THE INFLUENCE OF EGGSHELL WASHING ON BACTERIAL TRANSFER ACROSS THE SHELL WALL.

BY

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DECLARATION.

I declare that all the work in this thesis was undertaken by me.

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SUMMARY.

1. All three methods of washing action (brush, rotary and jet) investigated in this study caused damage to the cuticular surface of the egg shell.

2. The physical trauma to the egg caused by the washing procedure resulted in higher levels of protein in the post wash water. Not all of the protein was derived from the cuticular surface, some was also derived from the contents of broken eggs.

3. The pre wash water in both the brush and jet action machines contained bacteria, highlighting the difficulties associated with plant hygiene. The post water from all three washing machines contained a diverse population of bacteria, several of which were potential food pathogens.

4. The persistence of the bacterial population in the three washing systems, particularly the rotary and jet action machines underlined the inadequacies of the sanitiser regime. Eggs improperly rinsed displayed sanitiser residue on the shell surface. Chlorine from the sanitiser penetrated the thickness of the true shell.

5. In general terms, bacteria translocated across the shell wall of washed eggs more readily than the unwashed group. This trend was independent of the type of wash action although it did appear to be strain related.

6. As the bird aged, shell quality declined with a concomitant increase in bacterial transfer.

7. Infectious Bronchitis was verified during the course of this investigation and observed to have a profound effect on shell structure. This structural deterioration correlated with a rapid increase in bacterial penetration (56% in the unwashed eggs and 66% in the washed eggs). During the recovery phase bacterial penetration decreased.

INTRODUCTION.

1. EGG FORMATION.

The avian oviduct achieves an average length of 600mm. in it's active state (Gilbert 1979) and is divided into six spatially and temporaly distinct regions in which the forming egg spends different periods of time viz Infundibulum, (0.25-0.5hrs.); Magnum, (2-3hrs); Isthmus, (1.25hrs); Tubular Shell Gland and Shell Gland Pouch, (18-20hrs) and the Vagina, (0.25hrs).

Each region consists of six different layers viz: glandular epithelium; inner connective tissue layer; inner circular muscular layer; outer connective tissue layer; outer longitudinal muscular layer and a peritoneal covering (Hodges 1974; King 1975). The muscular layers lend support and firmness to the oviduct advancing the egg by peristalsis (Gilbert 1979) while the secretory cells identified as ciliated columnar, non ciliated goblet type and tubular glands (Romanoff & Romanoff 1949) are either active in the formation or passive in the transfer of all components of the egg with the exception of the yolk which is primarily of hepatic origin.

The ovum is released by surges of luteinising hormones and when it erupts at the avascular stigma the yolk mass surrounded by a pervitelline membrane enters the infundibulum. After the deposition of the multilayered albumen and paired fibres in the magum and isthmus respectively, the developing egg is ready for the process of shell formation.

This thesis sets out in the first instance to illustrate

diversity within the cuticular layer and to correlate diversity with inherent defects at the level of the mammillary layer which may be implicated in bacterial transfer. In this literature review detailed consideration is given to the distal portion of the oviduct. i.e. Tubular Shell Gland and Shell Gland Pouch.

Tubular Shell Gland.

The forming egg yolk, albumen and web of membrane fibres are at this point bathed in a supersaturated solution of calcium carbonate and on specially chemically modified areas of the membrane fibres calcium carbonate seeds to form the basal cap region from which the cone layer grows. The membranes act as a barrier to lateral crystal growth and subsequent mineralisation results in the formation of the palisade layer.

Shell Gland Pouch.

During the active phase of shell formation this area is bright red, the result of vascular engorgement. It has four functions: viz addition of plumping fluid; calcification; cuticle and pigment formation.

Addition of Plumping Fluid.

Approximately 15 gms of water is added to the albumen mass which has the effect of reducing the protein concentration of the latter from 20%-11% (Solomon 1979).

Calcification.

The true shell consists of 95% calcium carbonate and 5% organic material. The calcium for this process is derived from the diet, with some also being withdrawn from the special reserve

known as medullary bone which is laid down in the marrow cavities of the limb bones at the onset of the reproductive period. The breakdown of medullary bone results in a concomitant release of phosphate (Simkiss 1967). Solomon (1973) reported fluctuating tissue concentrations of acid phosphatase which correlates with the distribution of calcium in the active oviduct. Calcium and carbonate ions are assembled sandwich style. It has been postulated that there is an equilibrium between bound phosphates in the blood which do not cross the shell gland wall and free phosphates. These may substitute for carbonate ions in the calcite lattice and if present in excess can render the calcite lattice unstable. There appear to be two types of calcium reserve if dietary intake is insufficient. One is readily mobilised and Tullett et al. (1976) suggests that this, with it's high Ca:P ratio, is probably used at the beginning of shell formation. The other which is not so readily mobilised is used at the end and has a low Ca:P ratio. In the case of the domestic fowl increased phosphate levels are implicated in the termination of crystallisation.

Cuticle.

The organic cuticle, a protein/carbohydrate complex is the final secretory product of the oviduct and is intimately associated with the pigment.

Pigment Formation.

The pigment (ooporphyrin) is present in the shell as a polycrystalline complex which is not only deposited on the cuticular layer but occurs within the calcite matrix (Tamura & Fujii 1967). It has been identified as protoporphyrin (Solomon 1987).

The egg exits via the vagina which serves as a storage site for the spermatozoa which can remain there for 12-22 days before moving up the oviduct to the infundibulum where fertilisation takes place. Epithelial cells in the vagina secrete mucus which ensures the rapid expulsion of the egg via the cloaca. This region makes no contribution to shell formation.

Pores.

In order to perform it's function as an embryonic chamber, the shell must be sufficently porous to assist gaseous exchange yet resist excess water loss and microbial penetration. The aetiology of pore formation is still a matter of debate. Tyler & Simkiss (1959) propose that fluid transfer keeps the pore sites patent while Schmidt (1956) suggests that incomplete fusion of the calcium spherites on the mammillary layer results in spaces which correspond to the origins of the pores. In a later paper, Wyburn et al. (1973) put forward the theory that pore formation correlates with the secretory activity of the cells lining the distal oviduct. Ultrastructural analyses of the mammillary layer of the developing egg, which illustrate that the spatial arrangement of mammillary caps are dictated by available nucleation sites, tend to support the theory of Wyburn et al. (1973).

According to Tyler (1955) and Simkiss (1968) there are 7,000-17,000 pores per shell of which the greatest numbers are at the blunt end or the equator (Romanoff & Romanoff 1949) and are in a non random distribution tending towards uniformity away from aggregation although not remotely approaching perfect uniformity (Tyler 1969). Their diameters are described as ranging from 15μm-65μm at the mouth to 6μm-23μm at the inner aspect of the pore (Tyler 1956).

Cuticle.

The cuticle is the outermost covering of the eggshell, deposited just before oviposition. According to Wedral et al. (1974) it consists of 85%-87% Protein; 3.5%-4.4% Carbohydrate; 2.5%-3.5% Fat and 3.5% Ash. In terms of thickness it is variable: 5μm-10μm (Nathusius 1894); 3μm-5μm (Sajner 1955) and 10μm (Simkiss 1961). Schmidt (1962) hypothesised that this phenomenon reflected the variation in height of the underlying calcite columns. It serves a number of functions ranging from microbial defence to waterproofing (Williams & Whittemore 1967; Board & Halls 1973).

There is no such thing as the perfect egg (Solomon 1991). As stated by the former, structural diversity is to be anticipated in this dynamic biological system even under the most regulated conditions. Variations in husbandry and nutrition all exert an effect on egg shell quality and current methods of assessing quality, viz deformation and specific gravity are primarily useful as guides to variations in shell thickness and quality of internal contents. The literature is peppered with evidence to illustrate the effect of Housing (Mohumed 1986); Stocking Density (Watt 1989); Lighting (Roland et al. 1973); Temperature (Sauveur & Picard 1985); Humidity (Sauveur & Picard 1985); Age (Izat et al. 1985: Solomon 1991); Disease (Hanson 1968) and Diet (Gilbert & Wood-Gush 1971) on bird performance.

The interpretation of quality is highly subjective and variations in shell colour and yolk colour are essentially personal perferences. Shell thickness as currently assessed using specific

gravity is now recognised as an inadequate measure of quality in so far as on its own it gives no indication of the structural integrity of the product being measured. In recent years considerable evidence has been accumulated to illustrate the structural diversity within the egg shells of laying hens (Solomon 1991). Prime amongst these variations are those which occur in the mammillary layer and which Bain (1990) has correlated with increased/decreased resistance to crack growth. Solomon (1988) put forward the hypothesis that certain structural changes in the mammillary layer are indicative of external influences. These structural variants will be described in detail subsequently.

Many of the defects or variations initiated at the level of the mammillary layer reflect earlier changes in the quality of the egg white (Solomon 1983), the chemical composition of the paired membrane fibres (Watt 1989) and/or changes in the rate of mineralisation in the Shell Gland Pouch (Solomon 1991). Such variations during the early stages of mineralisation can have a knock on effect during the growth of the palisade columns.

Reid (1985) illustrated that shell formation was poisoned by the mercurial compound Panogen M. Both the organic and inorganic fractions of the shell were affected. i.e. shell structure was impaired and the cuticular layer was absent.

Under normal conditions many eggs are oviposited in a cuticleless state (Board & Halls 1973). According to Solomon (1991) the cuticle is rarely deposited as a thick and even covering over the surface of the egg. Indeed Diet, Age, Housing etc. have all been shown to influence the extent of this layer. Sparks (1985) illustrated the paucity of protection afforded by the cuticle as a

barrier immediately succeeding oviposition. He also demonstrated the maturation phenomenon of the latter as it dries.

2. SALMONELLA.

In recent times in this country most scientific effort has been directed towards Salmonella enteritidis phage type 4 as the causative agent in Salmonellosis. Whether this particular phage type can be cited exclusively on a worldwide basis is a matter of debate since there is a lack of standard phage typing (Hellig 1989).

These pathogenic bacteria are present mainly in the intestinal tract of animals and birds but are capable of being transferred via the food chain to humans. Salmonella food poisoning or Salmonellosis usually develops 12-48 hours after eating contaminated food and presents itself as abdominal pain, vomiting, diarrhoea and dehydration. The very young, the very old and patients already weakened by some other illness are particularly at risk. In a few cases the bacterium can spread from the gut to the bloodstream (bacteraemia) which may also lead to more serious complications such as kidney failure and meningitis. The debility may last from a few days or as long as a few weeks but it has been reported that half the patients who have had Salmonellosis may continue to excrete the bacteria for four to six weeks or longer and therefore are still capable of spreading the disease. Bacteraemia or "blood poisoning" can be fatal.

Salmonella enteritidis is a gram negative rod measuring 2-4 μm in length and 0.5μm in width. It possesses peritrichous flagellae, it is actively motile and is known to develop fimbriae.

The hen can become infected with Salmonella either by eating

contaminated food, drinking contaminated water or by inhalation. Strict health and sanitation programmes are followed to keep the flock disease free. The raw materials are sampled several times a week and the feed in addition to treatment with a good mould inhibitor is heat treated.

Evidence exists that Salmonella enteritidis is an invasive organism capable of penetrating the gut wall and infecting the hen's internal organs and it has been suggested that hens with infected ovaries can lay intact eggs whose contents are already infected with the bacteria and that the emergent strain phage type 4 (PT 4) may have an affinity for the genital tract. Baker et al. (1980) do not agree with this since they found no contamination of eggshell or contents (yolk and white) from hens that had been innoculated either orally or intravenously via the basalic vein of the wing, although they did find organisms in the faeces of orally infected hens.

The infected faeces is a source by which the egg itself can become infected (Forsythe et al. 1967). The moist recently oviposited egg is more susceptible to bacterial penetration (Sparks 1985). The route of infection can be via exposed patent pores from which the cuticular plug has been removed or damaged, if indeed it ever existed. Penetration is greatest at the blunt end of the egg (Walden et al. 1956; Vadehra et al. 1970). It was originally thought that the vulnerability of an egg to infection was associated with the presence of a highly porous shell (Walden et al. 1956) and a relationship between pore numbers and bacterial infection was suggested by Kraft et al. (1958). This was refuted by Board and Halls (1973) who stated that there was no correlation between shell porosity and water uptake and therefore by inference

bacterial infection. Sparks (1985) and Nascimento (1990) both agree with this finding. Once the initial barrier has been breached, the Salmonellae enter the shell and reach the shell membranes which behave like mechanical membranes rather than bacteriocidal barriers. Under suitable conditions of temperature and moisture the membranes can be breached by the Salmonellae and this can lead eventually to extensive bacterial multiplication in the highly nutritious yolk (Stokes et al. 1956). According to Lifshitz et al. (1964) the most important barrier to bacterial penetration is the inner shell membrane, then the shell itself, the least important being the outer membrane. Board and Fuller (1974) identified two forms of non-specific microbial defence systems. Physical defence comprising of the shell, shell membranes and the albumen sac and Chemical defence comprising of albumen plus possibly the shell membranes. Two distinct phases are mentioned in the course of infection. The first being confined to the shell membranes and is dependent on the storage temperature - a lower temperature giving a longer confinement time terminating when the yolk makes contact with the shell membrane. The second phase is when bacterial multiplication takes place (Board and Ayres 1965). Temperature and moisture influence penetration. Simmons et al. (1970) state that the greatest penetration is at humidity of 97% and temperatures above 15^0C and this has been corroborated by Moursy and Ahmed (1971) who found that eggs held at room temperature had a higher contamination than those held at refrigeration temperatures. It follows that eggs should be stored below 10^0C. to avoid the penetration and growth of Salmonella as this not only inhibits but eventually leads to their destruction (Stokes et al. 1956).

A higher incidence of bacteria in washed eggs as opposed to unwashed was reported by March (1969) - a view which was not shared by Williams and Dillard (1973) who stated that washing was an important factor in preventing Salmonella. However, if an egg is in a fluid at a lower temperature then this fluid is drawn into the egg (Haines and Moran 1940) and since the Salmonella organism can survive on the egg shell then it can possibly be drawn into the egg. Cantor and McFarlane (1948) suggested that this surface contamination could be the source of spoilage of egg products. This hypothesis was corroborated by Ager et al. (1967) who stated that the level of contamination in frozen, unpasturised eggs ranged from 25%-32% and by Garibaldi et al. (1969) who when examining samples from bulk tanks containing broken out eggs found the contamination to be 7% in the winter and 54% in the summer.

3. EGG WASHING.

It has been reported that the current restrictions imposed on the egg industry with regard to egg washing are "outdated" (Kuhl 1989). The author states that if eggs are properly washed on a continuous type washer, sanitised and dried then the consumer will be presented with a better quality, economically priced clean egg. The practice of washing has been in vogue for many years in the U.S.A. In that country it has progressed from single immersion washing through nozzle wash, to brushes, then brushes with water. It is important to note that in the U.S.A. all stages in egg handling from the bird to consumer involve "cooling".

The scientific community is divided in it's opinion as to the value of the process. Haines (1938) found that eggs washed under sanitary conditions were more susceptible to penetration of bacteria although the eggs did not show an increase in spoilage. Fromm (1960) found that washing increased the permeability of the shell to bacteria.

A report by the Egg Producers Council and the Council for Scientific Research of New South Wales concluded that rotting of eggs was almost certainly due to washing (Moats 1978).

Gillespie et al. (1950a) reported that the removal of the cuticle did not enhance wastage and conversely it's retention on unwashed eggs did not always prevent invasion. However Board (1975) advised those people responsible for the cleaning of eggs for consumption or hatching to isolate cuticleless shells since

these eggs are highly susceptible to infection by rot producing and/or pathogenic micro-organisms (Board and Fuller 1974).

Machines.

The nature of the washing apparatus may contribute to shell contamination (Gillespie et al. 1950b) introducing flora not commonly associated with the shell surface. Likewise stagnant water as opposed to constant flow will encourage bacterial ingress given the right temperature conditions (Haines and Moran 1940). When detergent is added to the water, cuticular changes occur (Simons and Wiertz 1966).

The cuticular plugs in the pores in the hen's egg are adapted to provide water resistance to the shell when it is exposed to hydrostatic pressures. In egg washing there must be an requirement to ensure that the force generated by the sprays of the machine is below the level at which the shell's resistance would be overcome. Water that contained iron increased the rate and extent of spoilage (Garibaldi and Bayne 1962). This was verified by Brant and Starr (1962) who found higher rates of spoilage when eggs were dipped in a bacterial suspension containing ferrous sulphate. Board et al. (1968) also observed that there was a lag in multiplication of organisms in contact with the shell membrane unless ferrous iron was present in the innoculum.

Sanitisers.

There are conflicting views on the various sanitisers. Their effect on the micro flora of the shell is dependent on the type of organism present, plus the dilution and temperature at which they are used. Moats(1978) stated that eggs washed with some type of sanitising chemical in the washwater invariably kept better than

eggs washed in water alone, however after eggs have been contaminated with bacteria, washing in sanitisers or using post washing sanitising rinses will not redress the balance.

At present in this country, egg washing is not mandatory, therefore there is no standardisation with respect to the nature of the washing machine procedures. Even in the most stable environment, shell quality declines with age and if during the laying year birds are exposed to "stress" factors then altered shell structure will reflect the attendant physiological disturbance. In the light of this knowledge it is therefore questionable whether one can justify imposing further mechanical stress on this fragile product.

This thesis also describes the results of a study designed to assess the effects of washing on the cuticular surface of the shell. Three commercially available systems were tested and the eggs thus treated were then exposed to *Salmonella enteritidis* to ascertain whether washing encouraged bacterial penetration. The process of shell formation must be seen as a continuum, thus changes in the cuticular surface are frequently associated with intra shell deficiencies. The work also discusses the implications of the "true" shell as an effective barrier to bacterial ingress.

MATERIALS AND METHODS.

1. EGGS.

The eggs used in the trials were produced by two strains of battery reared brown egg layers designated Strain A and B. Both strains were fed on a commercial layers diet. Strain A eggs were collected from the birds at the beginning (28 weeks), middle (48 weeks), peak shell quality, and end (65 weeks) of lay, whereas Strain B eggs were collected at 28, 48 and 60 weeks. (The re-scheduling was the result of a policy change at the farm from which the eggs were collected).

A total of 450 randomly selected eggs were used; 150 per treatment; 50 at each period of lay. Within each group of 50, 25 were washed. The passage of Salmonella enteritidis was monitored in both washed and control groups.

2. WASHING APPARATUS AND SANITISERS.

2-1 BRUSH ACTION MACHINE.

The eggs (Strain A.) are brought via a conveyor belt direct from the housing system to the washing machine. The machine itself is enclosed and the water plus 2% sanitiser (TEGO-diocto S) is constantly circulating at a temperature of 45°C. As the eggs pass through the machine on a conveyor belt of rollers they are cleaned by the lateral movement of plastic brushes. The eggs are subsequently blown dried by an air jet before being transported on a moving belt to the adjoining room to be graded and packed.

2-2 ROTARY ACTION MACHINE.

The rotary action washer consists of an oscillating base on which sits a galvanised bucket fitted with a preset thermostatically controlled (40°C) immersion heater. The bucket

is filled with water up to the suage ring, the immersion heater switched on and when the wash temperature has been reached, indicated by the automatic switching off of the control light the sanitiser (three level measures of Nusan) is added. The polythene coated basket is filled with eggs (Strain B) with the dirtiest placed peripherally since, in this position they are subjected to the greatest movement. When clean, the eggs plus bucket are removed and placed to dry in a good current of air.

2-3 JET ACTION MACHINE.

The principle of action is similar to the brush action machine. It is an enclosed system with circulating sanitiser at a controlled temperature. The eggs (Strain B) are sprayed with the sanitiser by means of spray nozzles as they pass through the machine, then blown dried with warm air. Information concerning the nature of the sanitiser was not provided.

3 WATER QUALITY.

3-1 PROTEIN ANALYSIS.

A sample of the water and sanitiser was taken from the washing machines before and after the washing process and analysed for protein using the Coomassie Brilliant Blue C250 method (Sedmak & Grossberg 1977). The readings were taken on a spectrometer using absorption peaks 620μm and 465μm. The ratio of 620:465 was calculated and plotted versus protein concentration.

3-2 BACTERIOLOGICAL ANALYSIS.

A sample of the water and sanitiser was taken from all three washing machines before and after the washing process and analysed for bacteria. The sample was spun down in a M.S.E. micro centaur at low speed for 2 minutes, the supernatant was pipetted off and discarded. The pellet was resuspended in a small amount of sterile normal saline, plated out on a 90mm. petri dish of McConkey's agar and incubated at 37°C for 24 hours. Resulting colony forming units were identified by the API-20 E system.

4 VISUAL EXAMINATION.

4-1 CUTICULAR STAINING.

