uterus. Wld's Poult. Congr.
Mitchell, P. H., 1946. A textbook of biochemistry. McGraw-Hill, New York and London.
Mongin, P., 1966. Le metabolisme calcique chez la pondeuse. Revue Elev. 21 : 121, 122, 125 and 127.
Mongin, P. & L. Lacassagne, 1964. Physiologie de la formation de la coquille de l'oeuf de poule et équilibre acido-basique du sang. C. r. hebd. Séanc. Acad. Sci., Paris 258 : 3093–3094.
Mongin, P. & L. Lacassagne, 1967. Excrétion urinaire chez la poule au moment de la ponte de son premier oeuf. C. r. hebd. Séanc. Acad. Sci., Paris 264 : 2479–2480.
Moran, T. & H. P. Hale, 1936. Physics of the hen's egg. I. Membranes in the egg. J. exp. Biol. 13 : 35–40.
Mueller, C. D. & H. M. Scott, 1940. The porosity of the egg-shell in relation to hatchability. Poult. Sci. 19 : 163-166.
Nathusius, W. von, 1868. Ueber die Hüllen, welche den Dotter des Vogeleies umgeben. Z. wiss. Zool. 18 : 225–270.
Nathusius, W. von, 1869. Nachträge zu der Arbeit 'Ueber die Hüllen, welche den Dotter des Vogeleies umgeben'. Z. wiss. Zool. 19 : 322–348.
Nathusius, W. von, 1871. Ueber die Eischalen von Aepiornis, Dinornis, Apterix und einigen Crypturiden. Z. wiss. Zool. 21 : 330–355.
Nathusius, W. von, 1882, Untersuchungen von Eischaalen, namentlich von Opisthocomus, Turnix, und der sogen. Ueberzüge bei den Steganopoden und anderen Eiern, nebst Bemerkungen über die systematische Bedeutung dieser Structuren. J. Orn., Lpz. 30 : 255–315.
Nathusius, W. von, 1885. Ueber die charakterischen Unterscheidungszeichen verschiedener Straussen-Eier. J. Orn., Lpz. 33 : 165–178.
Nathusius, W. von, 1887. 2. Die Kalkkörperchen der Eischalen-Überzüge und ihre Beziehungen zu den Harting'schen Calcosphäriten. Zool. Anz. 10: 292–296.
Nathusius, W. von, 1893. Die Entwicklung von Schale und Schalenhaut des Hühnereies im Oviduct. Z. wiss. Zool. 55 : 576–584.
Nathusius, W. von, 1894. 4. Über Farben der Vogeleier. Zool. Anz. 17 : 440–445.
Oosterwoud, A., 1970. Personal communication.
Partridge, S. M., 1962. Elastin. Adv. Protein Chem. 17 : 227–302.
Pearse, A. G. E., 1961. Histochemistry, Theoretical and applied, 2nd ed. Little, Brown and Company, Boston.
Petersen, J. & C. Tyler, 1967. The strength of guinea fowl *(Numida meleagris)* egg shells. Br. Poult. Sci. 7 : 291–296.
Plas, L. van der, 1966. De kristallijne struktuur van een eierschaal. Lecture held at the Central Institute for Poultry Research 'Het Spelderholt', Beekbergen, the Netherlands.
Pohlmann, G. & O. H. Volk, 1959. Tylose, ein wasserlösliches Abdruckmaterial für Oberflächenuntersuchungen. Z. wiss. Mikrosk. 64 : 252–256.
Purkinje, J. E., 1830. Symbolae ad ovi avium historiam ante incubationem. Vossius, Leipzig.
Reynolds, E. S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17 : 208–212.

Richardson, K. C., 1935. The secretory phenomena in the oviduct of the fowl, including the process of shell formation examined by the microincineration technique. Phil. Trans. R. Soc. Series B. 225 : 149–195.

Rizzo, A., 1899. Sul numero e sulla distribution dei pori nel guscio d'ele ovo di gallina. Ricerche fatte nel Laborat, di Anatomia Normale, Univ. di Roma: ed. in altri Lab. biol 7 : 171 (Cited by Stewart, 1935).

Robinson, D. S. & N. R. King, 1963. Carbonic anhydrase and formation of the hen's egg shell. Nature, Lond. 199 : 497–498.

Robinson, D. S. & N. R. King, 1970. The structure of the organic mammillary cores in some weak egg shells. Br. Poult. Sci. 11 : 39–44.

Romankewitsch, N. A., 1932. Die Biomorphologischen Besonderheiten der Struktur der Membrana Testae des Hühnereies. Z. Zellforsch. mikrosk. Anat. 16 : 208–216.

Romankewitsch, N. A., 1934. Das Kutikulum des Hühnereies. Z. Zellforsch. mikrosk. Anat. 21 : 110–118.