Edicol Supra Pea Green H described by Board and Halls (1973) is no longer commercially available and so a solution of Green S (2.8gms/litre) and Tartrazine (7.2gms/litre) was used. The eggs were dipped into the solution for 1 minute, washed in distilled water, air dried and examined.

5 SCANNING MICROSCOPY.

5-1 CUTICLE

A piece of shell 1.5cm^2 was carefully cut with a circular dental drill from the blunt end of the egg. The contents were discarded and the inside of the shell rinsed several times with distilled water to remove any albumen adhering to the inner membrane. Pieces of shell ca 1.5cm^2 were cut from the equatorial region of the shell and attached, cuticular side up, to aluminium stubs using silver paint. The samples were gold palladium coated in a Emscope Sputter Coater and examined using a Philips 501B scanning electron microscope at an accelerating voltage of 15kv., spot size 1000-200, depending on magnification, and a working distance of 13mm.

5-2 MAMMILLARY LAYER.

Pieces of eggshell were prepared as previously described. The inner membrane was manually removed before the pieces of shell were plasma etched in a Nanotech Plasmaprep 100 (5-2a) to remove the outer membrane and so expose the mammillary layer. The residue dust was blown away with a Kenair Clean Air Duster before the pieces of shell were mounted on aluminium stubs and coated as previously described (5-1).

5-2a PLASMA ETCHING.

Plasma etching is a non destructive technique for removing the outer membrane of the shell (Reid 1983). Pieces of shell were prepared, as described under (5-1), the inner membranes manually removed before the shell was placed membrane side uppermost in the chamber of the Nanotech Plasmaprep 100 unit. The pressure in the chamber was reduced to approximately 13.3 Pascals and oxygen gas was leaked in at 10cc/minute until the pressure stabilised at 133.3 Pascals. A radio frequency power of 100 ohms was applied and balanced by using the RF controls until a situation was reached whereby there was a maximum forward power reading for the minimum reflected power reading. This effects ionisation of the gas to form the reactive plasma. At optimium working conditions, a pale lilac colour plasma is visible within the chamber. After four hours the organic component of any remaining membrane fibres was removed by volatilisation, leaving the crystalline shell completely intact.

5-2b ENERGY DISPERSIVE X-RAY ANALYSIS.

Analysis of trace elements within the shell was achieved using the E.D.A.X. analyser attached to the scanning electron microscope. Spectra were recorded photographically.

5-2c INFRA-RED ANALYSIS.

This technique was used to identify the nature of aberrant crystal forms detected during scanning sessions. Small pieces of shell were ground up with potassium bromide and then compressed into a 7mm. disc. The discs were examined in a Perkin-Elmer 580 IR Spectrophotometer to determine whether the calcium carbonate in the shell was in the form of calcite, aragonite or vaterite.

6 SALMONELLA enteritidis.

PREPARATION AND IDENTIFICATION.

6-1 THE INNOCULUM

Salmonella enteritidis phage type 4 was isolated from infected chickens, freeze dried and a working culture kept in sloppy agar at 4^0C. The phage type was identified by the National Collection of Type Cultures (N.C.T.C.). The colonies formed were checked morphologically by means of Gram Jensen stain and biochemically using the AP1-20 E system to ensure that they were the same as those innoculated.

To make the innoculum, a wire loopful of the sloppy agar was placed into a bottle containing 25ml. of Nutrient Broth no.2 which was then incubated in an Gallenkamp Orbital Shaker at 100 revolutions per minute at 37^0C for 24 hours. 1ml. of this was then added to 9ml. of 0.8% sterile saline to form a 10^{-1} dilution of the Salmonella enteritidis. From this, serial dilutions were made until a final dilution of 10^{-8} was achieved. This concentration was found to be ca. 10×10^{-2} colony forming units (C.F.U.) per ml. This was determined by spreading a 90mm. diameter petri plate of brilliant green agar, which is specific for Salmonella, with 0.1ml. of the final dilution, incubating at 37^0C for 24 hours and counting the colony forming units by means of a Gallenkamp Colony Counter.

6-2 GRAM JENSEN STAIN.

A smear of Salmonella enteritidis was made on a clean glass slide, air dried and flame fixed prior to staining with crystal violet. The stained smear was then mordanted with Gram's iodine before rinsing with acetone and finally staining with carbol

fuchsin. The smear was examined with a light microscope using oil immersion.

6-3 FLUORESCENT ANTIBODY STAIN.

A smear of Salmonella enteritidis was made on a clean glass air dried slide then fixed in acetone. Rabbit antisera was placed on top of the smear (30mins) which was washed thoroughly in phosphorus buffered saline (P.B.S.) before goat anti rabbit Fitc was added. (30mins) The slide was washed, mounted in P.B.S. and examined under ultraviolet light using a Leitz microscope.

6-4 NEGATIVE STAINING.

Salmonella enteritidis was prepared in the broth as previously described (6-1). 2ml. of this broth was spun down in a M.S.E. micro centaur at low speed for 2 minutes and the supernatant discarded The bacteria were gently resuspended in sterile water and the above process repeated. The bacteria were negatively stained in a fume cupboard as follows.

A 200mμ mesh copper grid coated with parlodium (see 6-4b) was held by means of forceps under an upturned transparent polystyrene weighing bottle which had an opening cut in its side. This constituted a makeshift fume cupboard which was then placed in a 90mm petri dish containing a filter paper soaked in 40% formaldehyde solution to fix the bacteria. By means of a micro pipette a drop of bacteria was carefully placed onto the grid and left for 2 minutes before the excess fluid was removed by touching the edge of the grid with blotting paper. The above process was repeated with the negative stain then the grid was immediately examined under a Jeol 100 CX2 Transmission Microscope at an accelerating voltage of 80 Kv.

6-4a NEGATIVE STAIN.

Freshly prepared aqueous solution of 2% phosphotungstic acid adjusted to pH 7.2 with 10M KOH.

6-4b TO COAT GRID WITH PARLODIUM.

The stock solution is 3% parlodium in amyl acetate. A clean slide was coated with 0.6% of the parlodium solution, dried, then the film was cut round the edge with a scalpel blade and floated onto water. The copper grids were carefully placed dull side down, onto the film, a piece of absorbent paper was placed over them, then lifted taking the now coated grids with it. Once dried, the grids were ready for use.

6-5 TRANSMISSION MICROSCOPY.

Salmonella enteritidis grown in nutrient broth was mixed with an equal amount of Karnovsky's fixative, spun down in a J2-21 ultra centrifuge using rotar type JA 21, 10K r.p.m. for 15 minutes. The supernatant was discarded, the pellet resuspended in Karnovsky's fixative, respun and the resultant pellet was carefully removed, post fixed in 1% osmium tetroxide, dehydrated through a graded series of acetones and transferred to propylene oxide before being embedded in emix. Once cured, the blocks were trimmed on a L.K.B. pyramitome and, using a glass knive, cut at 200°A at speed 2 on a L.K.B. ultratome 2. The sections were stained with Reynold's Uranyl acetate/Lead citrate and examined in a Jeol 100 CX 2 Transmission Microscope at an accelerating voltage of 80 Kv.

7 SALMONELLA enteritidis.

MONITORING MOVEMENT THROUGH THE SHELL

7-1 PREPARATION.

Pieces of shell were cut, stripped of inner membranes, plasma etched and the residue dust blown away as previously described (5-2a). The shells were individually placed cuticle side up on the surface of 0.8% Brilliant Green Agar[1] in a 50mm. diameter petri dish taking care to eliminate any air trapped beneath the shell. The plates plus shells were placed in a 28°C incubator for 15 minutes to dry the cuticular surface before adding a ring of Silicone High Vacuum Grease[2] by mean of an Elastomer syringe with an attached tip. 0.01ml. of 10^{-7} Salmonella broth containing ca $10x10^{-1}$ was placed on the surface of the shell inside the grease ring and left at room temperature for 20 minutes to allow the bacteria to penetrate the shell after which the shells were carefully removed from the agar and autoclaved for 30 minutes at 121°C and 15lbs. pressure in a Sterilin National Autoclave to kill off any Salmonella enteritidis still present on the shell. The plates were incubated at 37°C for 24 hours after which time the number of colony forming units that had penetrated the shell and grown on the agar were counted. Control plates with bacteria free shells were similarly treated.

[1] Modified from 1.2% to make the agar soft enough to manipulate the shell but still to be firm enough to support forming colonies.

[2] This was to contain the Salmonella Broth on the shell and also did not melt in the high temperature which is subsequently used to kill off the bacteria.

7-1a ANALYSIS OF DATA.

The mean bacterial penetration values were calculated for both washed and unwashed (controls) eggs associated with each machine according to the point of lay. These data were tested by using analysis of variance (Anova) to determine if

a) There was a difference in bacterial penetration between unwashed and the washed eggs.

b) If the incidence of bacterial penetration varied according to the age of the flock irrespective of whether the eggs were washed or unwashed.

c) If this was dependent on the type of washer used.

7-2 SCANNING MICROSCOPY.

In order to establish if a relationship exists between bacterial movement and shell ultrastructure 50 pieces of shell from the controls, 25 Strain A, 25 Strain B from (7-1) were further tested as follows. The area of shell inside the grease ring was cut out by means of a dental drill then plasma etched to remove any agar adhering to the mammillary side. The sample was then affixed, cuticular side up, to an aluminium stub, by means of silver paint on the corners of the shell, coated and examined as described previously (5-1). Following cuticular assessment, (see result 4-1) the shell was detached from the stubs and mounted mammillary layer uppermost. Analyses of structural variations therein were made according to methods developed by Bain (1990) and Solomon (1991).

7-2a ANALYSIS OF DATA

The incidence of structural variation within these shells was noted then expressed in the terms of the mean score for each structural variant. Likewise the degree of cuticular coverage was expressed in terms of mean score according to point of lay. These data were subsequently analysed using appropriate statistical tests. (T tests and Regression Analysis) to determine if

a) If different structural variations were influenced by age.

b) If there was a structural variation between Strain A and Strain B.

Also if bacterial penetration was related to

c) Structural variation.

RESULTS.

As stated in the introduction, this thesis set out in part to consider whether egg washing causes shell damage and so aids the translocation of bacteria. The work has also served to underline the diversity of structure which exists within the the egg shell at all levels and since these structural variants are crucial to the process of transfer they are presented in the first instance as a baseline.

1. CUTICULAR DIVERSITY.

Figure1 illustrates a normal cuticle and so with reference to this image, the following micrographs are presented to illustrate the structural diversity which existed within this layer.

1-1 ACCRETIONS.

These are defined as heavy calcareous deposits on the surface of the egg which give it a pimpled appearance. In many instances, these deposits penetrate the entire depth of the shell (Figures 2, 3). At ultrastructural level accretions can adopt a variety of forms, one of which is illustrated in figure 4. At the level of the mammillary layer, type B bodies (2-5) dominate and the shell is distinctly eroded (Figure 5).

1-2 TOE HOLE.

Toe hole damage disrupts the underlying layers, thus rendering the egg contents vulnerable to bacterial challenge (Figures 6, 7). It can be distinguished from pinholes of oviducal origin by the accumulation of shell debris within the hole.

1-3 CALCIUM SPLASHING.

Figures 8-11 show the cuticle with different degrees of extra calcium deposits thereon.

1-4 CUTICLELESS EGG.

Figures 12-15 illustrates the diversity of structure within the palisade layer.

1-5 MEMBRANOUS STRUCTURES.

These membranous-like structures were found lying on the surface of the cuticle (Figure 16) which appears to be completely disrupted (Figure 17).

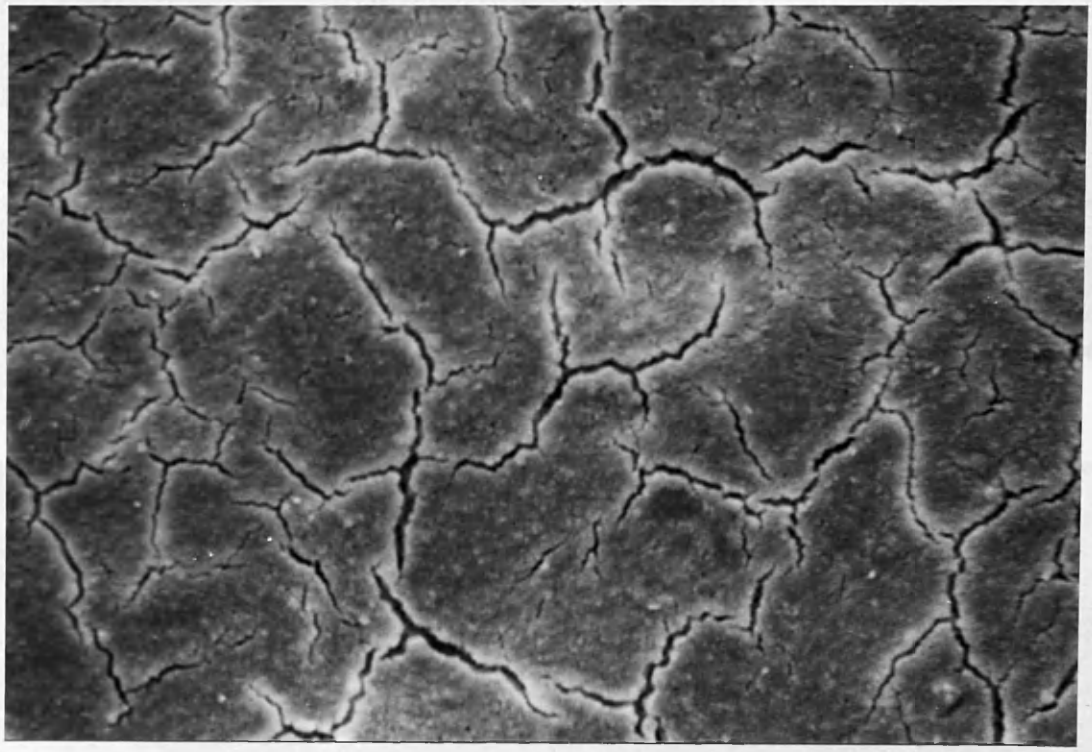


Figure 1. Normal cuticle. Note fissured appearance x 720

Figure 2. Large accretions on pole of eggshell.

Figure 3. Concavity beneath an accretion on the mammillary side of the shell.

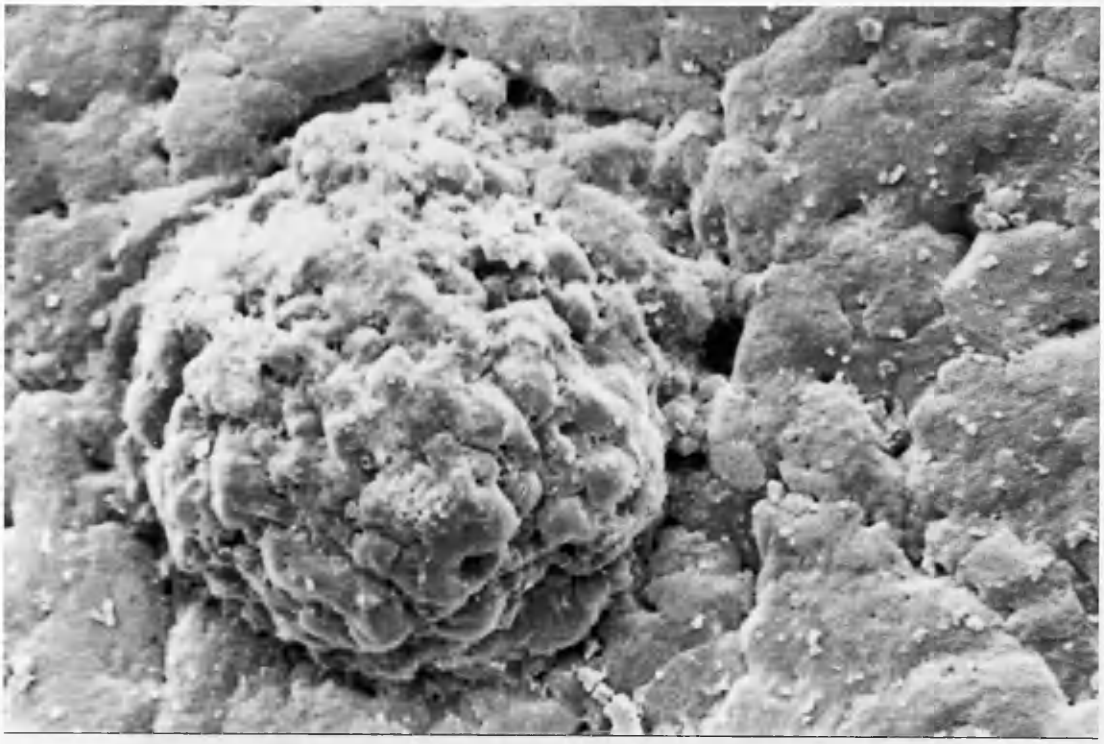


Figure 4. Accretion on cuticular surface x 360

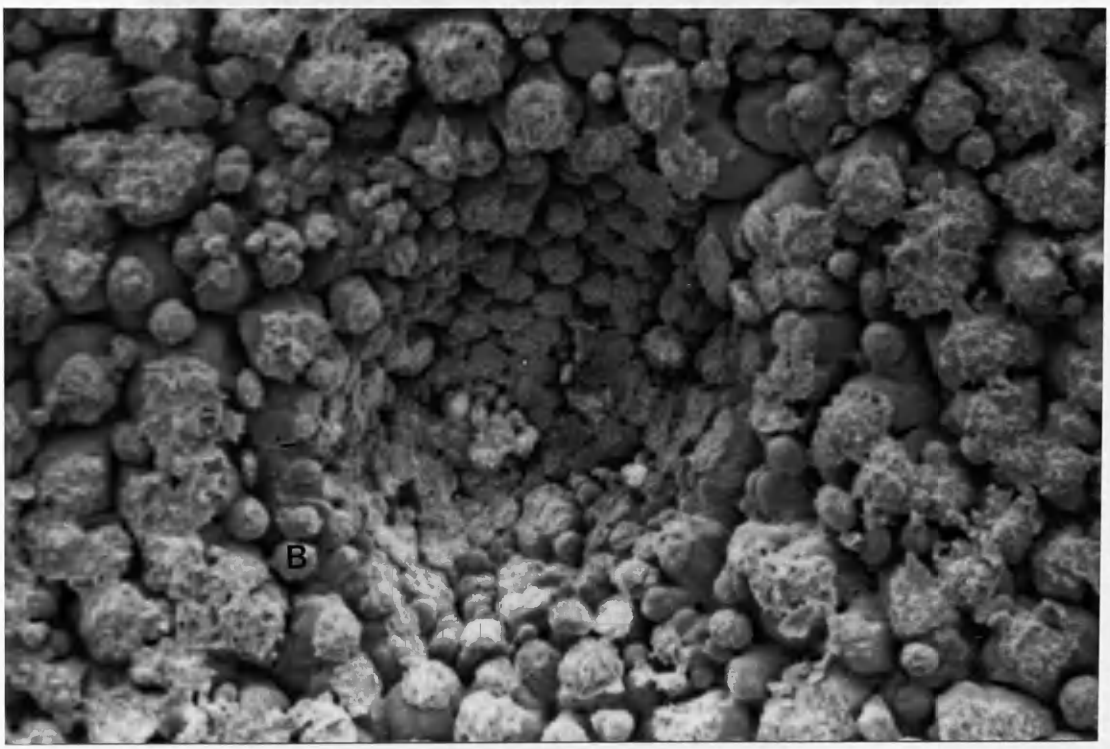


Figure 5. Mammillary layer directly below the accretion. Note the hollow appearance and the large numbers of Type B bodies (B) x 180

Figure 6. Toe hole damage.

Figure 7. Toe hole damage. The hole is filled with cuticular debris x 90

Figure 8. Calcium splash (arrow) x 360

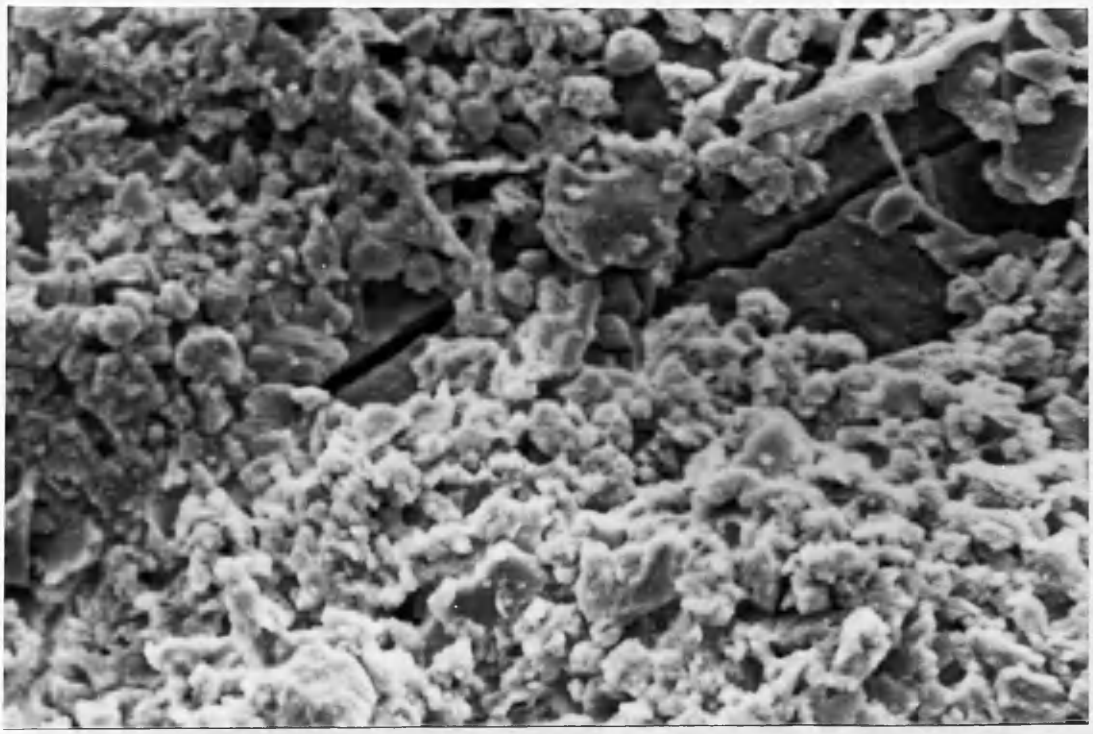


Figure 9. Calcium deposits covering the fissured cuticle x 1,440

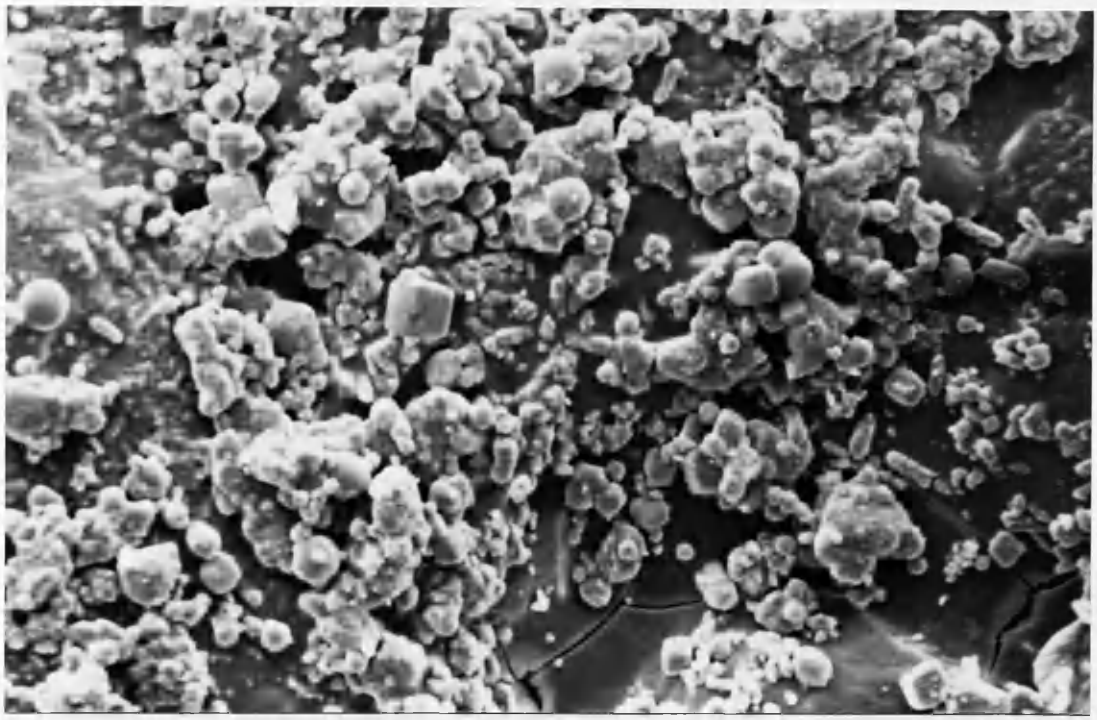


Figure 10. Calcium deposits x 720

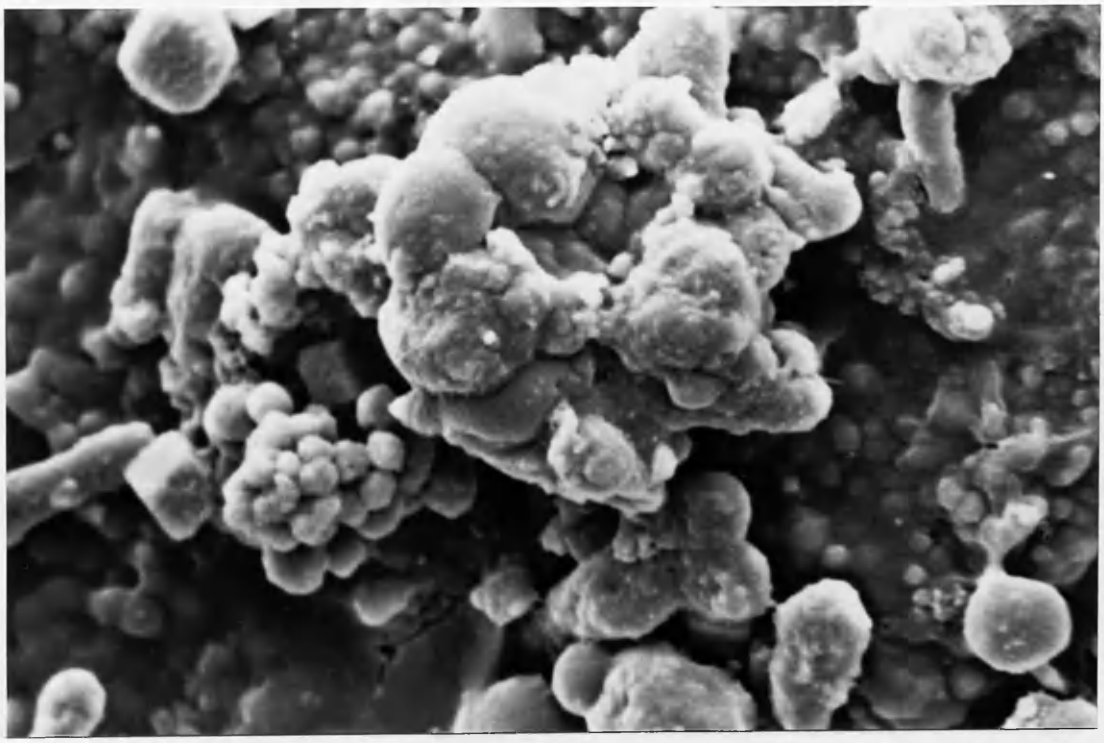


Figure 11. At higher magnification the diverse crystalline forms of calcium deposits are more distinctive x 2,800

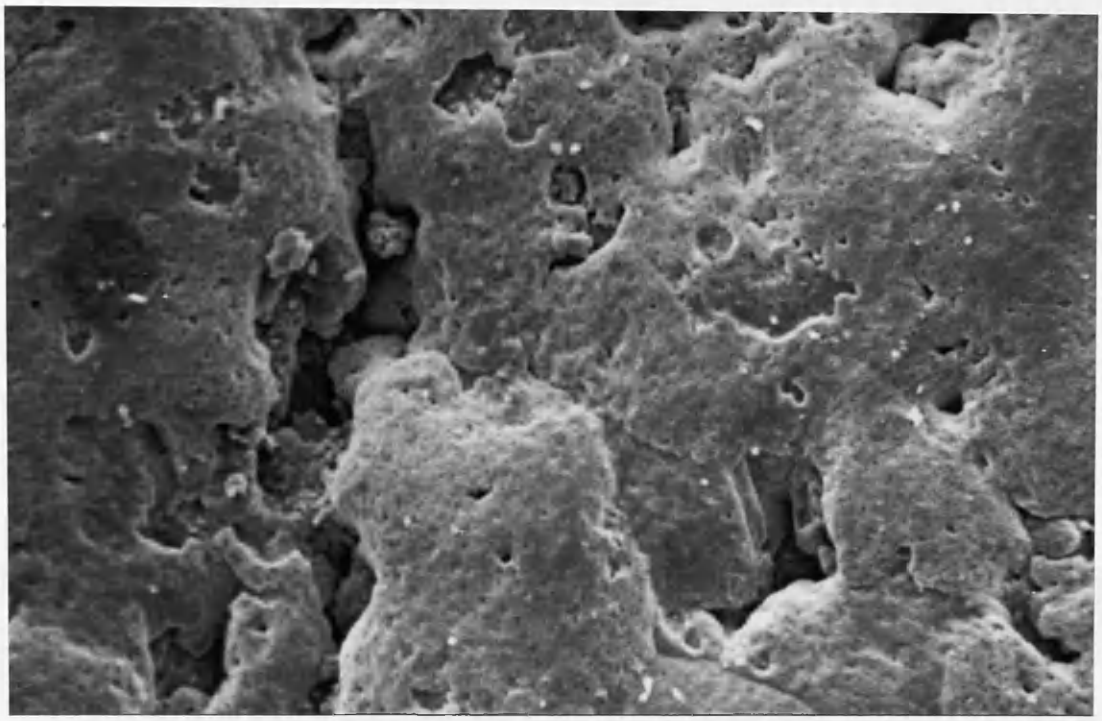


Figure 12. Cuticleless egg. The porous palisade columns are exposed x 720

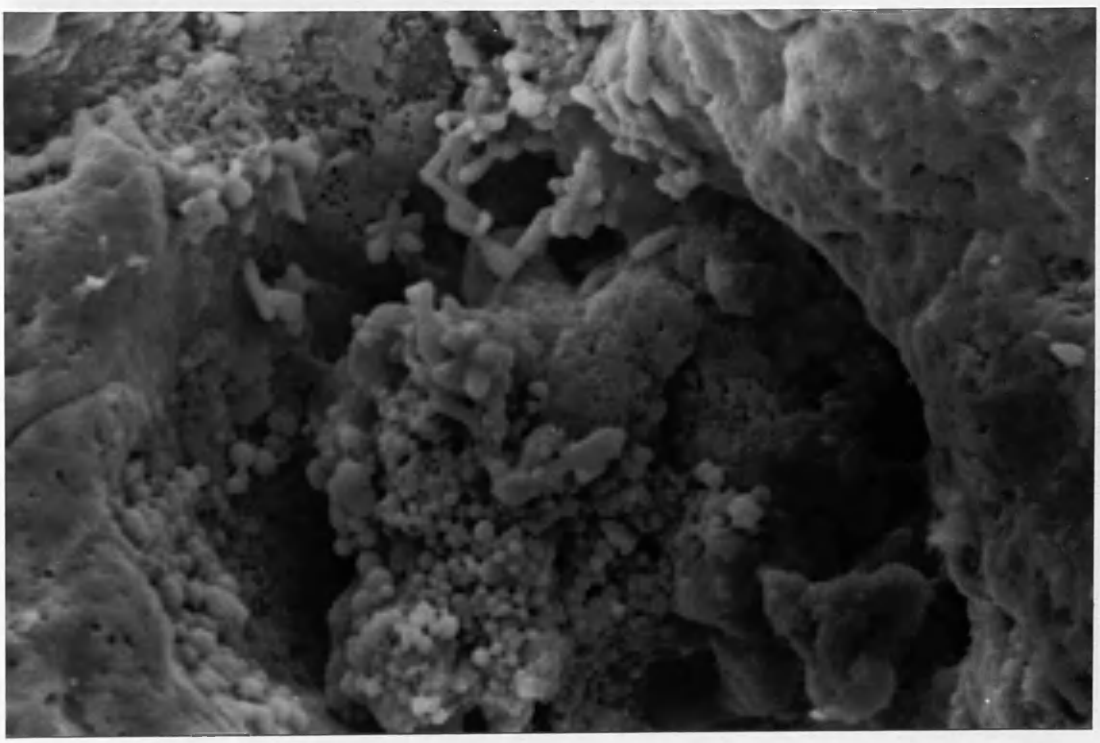


Figure 13. Cuticleless egg. A pore site is filled with inorganic material x 2,800

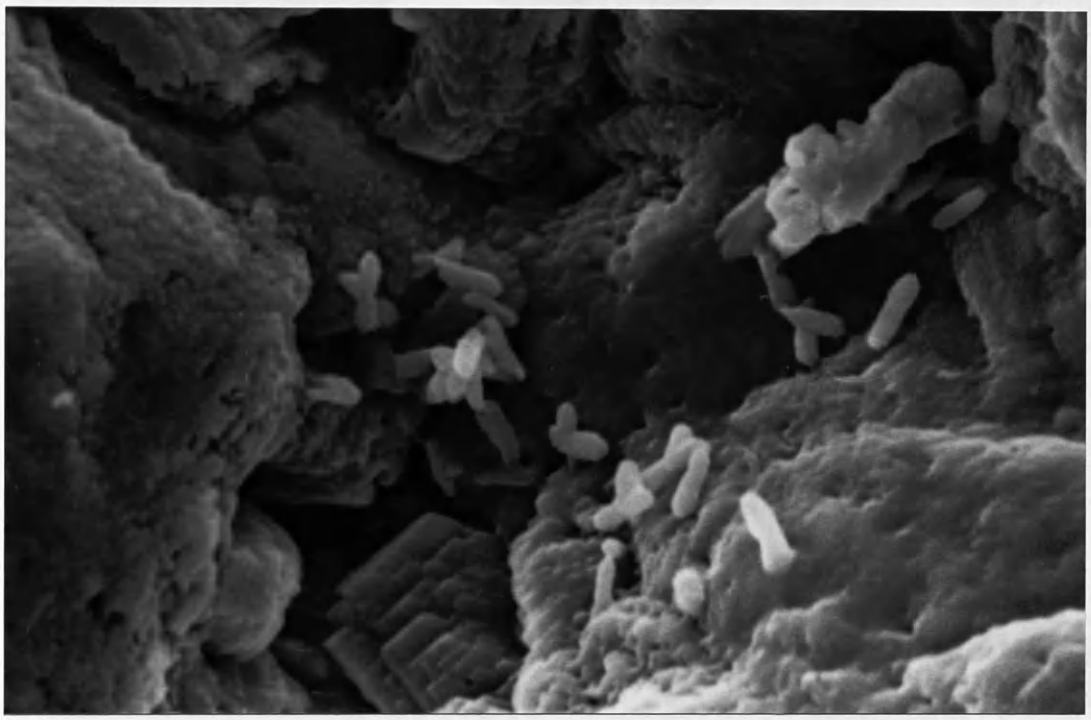


Figure 14. Cuticleless egg. The elongated forms have the dimensions of bacteria x 5,600

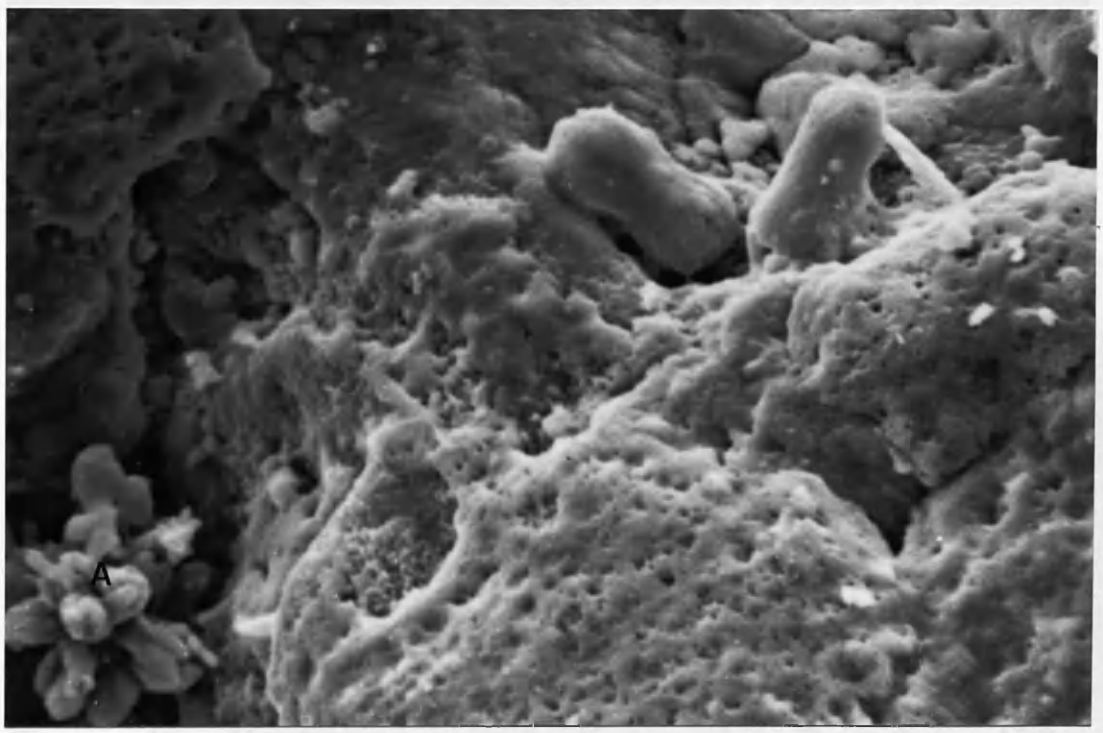


Figure 15. Cuticleless egg. At high magnification the layered appearance of the elongated forms confirms their inorganic composition. Note the presence of aragonite clusters (A) x 2,800

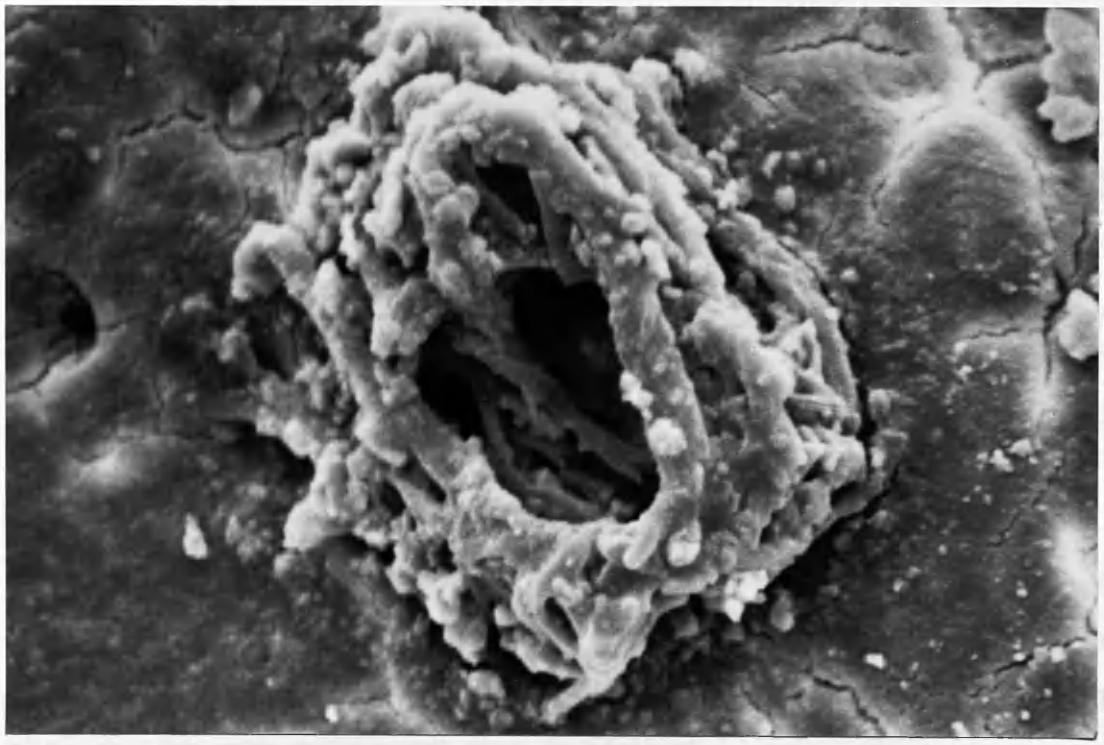


Figure 16. Membranous mass on cuticular surface x 2,800

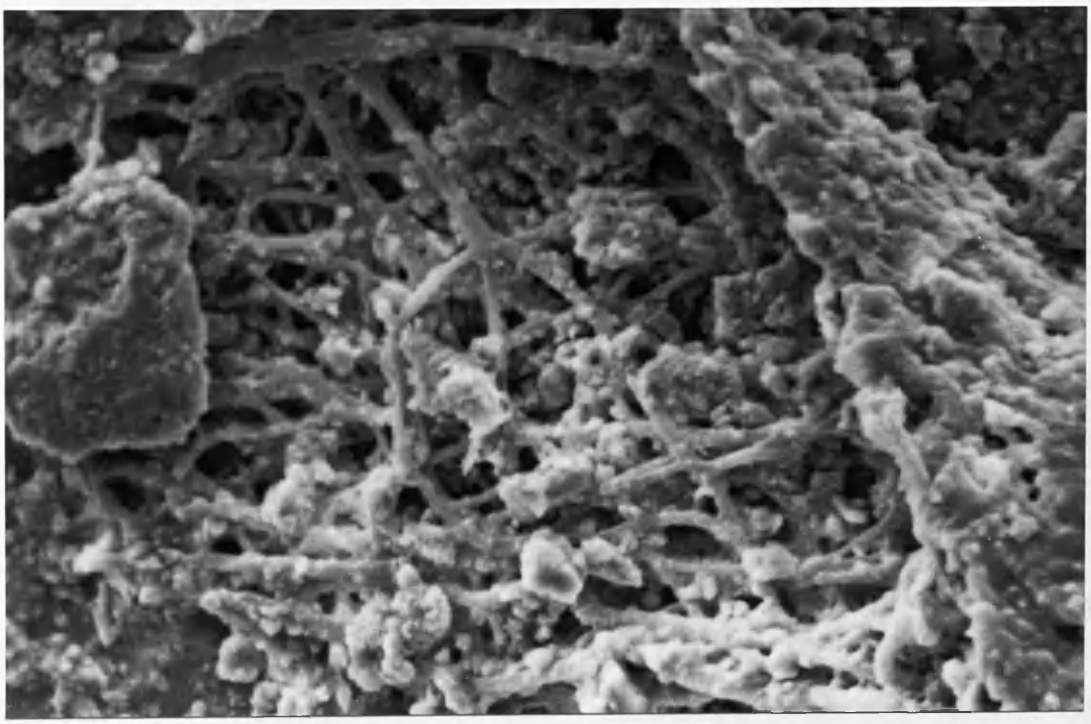


Figure 17. Membranous mass on cuticular surface. These fibres which are similar in size to the fibres of the soft shell membranes may reflect the transfer of debris from the isthmus subsequent to the process of mineralisation x 1,440

2 MAMMILLARY VARIATION.

Thirteen mammillary variations have been described by Reid (1985); Watt (1985); Bain (1990); Nascimento (1990) and Solomon (1991). These are as follows Mammillary Density, Confluence, Caps, Early Fusion, Late Fusion, Mammillary Organisation, Type B bodies, Pitting, Aragonites, Type A's, Cubics, Cuffing and Changed Membrane.