Romanoff, A. L. & A. J. Romanoff, 1949. The avian egg. Wiley, New York; Hall, London.

Romeis, B., 1968. Mikroskopische Technik. 16th ed., Oldenbourg, München and Vienna.

Sajner, J., 1955. Über die Mikroskopischen Veränderungen der Eischale der Vögel im Laufe der Inkubationszeit. Acta anat. 25 : 141–159.

Schmidt, W. J., 1956. Beiträge zur mikroskopischen Kenntnis der Farbstoffe in der Kalkschale des Vogeleies. Z. Zellforsch. mikrosk. Anat. 44 : 413–426.

Schmidt, W. J., 1957. Schraubiger Faserverlauf in der Schalenhaut des Vogeleies. J. Orn., Lpz. 98 : 432–440.

Schmidt, W. J., 1958 a. Schleim-, Kalk- und Farbstoff-Ablagerungen auf der Kalkschale von Vogeleiern. Z. Zellforsch. mikrosk. Anat. 47 : 251–268.

Schmidt, W. J., 1958 b. Über den Aufbau der Schale des Vogeleies nebst Bemerkungen über kalkige Eischalen anderer Tiere. Ber. oberhess. Ges. Natur- u. Heilk. Giessen, Naturwissenschaftliche Abteilung, 28 : 82–108.

Schmidt, W. J., 1960. Polarisationsoptik und Bau der Kalkschale des Hühnereies. Z. Zellforsch. mikrosk. Anat. 52 : 715–729.

Schmidt, W. J., 1961. Über das Schleimhäutchen der Vogeleischale, insbesondere bei Phoenicopterus, Aptenodytes und Spheniscus. J. Orn., Lpz. 102 : 456–467.

Schmidt, W. J., 1962 a. Liegt der Eischalenkalk der Vögel als submikroskopische Kristallite vor? Z. Zellforsch. mikrosk. Anat. 57 : 848–880.

Schmidt, W. J., 1962 b. Über die Basalkalotten der Vogeleischale. J. Orn., Lpz. 103 : 28–37.

Schmidt, W. J., 1964 a. Die Deckschicht auf der Eischale von *Cygnus olor*. Zool. Anz. 172 : 431–436.

Schmidt, W. J., 1964 b. Die Primärsphäriten der Hühner-Eischale. Z. Zellforsch. mikrosk. Anat. 62 : 48–52.

Schmidt, W. J., 1964 c. Nochmals über die primärsphäriten der Hühner-Eischale. Z. Zellforsch. mikrosk. Anat. 63 : 682–685.

Schmidt, W. J., 1964 d. Bemerkungen zur leichten Löslichkeit der Calcosphäriten-Zentren in der Vogel-Eischale. Z. Zellforsch. mikrosk. Anat. 64 : 726–732.

Schmidt, W. J., 1964 e. Das 'Fischgrätenmuster' auf dem Querschliff der kalkigen Eischale von Vögeln. Z. Zellforsch. mikrosk. Anat. 62 : 53–60.

Schmidt, W. J., 1964 f. Über die Struktur einiger abnormer Vogel-Eischalen nebst Bemerkungen zu neueren Auffassungen betreffend Bau und Bildung der Kalkschale. Z. Morph. Ökol. Tiere 53 : 311–361.

Schmidt, W. J., 1965 a. Goldfärbung der Schalenhautfasern des Vogeleies am Schliff. Z. wiss. Mikrosk. 67 : 51–54.

Schmidt, W. J., 1965 b. Die Eisosphäriten (Basalkalotten) der Schwanen-Eischale. Z. Zellforsch. mikrosk. Anat. 67 : 151–164.