The following micrographs as in those of the cuticular examination are all taken from the eggs studied in these experiments. Only those structural variations judged to threaten shell quality are discussed and illustrated.

Figure 18 shows a normal mammillary layer. Note the strong attachment of the outer membrane fibres to the basal cap which gives a good foundation for the build up of the palisade layer. Figure 19 shows the mammillary layer with the outer and the inner membranes attached.

2-1 MAMMILLARY NUMBERS.

Inter and intra shell variations in numbers of mammillae per unit area are the norm (Figures 20, 21).

2-2 MAMMILLARY ORGANISATION.

Figure 22 demonstrates mammillary alignment, and figure 23 illustrates a crack line following the path of alignment.

2-3 CAPS.

Figures 24-26 are examples of poor contact between the membrane fibres and the initial calcium carbonate crystals.

2-4 EARLY AND LATE FUSION.

Figures 27, 28 show early and late fusion of the palisade columns.

2-5 TYPE B BODIES.

Figure 29. These rounded bodies invariably grow from the side of adjacent cone layers. They do not contribute to the formation of the palisade layer and have been implicated as a causative factor in shell thinning.

2-6 PITTING.

Figures 30-32 illustrate depression and erosion. Pitting is variously catagorised according to the depth of the fault.

2-7 ARAGONITE.

Figures 33-37 demonstrate the different morphological forms of aragonite found in the eggs examined. Figure 38 shows aragonite on the basal cap.

2-8 TYPE A's.

Figure 39 This mammillary body has no membrane fibre attachment area although it does support a cone area and palisade column.

2-9 CUBIC.

Figure 40 illustrates several cubic crystals along with aragonite in the inter-mammillary spaces.

2-10 CUFFING.

Figures 41, 42 demonstrate the extra calcium cuff which, because it fills in the inter mammillary space, enhances the strength of the shell.

2-11 CHANGED MEMBRANE.

Figures 43, 44 show the remaining sulphur rich strands which can persist even after plasma etching.

2-12 COMBINATION OF FAULTS.

Figures 45-49 demonstrate that structural variations frequently occur in combination.

3 TRANSVERSE SECTIONS.

Figures 50-56 illustrate transverse sections through the eggshell.

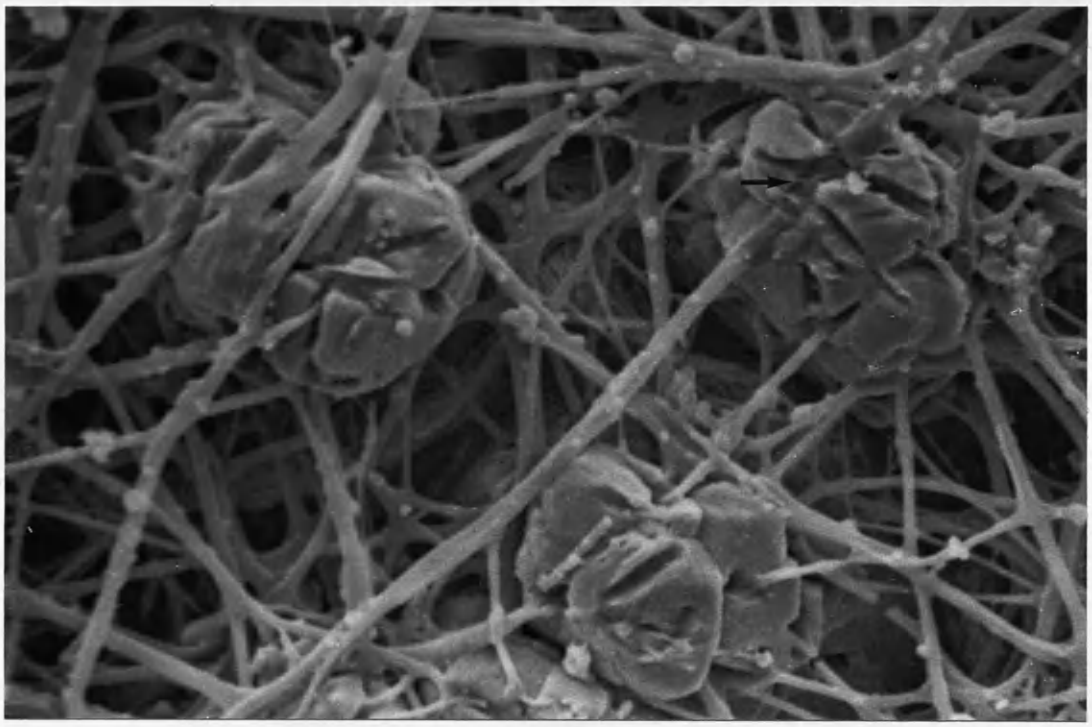


Figure 18. Outer membranes firmly attached to basal caps (arrow) x 1,440

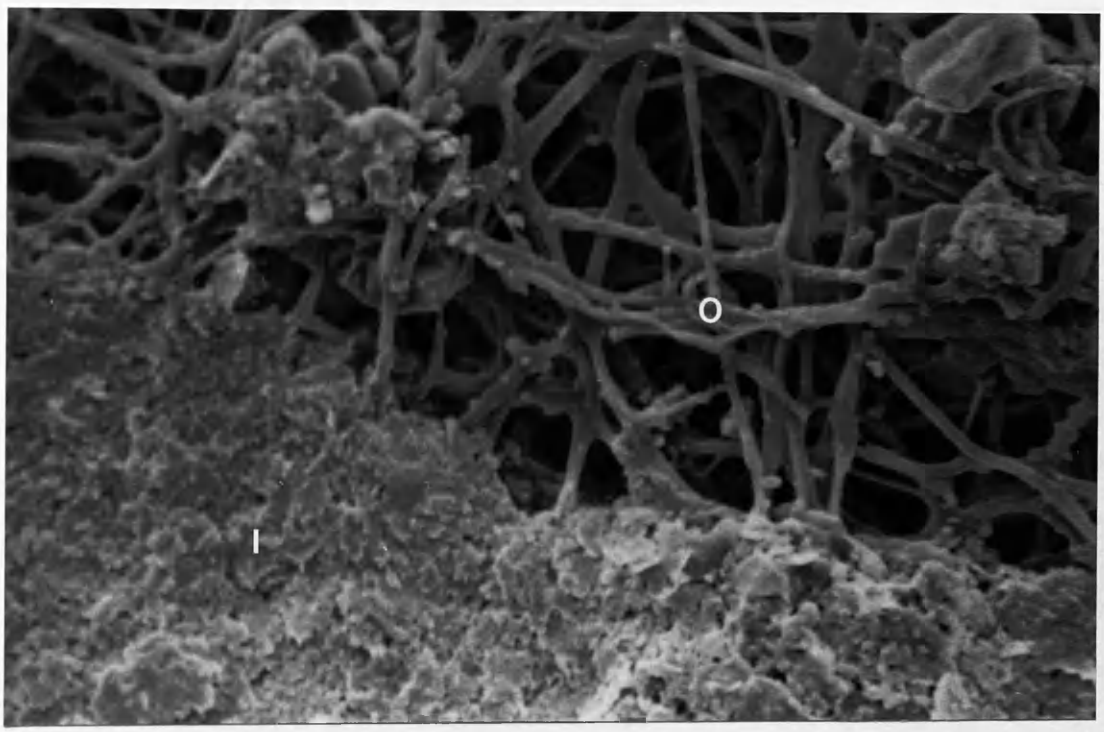


Figure 19. Inner surface of inner shell membrane (I) and outer shell membrane fibres (O) x 1,440

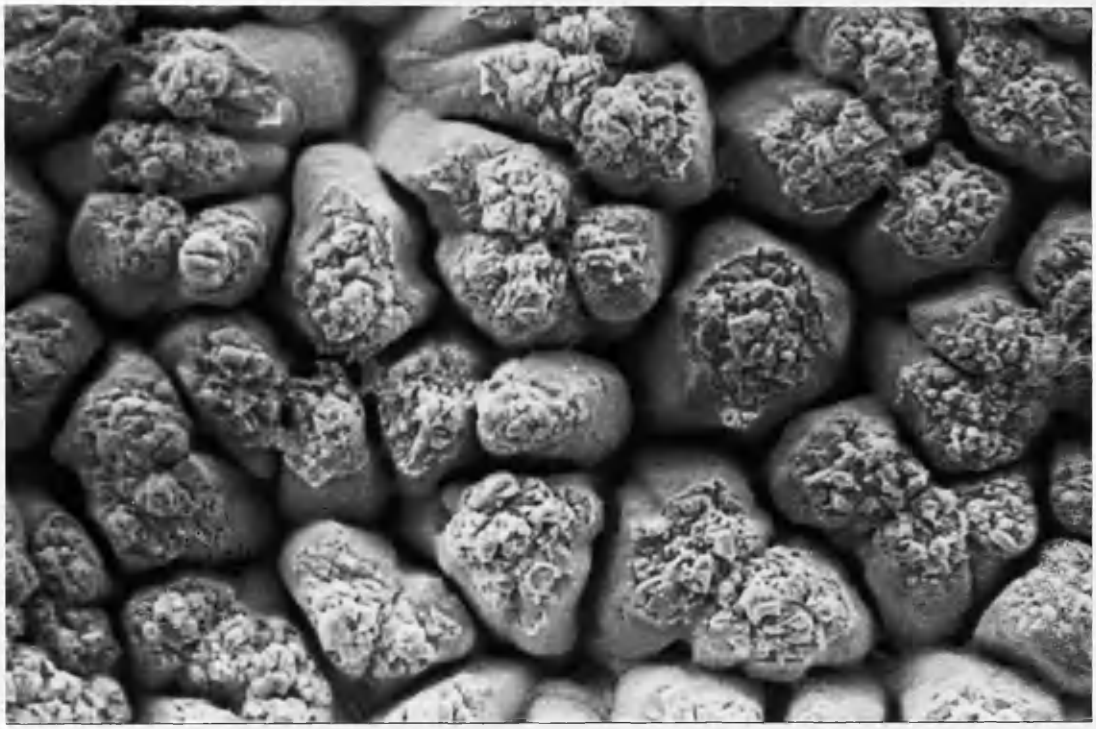


Figure 20. Low mammillary count. (44 per unit area of magnification) x 360

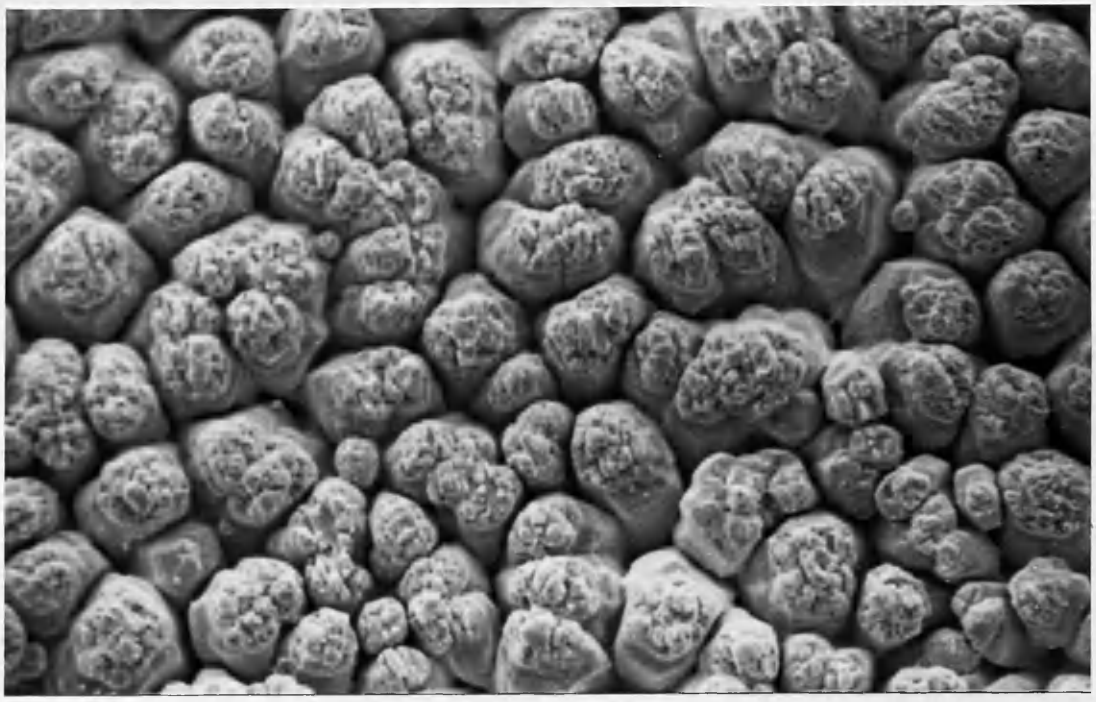


Figure 21. High mammillary count. (> 94 per unit area of magnification) x 360

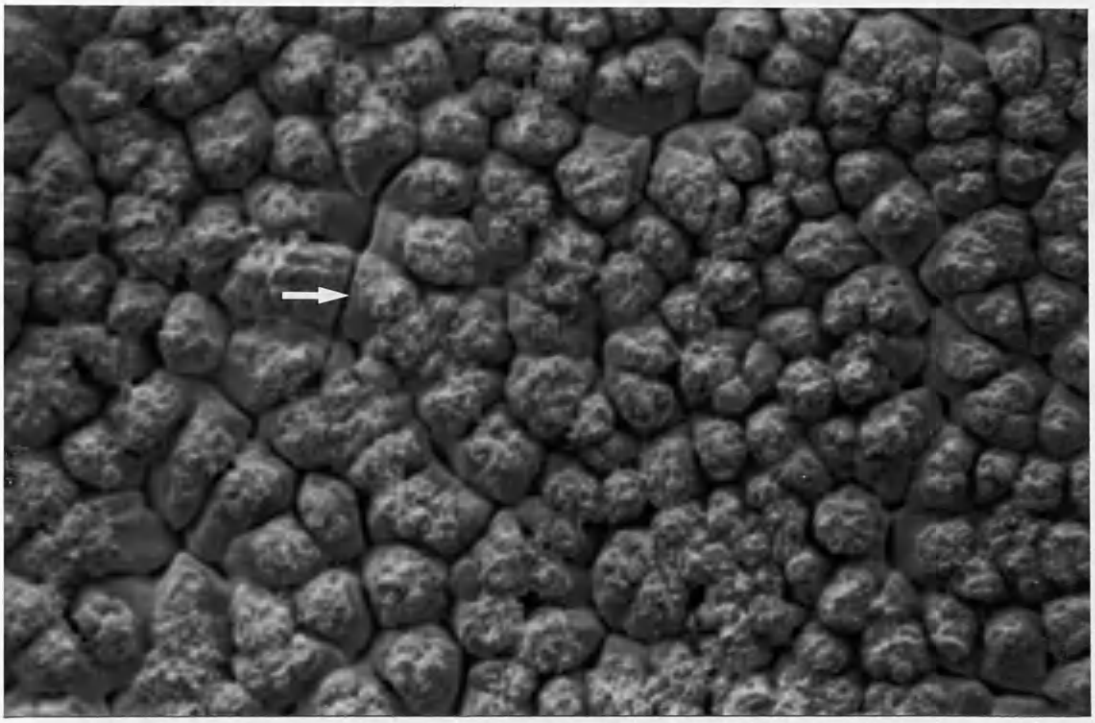


Figure 22. Mammillary alignment (arrow) x 180

Figure 23. Crackline following the path of alignment (arrow) x 180

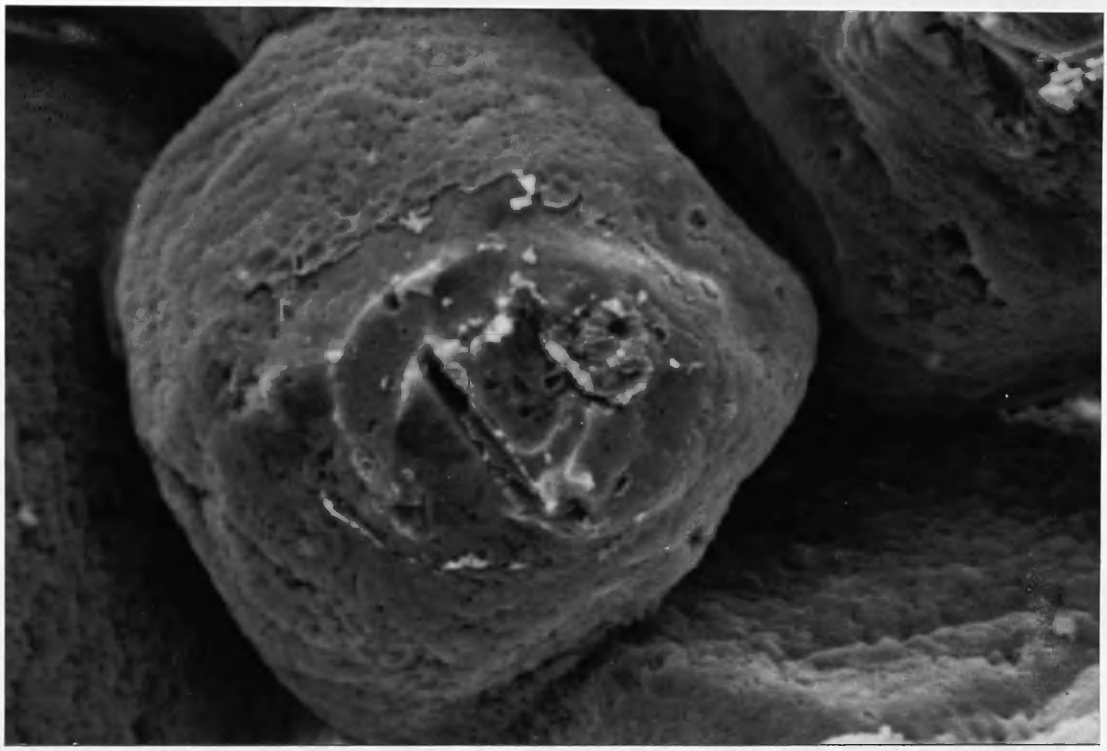


Figure 24. Poor cap. The cap area displays poor attachment with the membrane fibres. (now removed by plasma etching) x 2,800

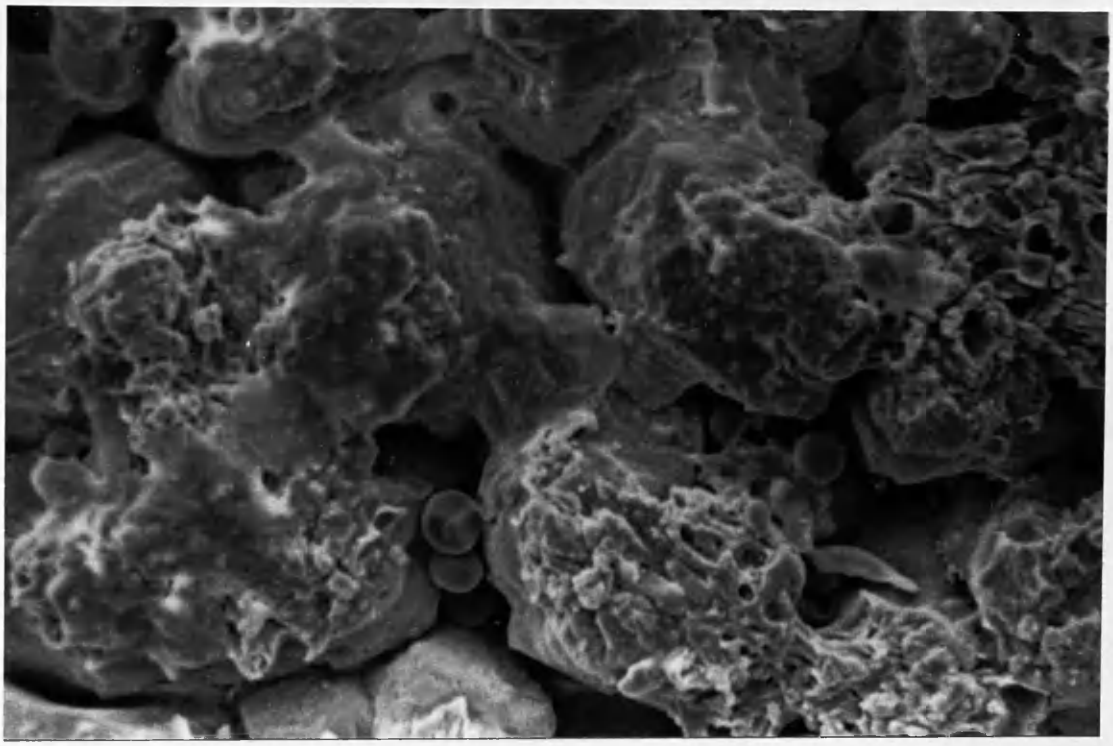


Figure 25. Poor caps. The cap areas are flattened and confluent. Note the absence of fibre tracts x 720

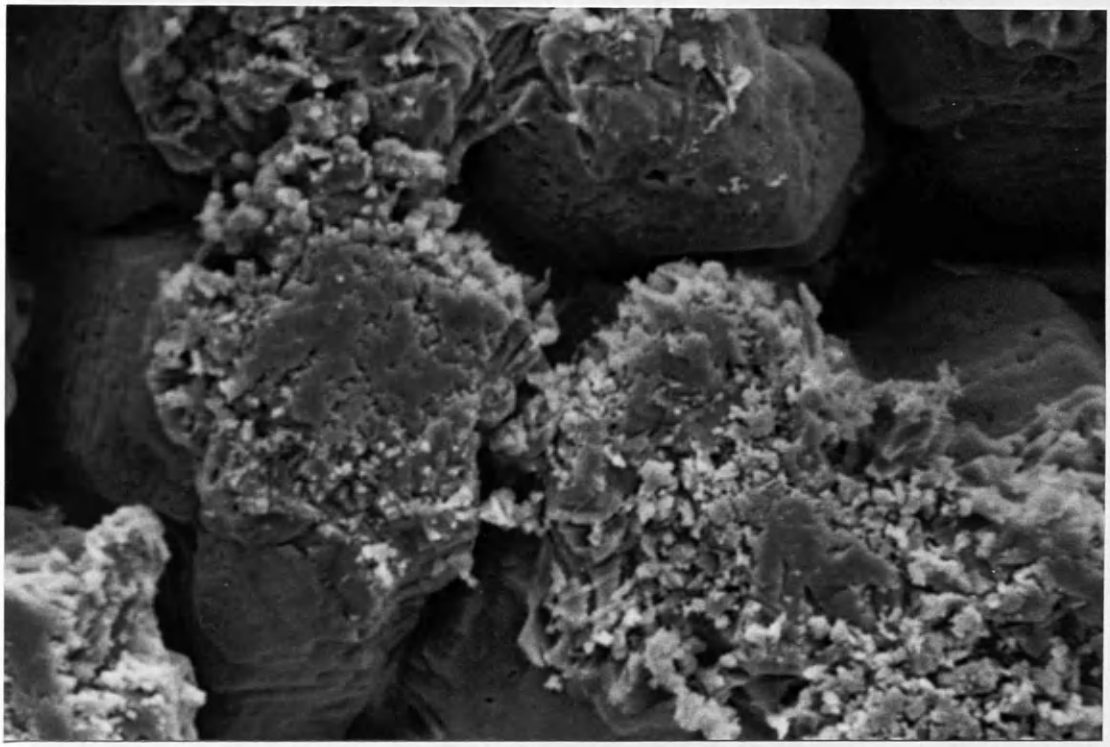


Figure 26. Fragmented mammillary cap x 1,440

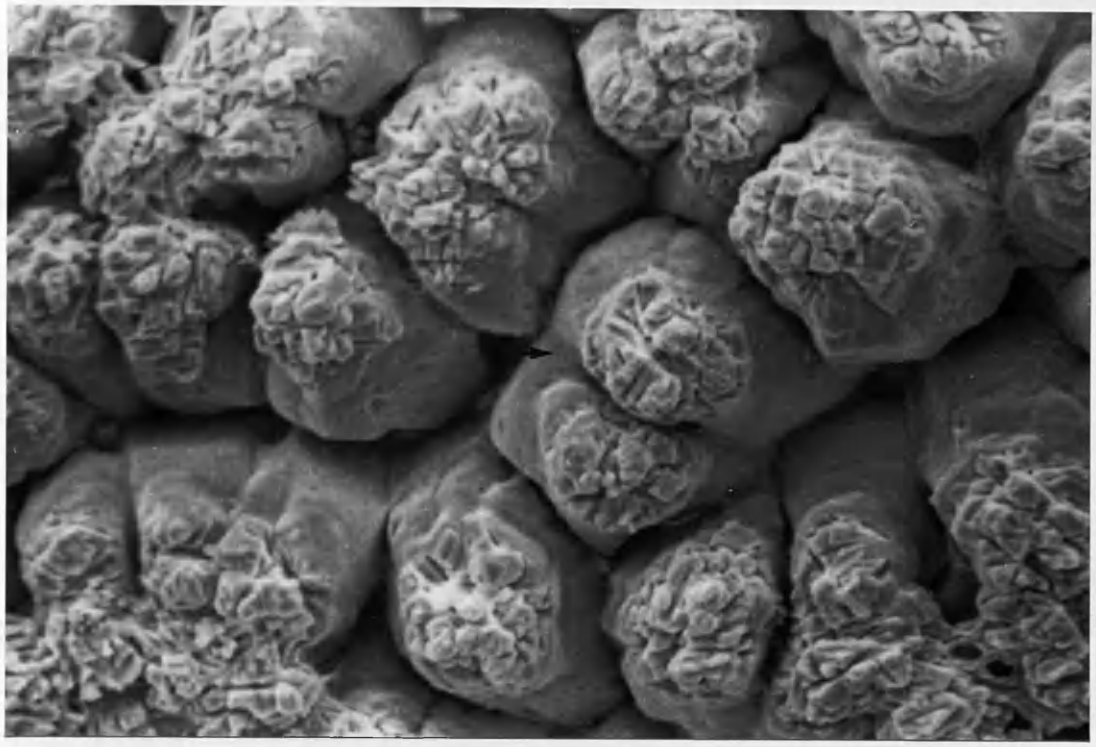


Figure 27. Early fusion (arrow) x 720

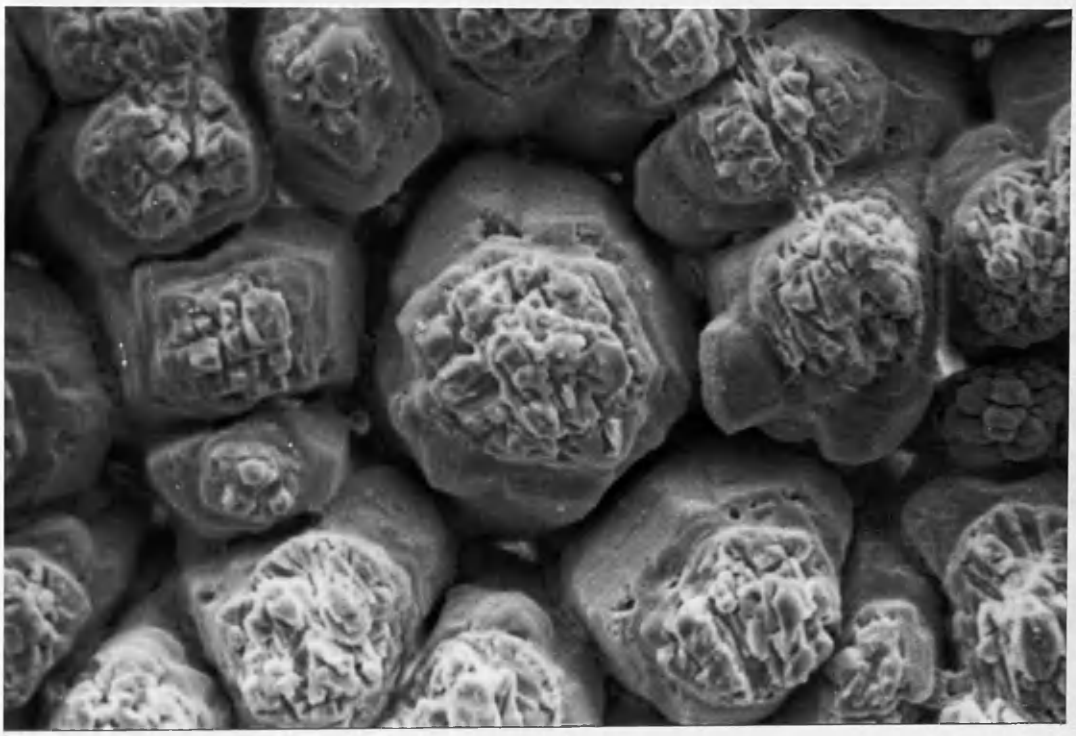


Figure 28. Late fusion. Note the clefts between adjacent palisade columns x 720

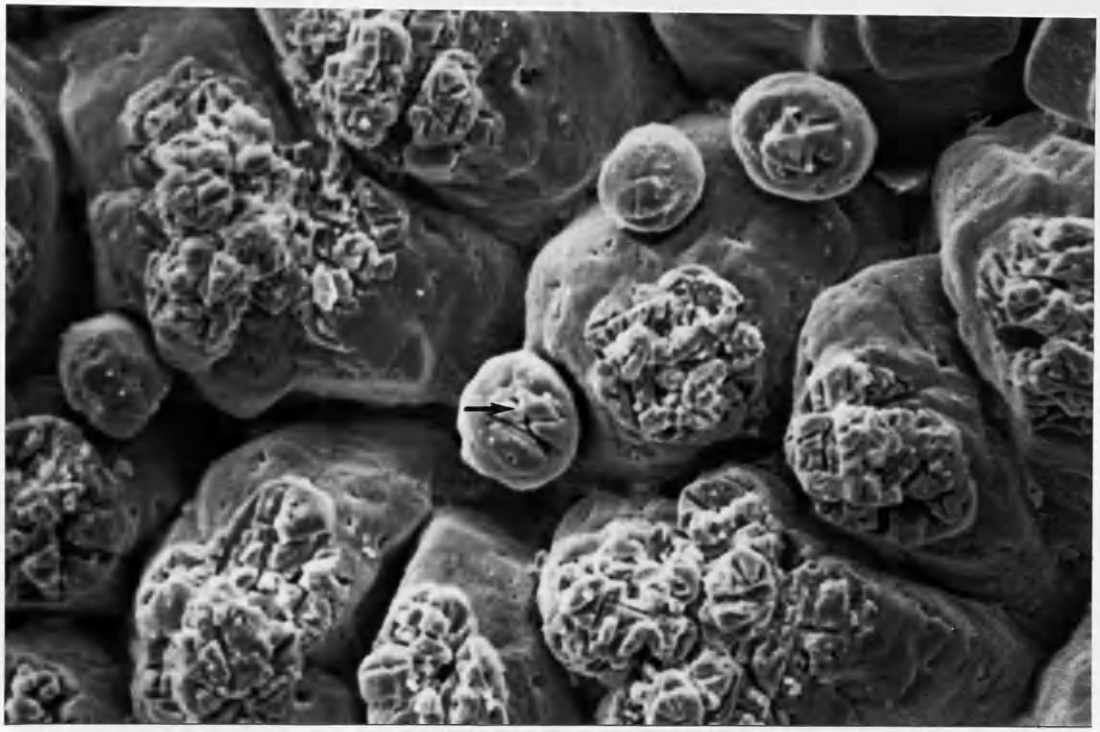


Figure 29. Rounded Type B bodies (arrow) x 720

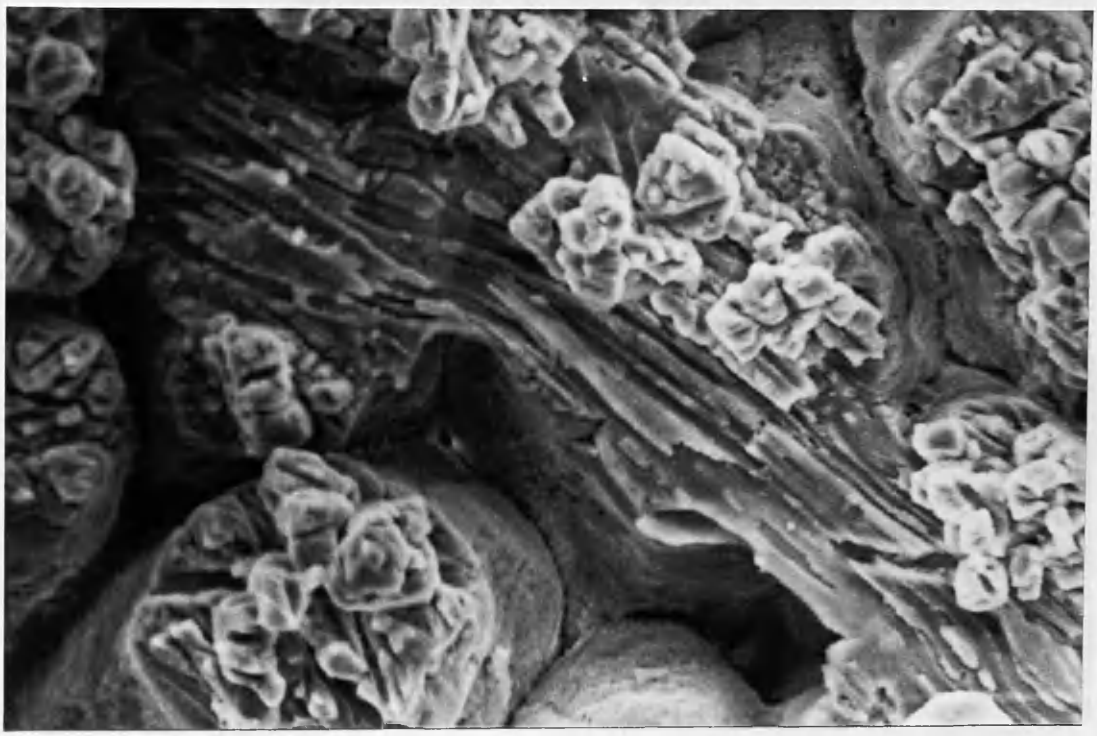


Figure 30. This depression in the mammillary layer, with its parallel configeration reflects the similar arrangement of the membrane fibres x 1440

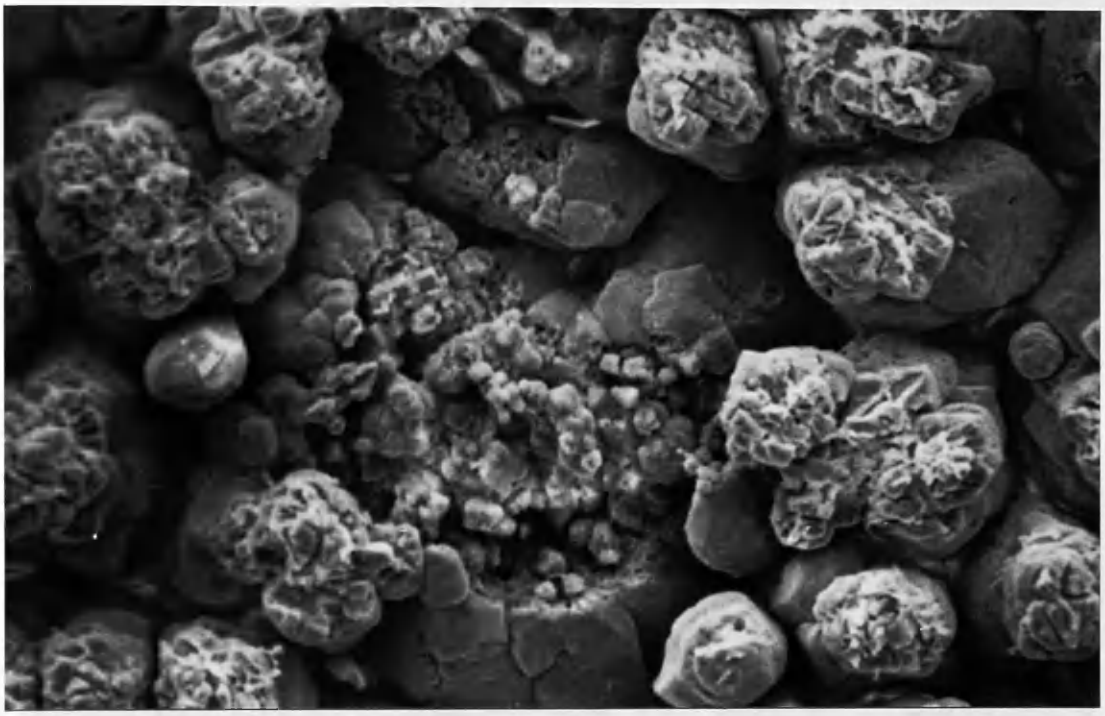


Figure 31. Erosion. The mammillary layer is eroded and filled with various crystal forms x 720

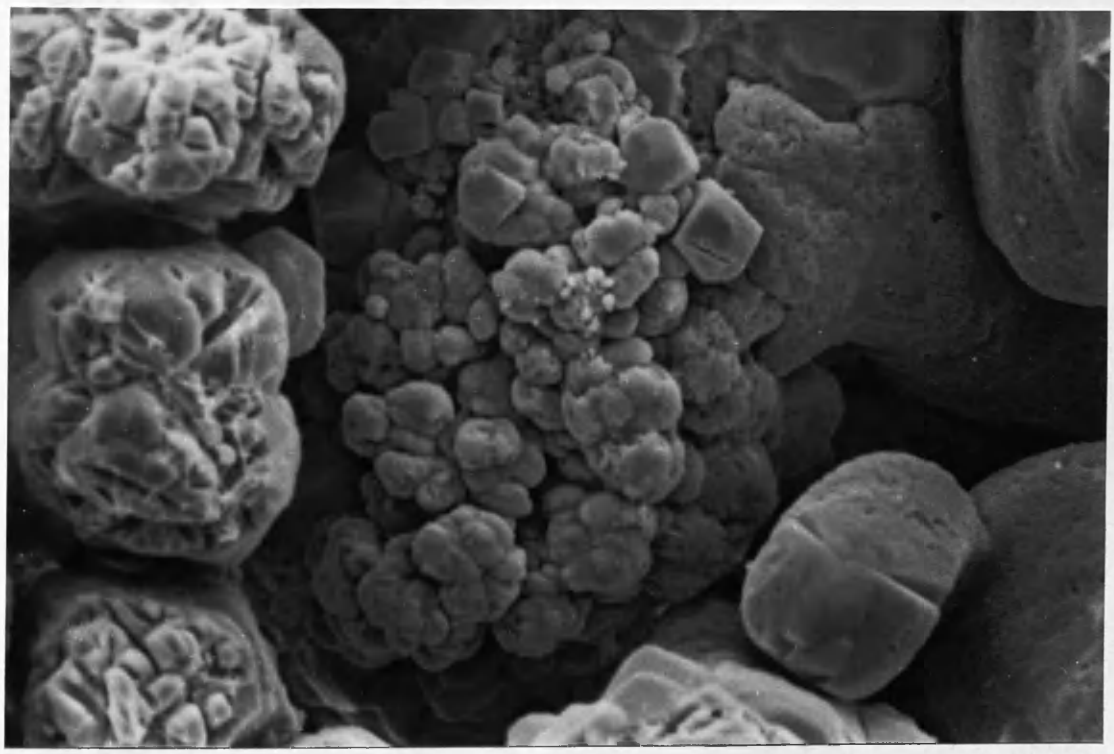


Figure 32. Erosion. This area of minimal contact with the membrane fibres represents a point of weakness x 1,440

Figures 33 - 38 illustrate the diverse forms of aragonite.