Schmidt, W. J., 1965 c. Das organische Fachwerk in 'Kegeln' und 'Kalotten' der Vogel-Eischale. Z. Zellforsch. mikrosk. Anat. 68 : 194–204.
Schmidt, W. J., 1965 d. Die organischen Kerne der Mammillen in den Frühstadien der Eischalenbildung bei der Lachmöwe. Z. Zellforsch. mikrosk. Anat. 65 : 713–718.
Schmidt, W. J., 1965 e. Morphologie der Kalkresorption an der ausgebrüteten Vogel-Eischale. Z. Zellforsch. mikrosk. Anat. 68 : 874–892.
Schmidt, W. J., 1966 a. Nachtrag zu meiner Arbeit 'Schraubiger Faserverlauf in der Schalenhaut des Vogeleies'. J. Orn., Lpz. 107 : 236.
Schmidt, W. J., 1966 b. Das Verwachsen der Calcosphäriten bei der Schalenbildung des Vogeleies. Z. Morph. Ökol. Tiere 56 : 252–258.
Schmidt, W. J., 1966 c. Das Rhomben- und Blockmuster im Eischalenkalk von *Cygnus olor*. Z. Zellforsch. mikrosk. Anat. 72 : 475–486.
Schmidt, W. J., 1967 a. Schliffe von Vogel-Eischalen im Dunkelfeld. Z. Morph. Ökol. Tiere 60 : 17–34.
Schmidt, W. J., 1967 b. Das 'Globularmuster' im Eischalenkalk von Diomedea. Z. Zellforsch. mikrosk. Anat. 77 : 518–533.
Schmidt, W. J., 1967 c. Das 'Muster' im Eischalenkalk der Vögel dargestellt durch Schliffätzung. Z. Zellforsch. mikrosk. Anat. 79 : 446–458.
Schmidt, W. J., 1967 d. Untersuchung der Kalkresorption an der Vogel-Eischale während der Bebrütung. Z. wiss. Mikrosk. 68 : 51–58.
Schmidt, W. J., 1968. Zur Eischalenstruktur beim Perlhuhn *(Numida meleagris)*. Z. Zellforsch. mikrosk. Anat. 86 : 444–452.
Schoorl, P., 1965. Red fluorescence of eggs in U.V. light after washing. Int. Congr. Refrig. Inds.
Schoorl, P., 1968. Annual report Processing Department, Central Institute for Poultry Research 'Het Spelderholt', Beekbergen, the Netherlands.
Schoorl, P. & H. Y. Boersma, 1962. Research on the quality of the egg-shell. A new method of determination. Wld's Poult. Congr.: 432–435.
Schoorl, P. & G. Mos, 1968. Porositeit – gewichtsverlies en poriënaantal – bij kippe- en eendeeieren. Pluimveeonderzoek, Communication no. 155, Central Institute for Poultry Research 'Het Spelderholt', Beekbergen, the Netherlands.
Schwarz, L. & F. Fehse, 1957. Beobachtungen an Eierschalen und Eiern. I. Mitteilung. Über Unterschiede der Schalen unbebrüteter und ausgebrüteter Eier. Zool. Anz. 159 : 268–284.
Shrimpton, D. H. & C. M. Hahn, 1968. An investigation into the value of measurements of egg shell deformation in predicting breakage due to transport and handling. Expl Husb. 17 : 29–35.
Simkiss, K., 1958. The structure of the eggshell with particular reference to the hen. Ph.D Thesis, University of Reading.
Simkiss, K., 1961. Calcium metabolism and avian reproduction. Biol. Rev. 36 : 321–367.
Simkiss, K., 1967. Calcium in reproductive physiology. A comparitive study of vertebrates. Chapman and Hall, London; Reinhold, New York.
Simkiss, K., 1968. The structure and formation of the shell and shell membranes. In: Carter, T.C. (Ed.): Egg quality: A study of the hen's egg. Oliver and Boyd, Edinburgh 4 : 3–25.
Simkiss, K. & C. Tyler, 1957. A histochemical study of the organic matrix of the hen egg-shells. Q. Jl microsc. Sci. 98 : 19-28.
Simkiss, K. & C. Tyler, 1958. Reactions between egg-shell matrix and metallic cations. Q. Jl microsc. Sci. 99 : 5–13.
Simons, P. C. M., 1962. De bouw en de chemische samenstelling van schaalvliezen, schaal en cuticula van het kippeëi en enige opmerkingen over de schaalsterkte. Laboratory of Animal Physiology, Agricultural University, Wageningen.
Simons, P. C. M. & G. Wiertz, 1963. Notes on the structure of membranes and shell in the hen's egg. An electron microscopical study. Z. Zellforsch. mikrosk. Anat. 59 : 555–567.