Figure 33. Aragonite x 2,800

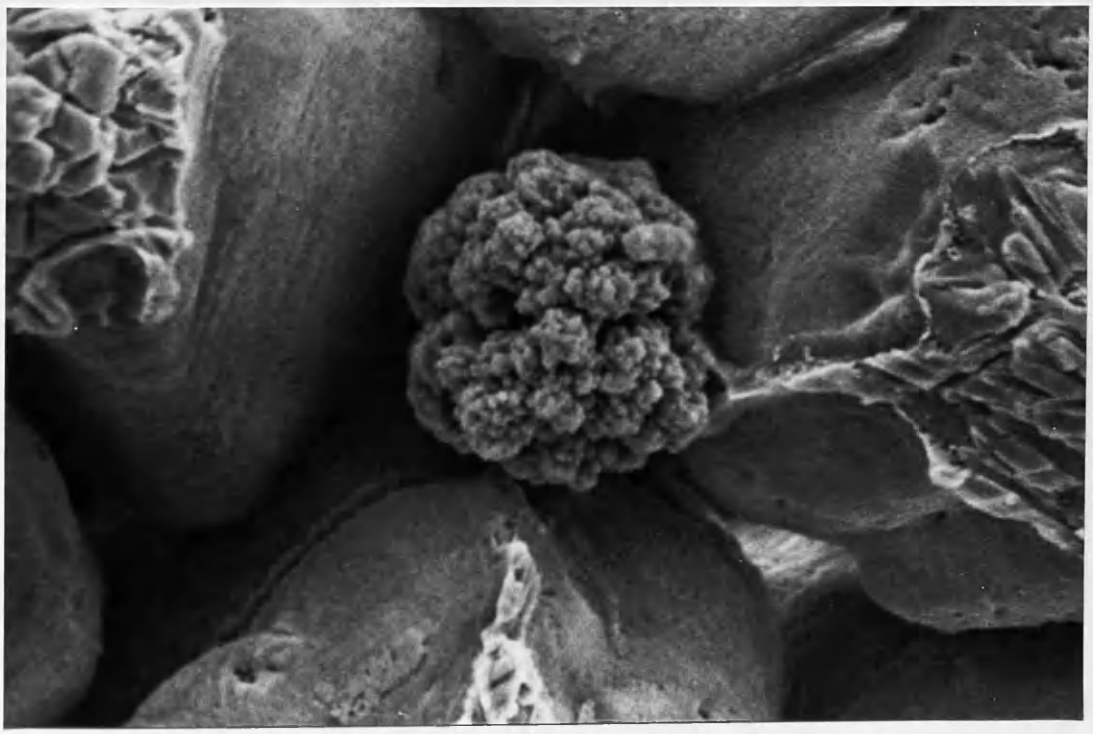


Figure 34. Aragonite x 1,440

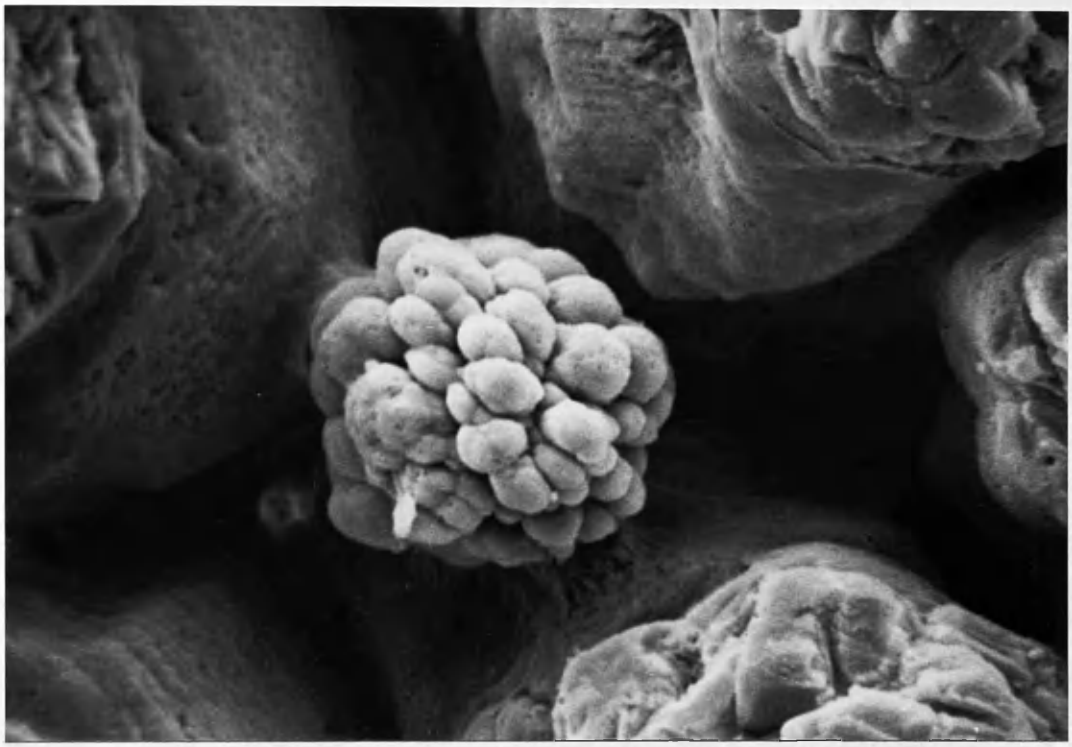


Figure 35. Aragonite. These leaf shaped forms have been observed on the surface of the eggshell of the green turtle where their presence is also abnormal x 2,800

Figure 36. Aragonite. The corn sheaf arrangement is the typical crystal form of the "normal" eggshell of the green turtle x 2,800

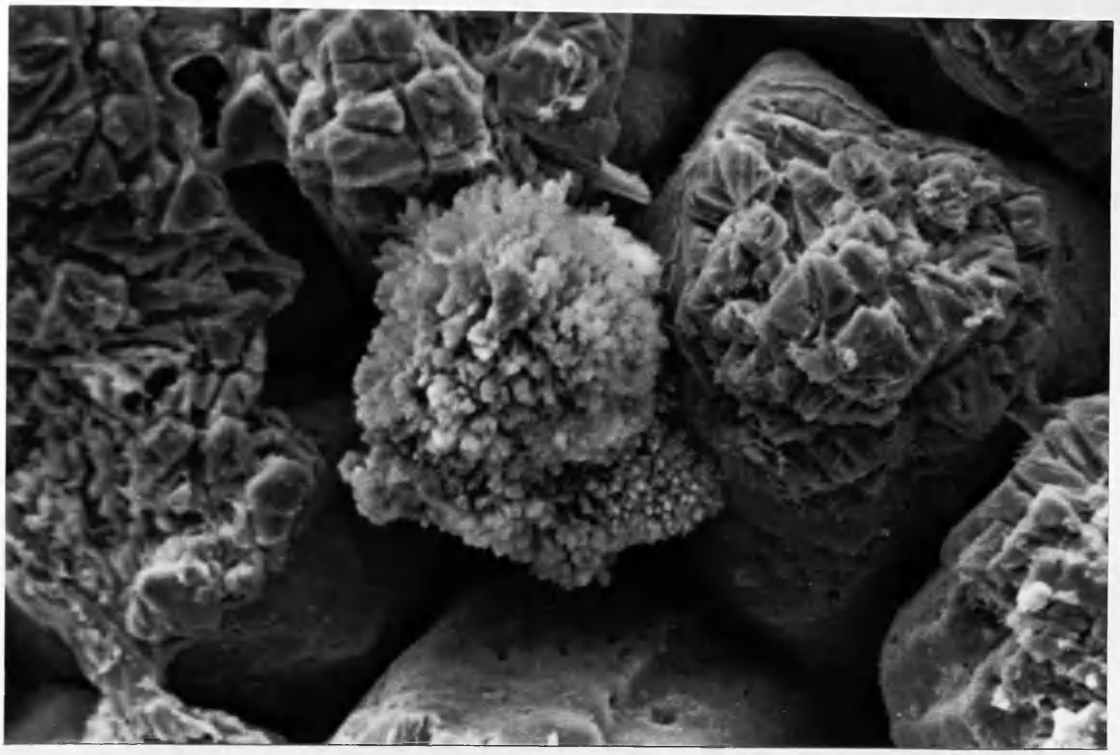


Figure 37. Aragonite x 1,440

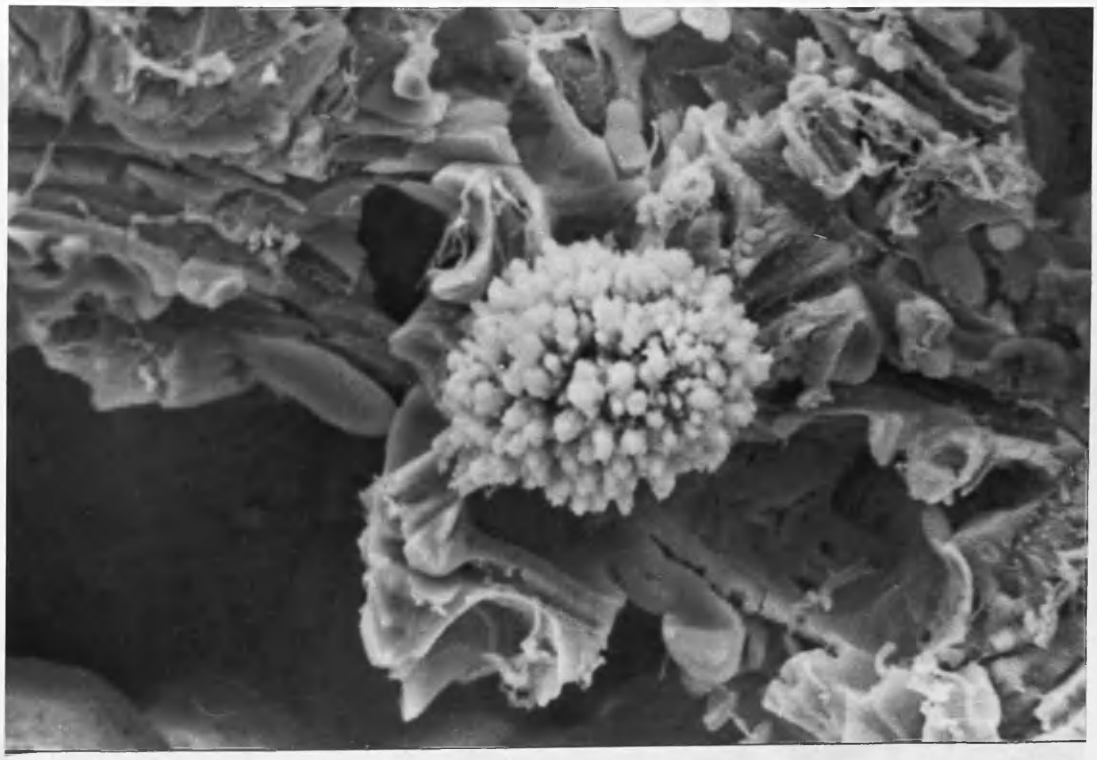


Figure 38. Aragonite on the mammillary cap x 2,800

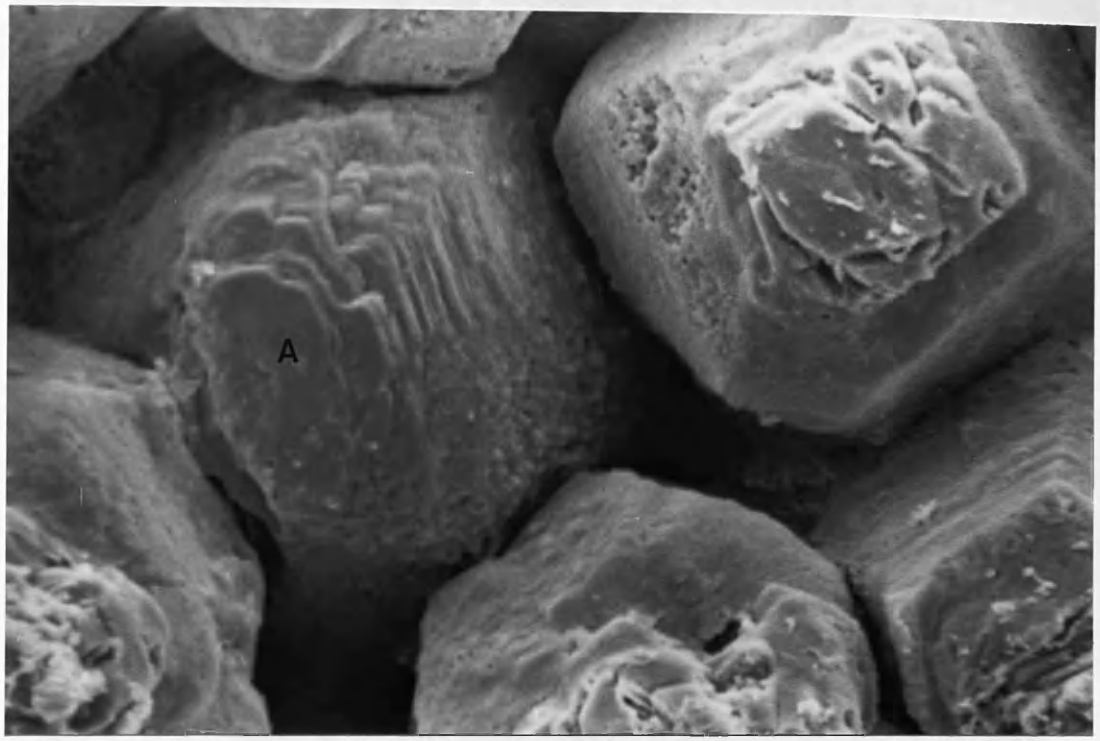


Figure 39. Type A (A) displaying no point of attachment with the membrane fibres x 1,440

Figure 40. Cubic calcite crystals together with aragonite x 2,800

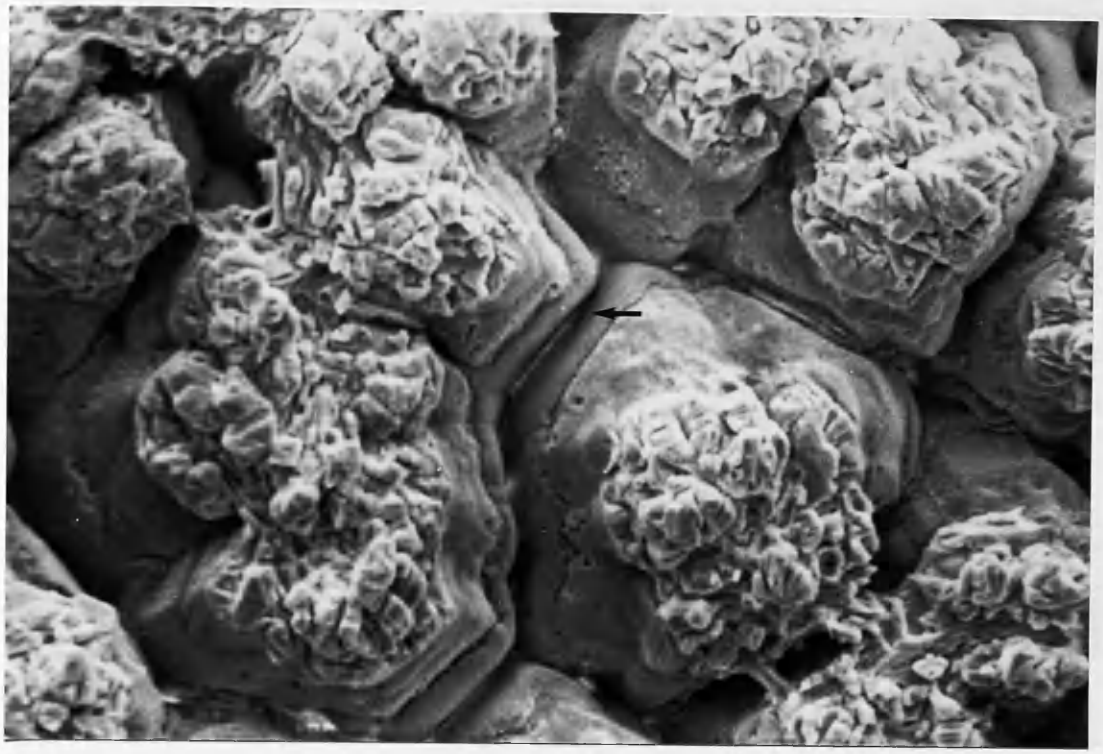


Figure 41. Cuffing (arrow) encourages early fusion of the mammillary columns x 720

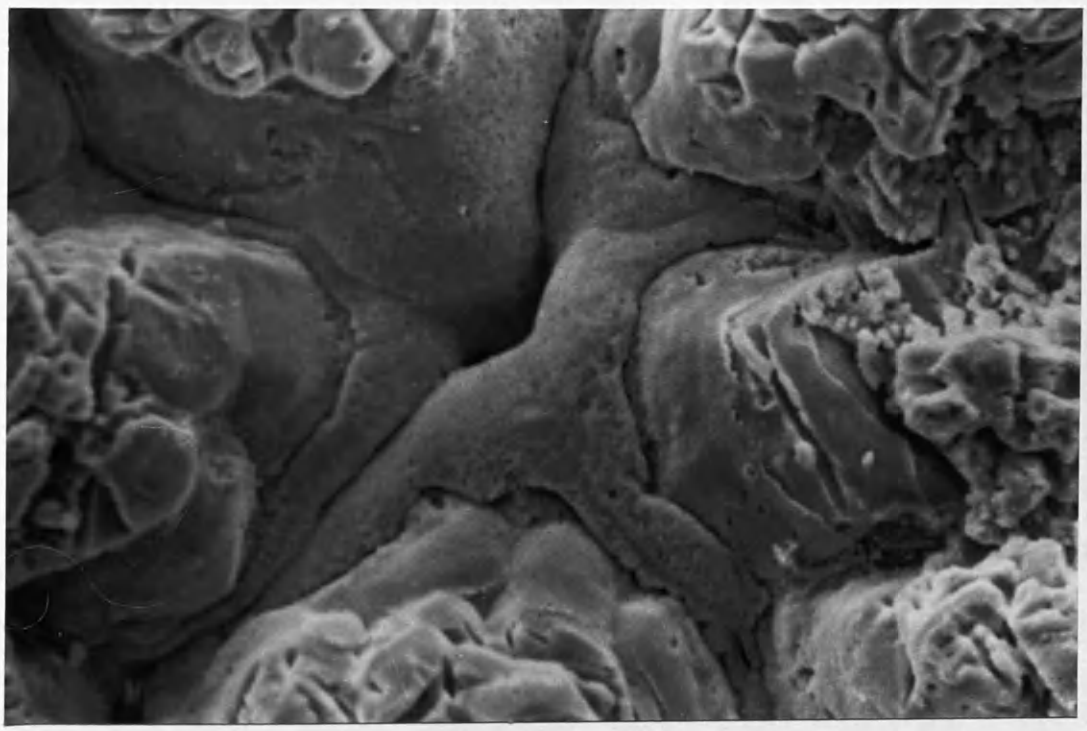


Figure 42. Cuffing. High magnification serves to illustrate the grouting effect of the cuffing phenomenon x 1,440

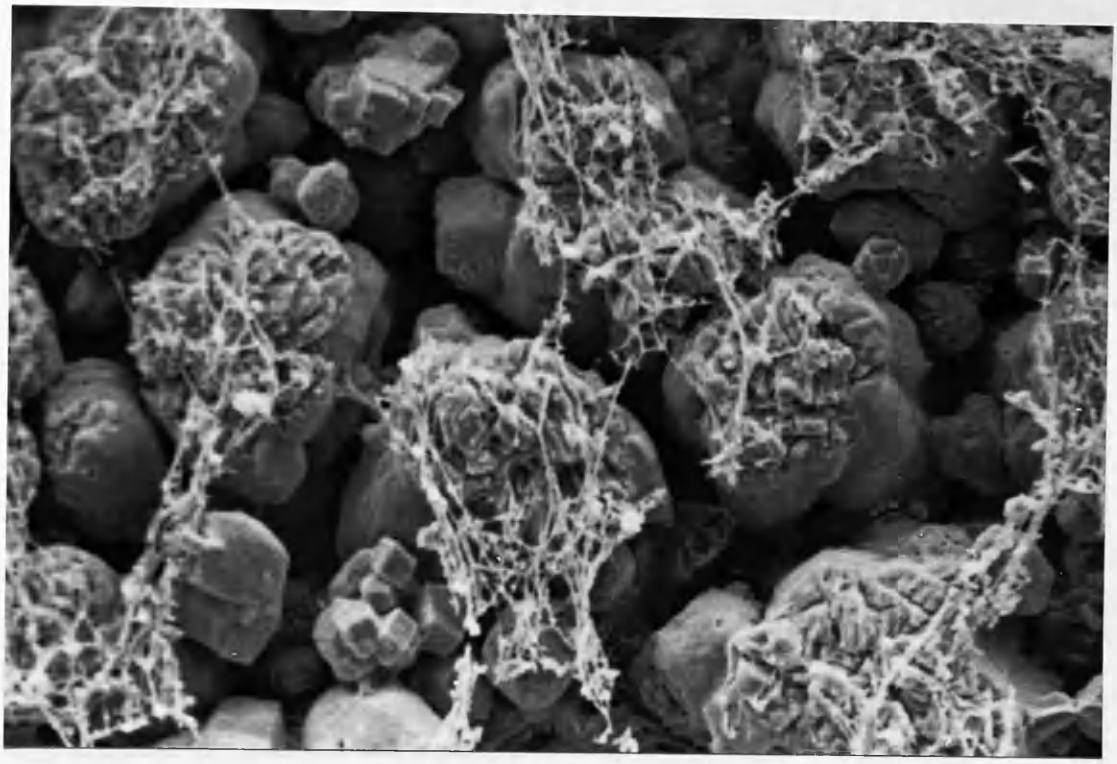


Figure 43. Sulphur rich membrane fibres x 720

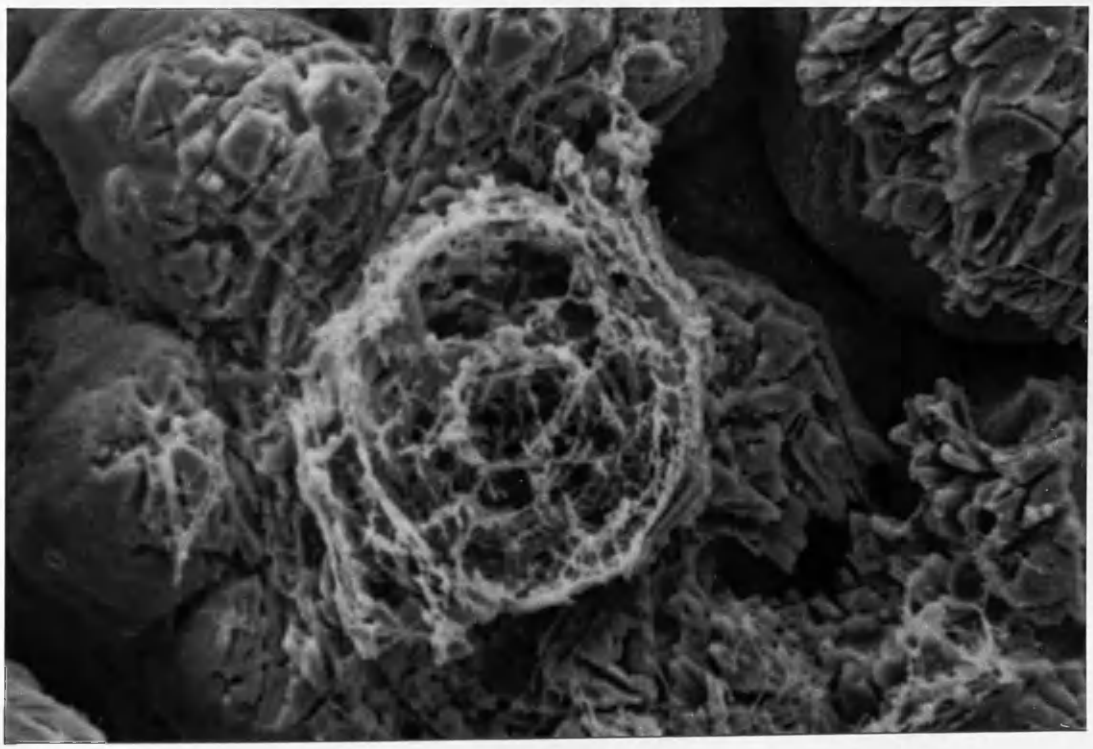


Figure 44. A whorl arrangement of changed membrane fibres x 1,440

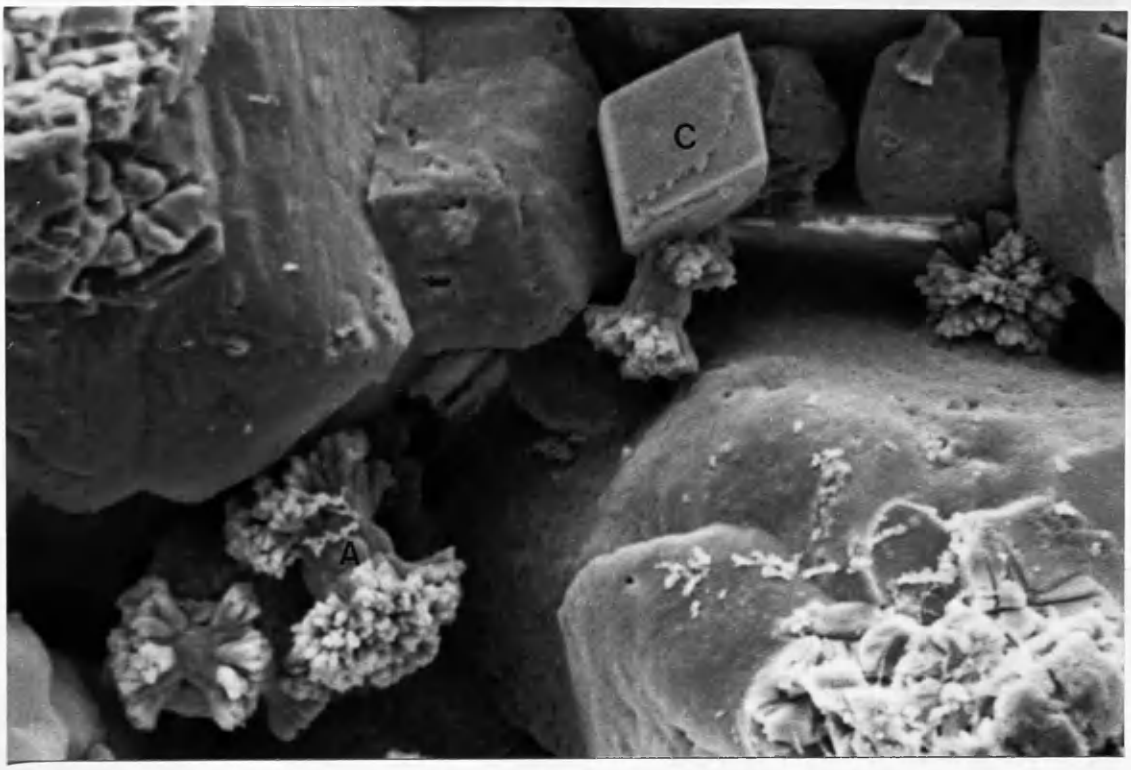


Figure 45. Aragonite (A) and cubic calcite (C) x 1,440

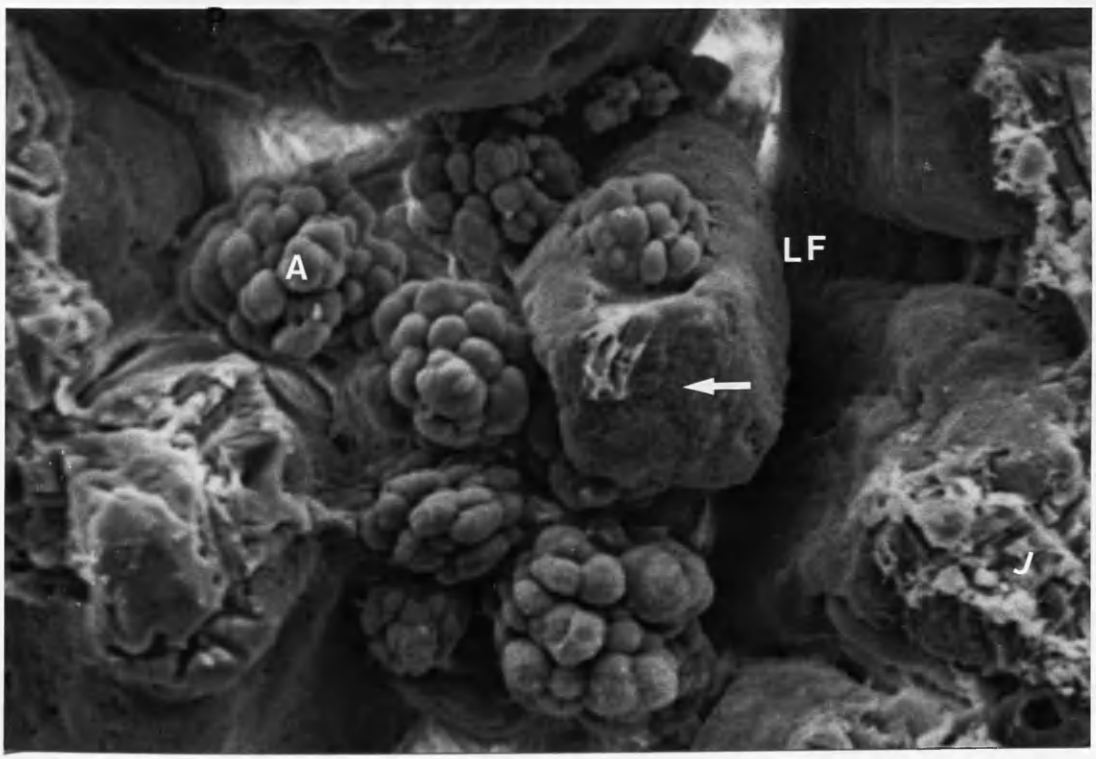


Figure 46. Aragonite (A), late fusion (L F) and type A (arrow) x 2,800

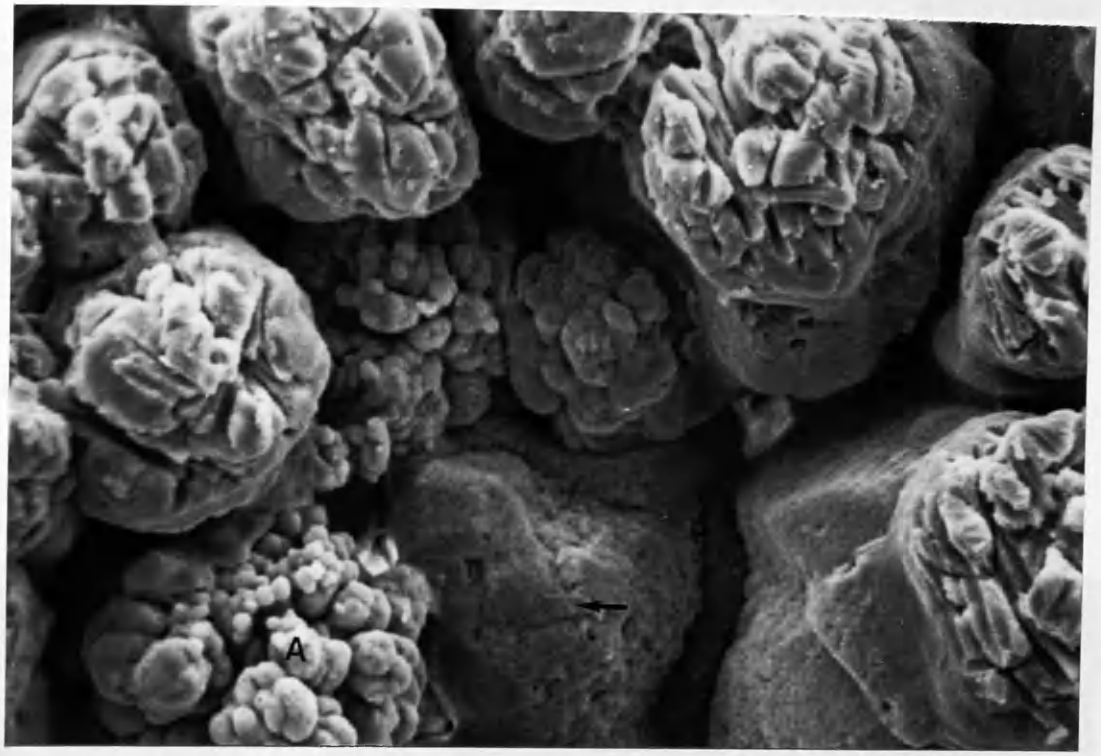


Figure 47. Aragonite (A) and type A body (arrow) x 1,440

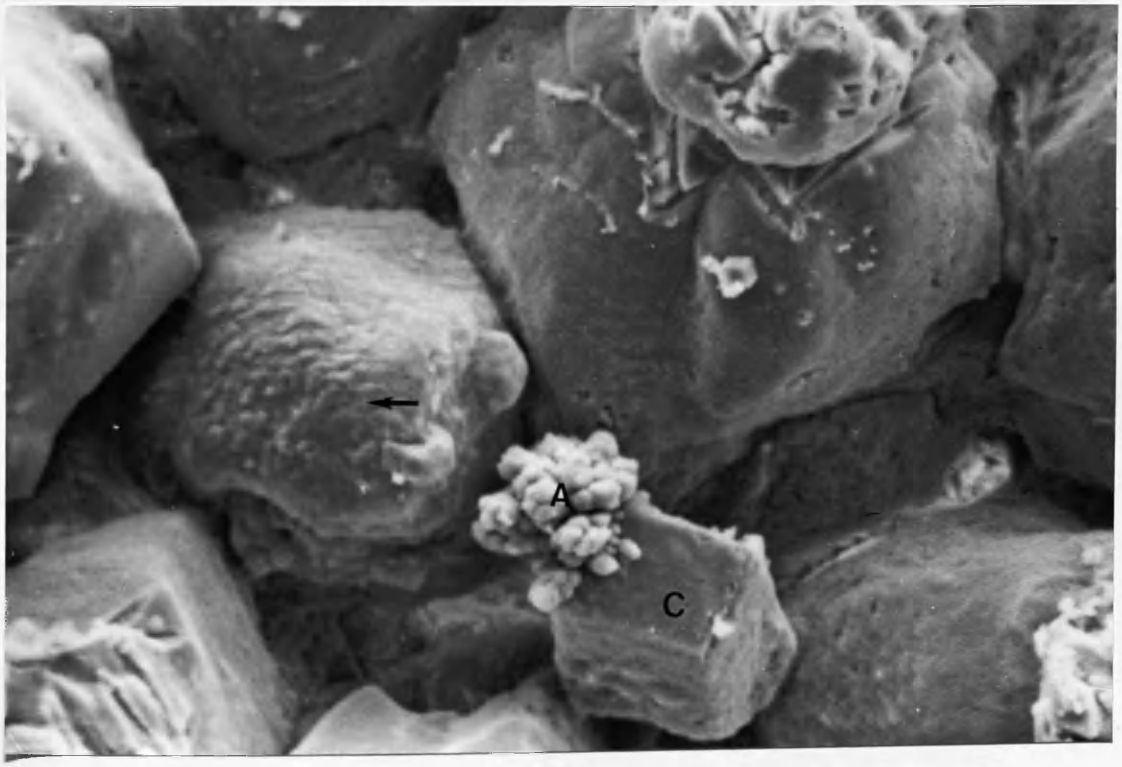


Figure 48. Type A (arrow), cubic calcite (C) and aragonite (A) x 1,440

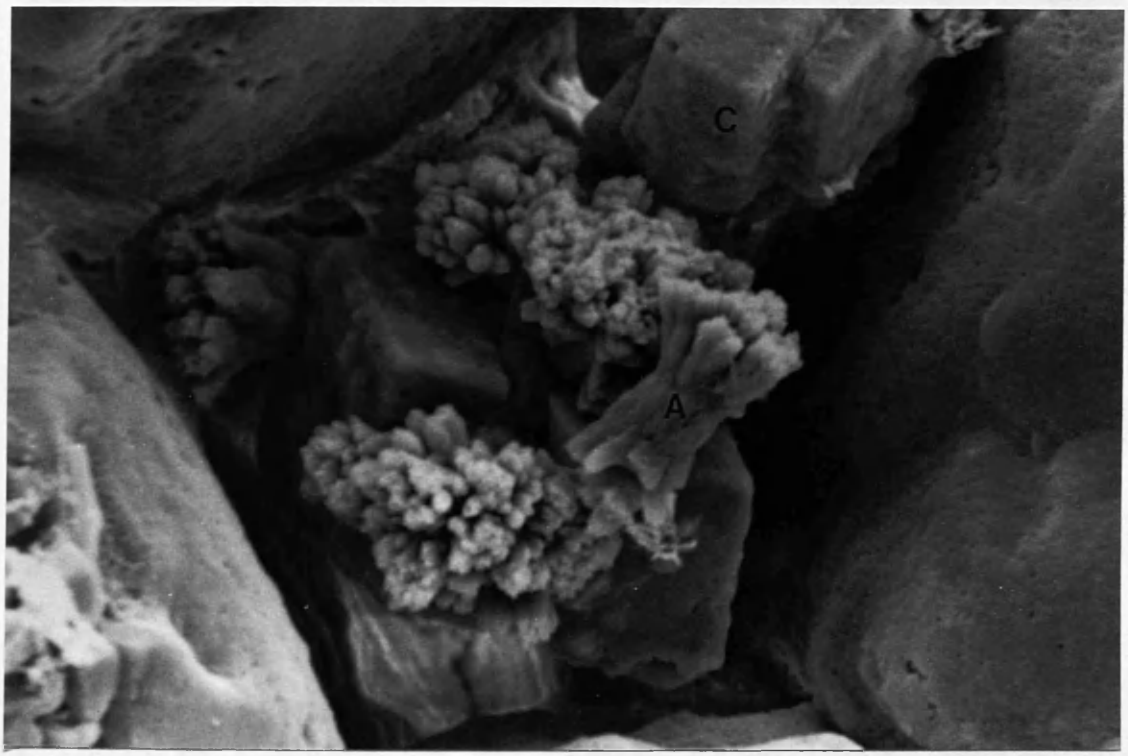


Figure 49. Aragonite (A) and cubic calcite (C) x 2,800

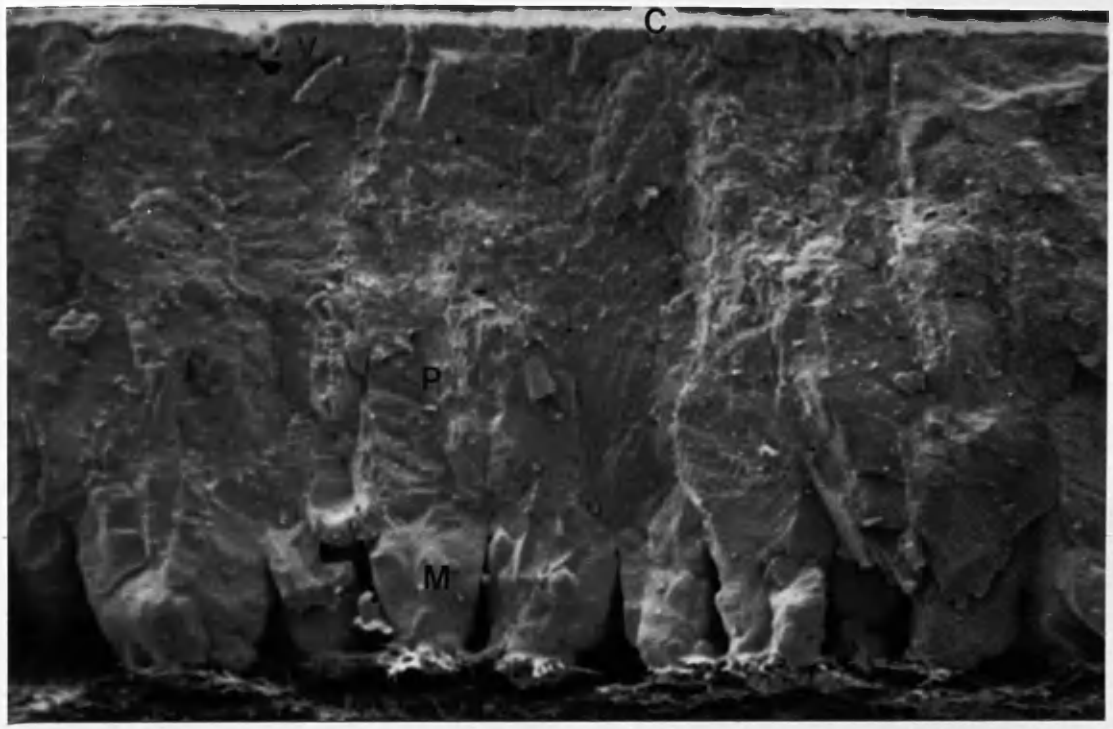


Figure 50. Transverse section through the eggshell. Mammillary layer (M), palisade layer (P), vertical crystalline layer (V), and cuticle (C) x 360