Simons, P. C. M., C. Tyler & H. P. Thomas, 1966. The effect of sodium hydroxide and sodium sulphide on the snapping strength of egg shells. Br. Poult. Sci. 7 : 309–314.
Smith, A. H., C. M. Winget & J. R. Blackard, 1954. The transfer of phosphorus to the hen's egg, under controlled environment, as traced with radiophosphorus (P^{32}). Poult. Sci. 33 : 908–919.
Stewart, G. F., 1935. The structure of the hen's egg shell. Poult. Sci. 14 : 24–32.
Stresemann, E., 1927/1934. Handbuch der Zoologie, Bd. 7, 2. Hälfte, Sauropsida: Aves. W. de Gruyter und Co, Berlin and Leipzig.
Szielasko, A., 1913. Die Bedeutung der Eischalenstruktur der Vögel für die Systematik. J. Orn., Lpz. 6 : 52–117.
Taylor, T. G., 1962. Symposium on the quality of the consumption-egg. Central Institute for Poultry Research 'Het Spelderholt', Beekbergen, the Netherlands.
Taylor, T. G., 1965. Personal communication.
Taylor, T. G., 1966. The egg shell problem: what of the future? Poult. Fmr, Lond. 154 : 7 Dec., 55.
Taylor, T. G., 1970. How an eggshell is made. Sci. Am., 222 : 89–95.
Terepka, A. R., 1963 a. Structure and calcification in avian egg shell. Expl Cell Res. 30 : 171–182.
Terepka, A. R., 1963 b. Organic-inorganic interrelationships in avian egg shell. Expl Cell Res. 30 : 183–192.
Tijen, W. F. van, 1963. A method to determine collectively the average specific gravity of a number of eggs. Veeteelt- en Zuivelber., 6 : 574–578.
Tukker, J. G., 1957. Het ei van producent tot consument. Cuperus, De Bilt, the Netherlands.
Tyler, C., 1953. Studies on egg shells. II. A method for marking and counting pores. J. Sci. Fd Agric. 4 : 266–272.
Tyler, C., 1955. Studies on egg shells. VI. The distribution of pores in egg shells. J. Sci. Fd Agric. 6 : 170–176.
Tyler, C., 1956. Studies on egg shells. VII. Some aspects of structure as shown by plastic models. J. Sci. Fd Agric. 7 : 483–493.
Tyler, C., 1958. Studies on egg shells. X. A method for determining variations in characteristics in different parts of the same shell. J. Sci. Fd Agric. 9 : 584–590.
Tyler, C., 1961. Studies on egg shells. XVII. Variations in membrane thickness and in true shell nitrogen over different parts of the same shell. J. Sci. Fd Agric. 12 : 470–475.
Tyler, C., 1964. A study of the egg shells of the Anatidae. Proc. zool. Soc. Lond. 142 : 547–583.
Tyler, C., 1965 a. Personal communication.
Tyler, C., 1965 b. A study of the egg shells of the Sphenisciformes. J. zool. Res. 147 : 1–19.
Tyler, C., 1966. A study of the egg shells of the Falconiformes. J. zool. Res. 150 : 413–425.
Tyler, C., 1969. Structural flaws in some very strong egg shells. Br. Poult. Sci. 10 : 371–374.
Tyler, C. & J. R. Coundon, 1965. Apparatus for measuring shell strength by crushing, piercing or snapping. Br. Poult. Sci. 6 : 327–330.
Tyler, C. & F. H. Geake, 1958. Studies on egg shells. IX. The influence of individuality, breed and season on certain characteristics of egg shells from pullets. J. Sci. Fd Agric. 9 : 473–483.
Tyler, C. & F. H. Geake, 1961. Studies on egg shells. XIV. Variations in egg weight, shell thickness and membrane thickness between eggs within a clutch. J. Sci. Fd Agric. 12 : 273–280.
Tyler, C. & F. H. Geake, 1964. The effect of water on egg shell strength including a study of the translucent areas of the shell. Br. Poult. Sci. 5 : 277–284.
Tyler, C. & K. Simkiss, 1958. Studies on egg shells. VIII. The determination of membrane-, pore- and matrix-protein – a reassessment. J. Sci. Fd Agric. 9 : 375–380.
Tyler, C. & K. Simkiss, 1959 a. Studies on egg shells. XII. Some changes in the shell during incubation. J. Sci. Fd Agric. 10 : 611–615.
Tyler, C. & K. Simkiss, 1959 b. A study of the egg shells of ratite birds. Proc. zool. Soc. Lond. 133 : 201–243.
Tyler, C. & N. Standen, 1969. The artificial production of translucent streaks on egg shells and various factors influencing their development. Br. Poult. Sci. 10 : 359–369.

Tyler, C. and H. P. Thomas, 1966. A study of the snapping strength of egg shells and the effect of various factors on it. Br. Poult. Sci. 7 : 227–238.
Völker, O., 1940. Die Verbreitung von Protoporphyrin in Vogeleischalen. J. Orn., Lpz. 88 : 604–612.
Warren, D. C. & R. M. Conrad, 1942. Time of pigment deposition in brown shelled hen eggs and in turkey eggs. Poult. Sci. 21 : 515–520.
Warren, D. C. & H. M. Scott, 1935. The time factor in egg formation. Poult. Sci. 14 : 195–207.
Wittich, von, 1851. Ueber Pilzbildung im Hühnerei. Z. wiss. Zool. 3 : 213–219.
Wolken, J. J., 1951. Structure of hen's egg membranes. Anat. Rec. 111 : 79–84.
Young, J. D., 1950. The structure and some physical properties of the testudinian egg-shell. Proc. zool. Soc. Lond. 120 : 455–469.