Figure 51. The vertical crystalline layer (V) x 2,800

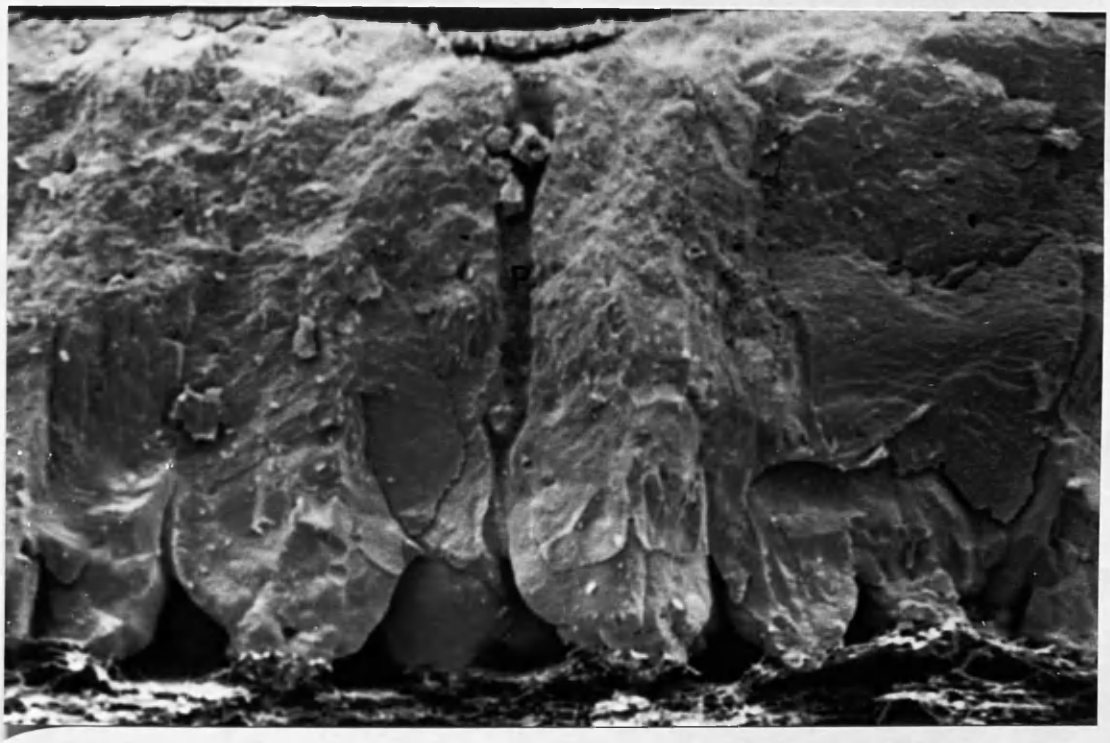


Figure 52. Transverse section through a patent pore (P) x 360

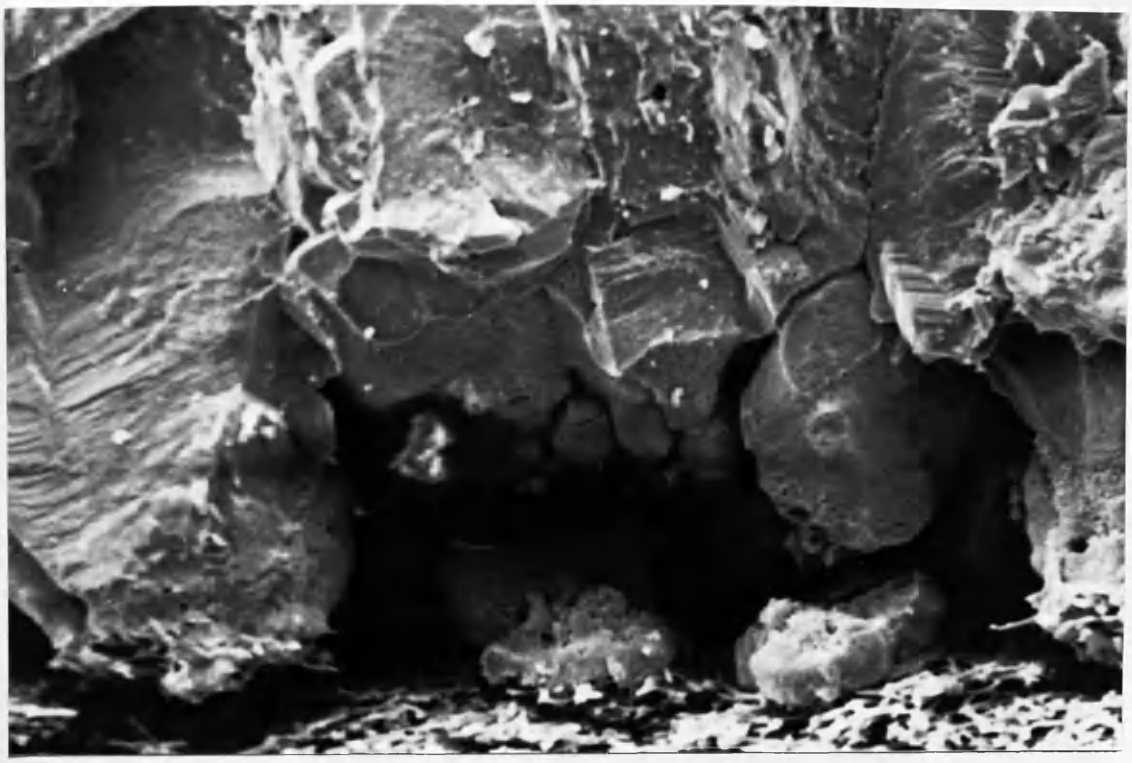


Figure 53. Discontinuity between the true shell and the shell membranes x 720

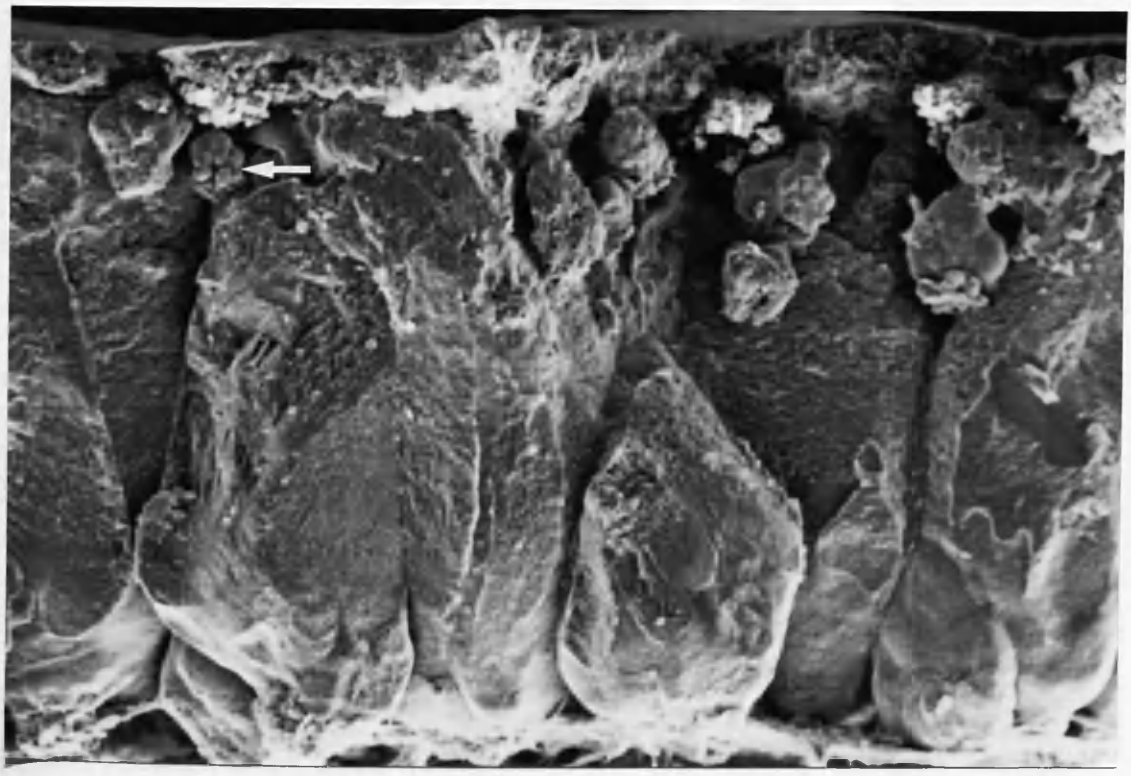


Figure 54. Aberrant crystal forms at the cuticular surface (arrow) x 360

Figure 55. Structural diversity at the cuticular surface x360

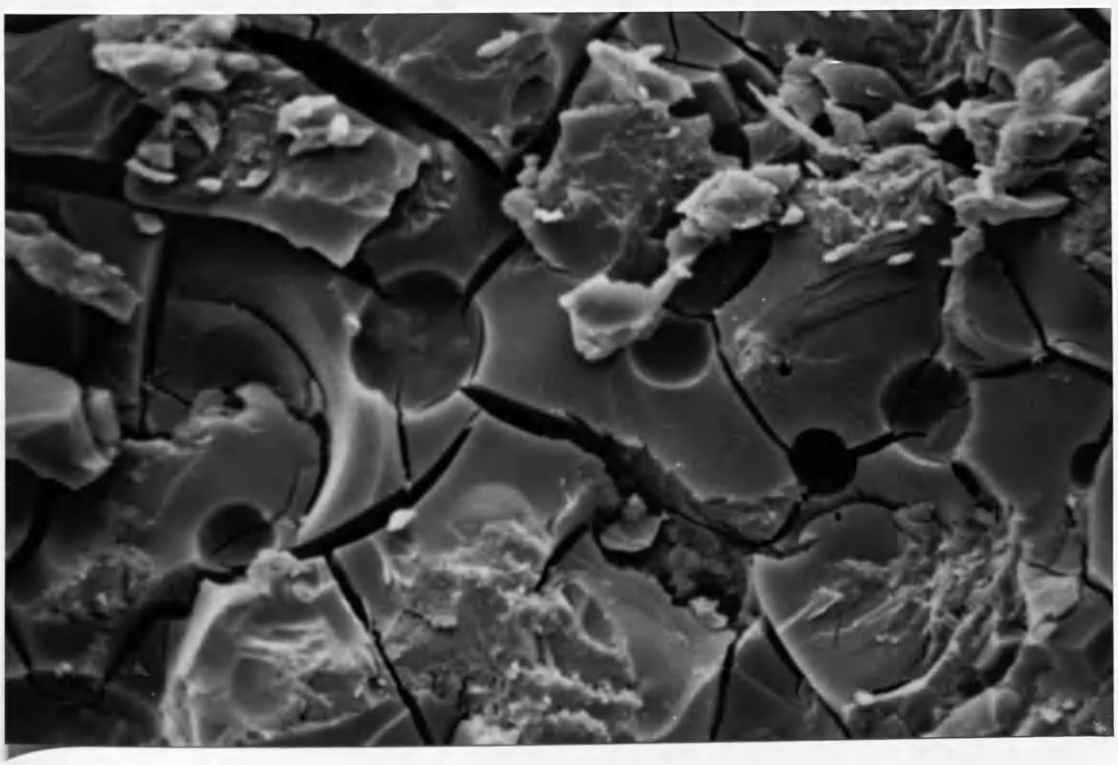


Figure 56. High magnification illustrates the pitted molten appearance of this outer layer x 1,440

4 BRUSH ACTION MACHINE.

4-1 CUTICLE DAMAGE- VISUAL.

An even green colouring is evidence of the presence of a similarly disposed cuticular layer. The cuticle deteriorates with age (Figure 57). Figure 58 shows the unwashed eggs at end of lay and as expected the cuticle ranges from sparse to absent. Figures 59, 60 demonstrates the effect of washing on eggs at the end of lay. 5 of the eggs display visible bristle damage where the cuticle has been abraded.

4-1 a CUTICLE DAMAGE - SCANNING MICROSCOPY.

Given that the cuticular layer is frequently patchy in its distribution and that the washed eggs were only examined after the washing procedure, due care has to be taken to ensure that the damage observed is the result of the latter and not merely evidence of the vagaries of this part of egg formation.

When the cuticle is absent, because of a defect in oviducal function, it rarely affects the organisation of the underlying layers and so with the exception of machine 1, where the damage clearly correlates with bristle action, the gouges and associated debris on the eggs washed by rotary or jet action have been interpreted as originating from the process of washing.

Using the scoring system below, graph 1 shows that the cuticle has been damaged by all three washing actions.

CUTICLE	SCORE
GOOD	1
GOOD -	2
POOR	3
CUTICLELESS	4

At the beginning of lay 22 of the 25 eggs displayed evidence of brush damage ranging from broad parallel gouges and deep intersecting striations to narrower striations on the surface layer. This observation was reported to the company concerned and it was interesting to note that at the middle of lay only 13 out of the 25 exhibited structural damage while at the end of lay this number had fallen to 5 out of 25. Figure 61 illustrates cage damage. These depressions in the cuticle are possibly the result of wire contact and Figures 62-72 demonstrate the range of cuticular damage resulting from the washing procedure.

4-2 SANITISER.

The cuticular layer of one of the eggs displayed a mesh like deposit (Figure 73). X-ray analysis identified the latter as phosphorus. The mammillary layer of an unwashed and washed shell was analysed and as Figure 74 reveals there was more chlorine and phosphorus present in the washed shell.

4-3 BACTERIOLOGICAL ANALYSIS.

Pseudomonas spp. which rarely causes disease was the only organism found in the culture (Table 1).

4-4 PROTEIN ANALYSIS.

The results presented in Table 2 indicate that at the three periods of lay the protein concentration in the wash water increased succeeding the washing procedure. It is deduced that the prewash protein originates partially from the sanitiser and also from debris adhering to the unit. The elevated levels after washing not only reflect abraded cuticular material but also albumen protein from leaking eggs and faecal material.

4-5 INFRA-RED ANALYSIS.

Graph 2 illustrates the presence of both calcite and aragonite in the sample. Calcite peaks (C) at 879 and 715. Aragonite peaks (A) at 1100 and 675.

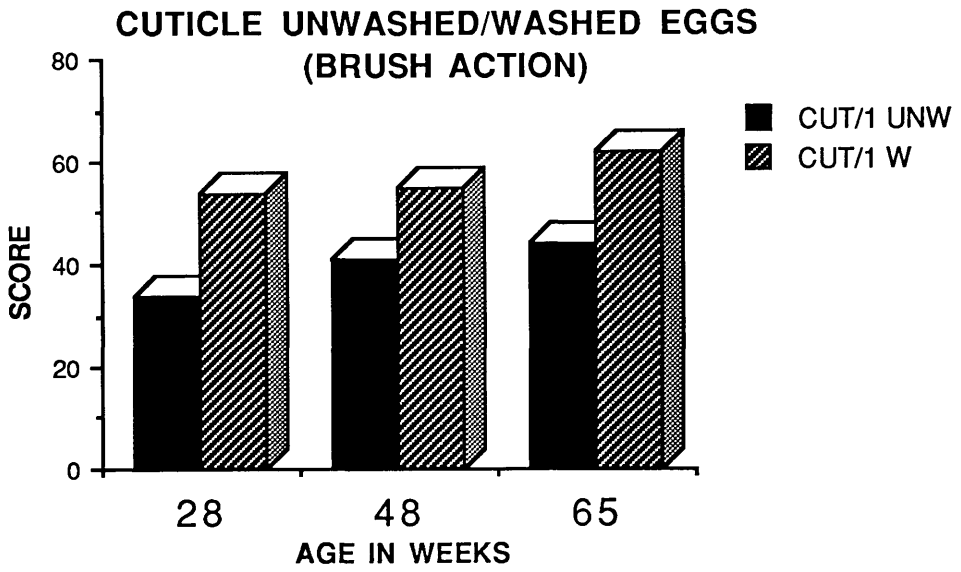
Figure 57. Eggs stained with edicol supra green demonstrate the deterioration of the cuticle during the laying period. From right to left is beginning, middle and end of lay.

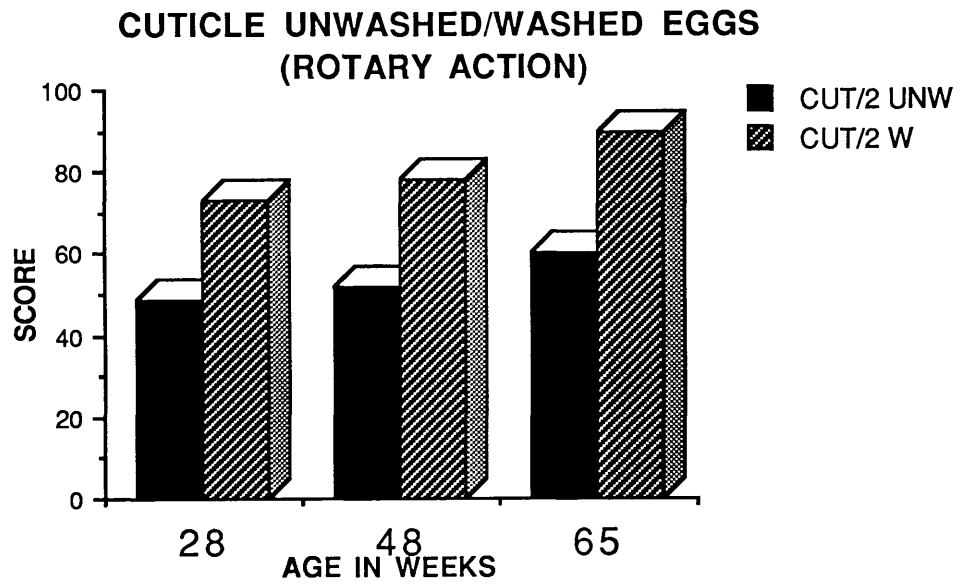
Figure 58. Eggs stained with edicol supra green - Brush wash controls - End of lay. Variation in staining intensity reflects variation in degree of cuticular coverage.

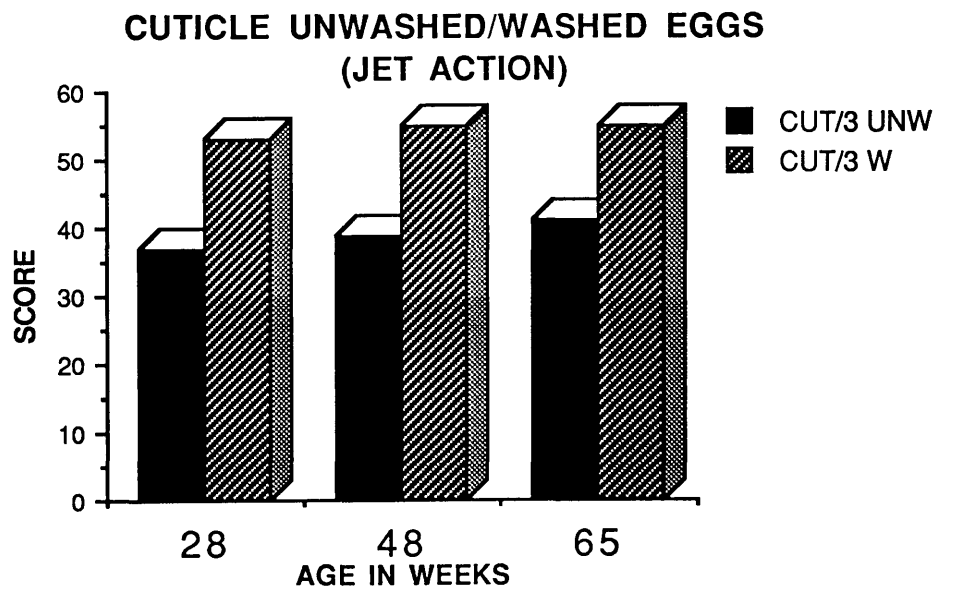
Figure 59. Eggs stained with edicol supra green - Brush wash - End of lay.

Figure 60. Egg stained with edicol supra green - Brush wash - End of lay. The egg is scored by brush action.

GRAPH 1. UNWASHED AND WASHED CUTICLE AT THE DIFFERENT PERIODS OF LAY.







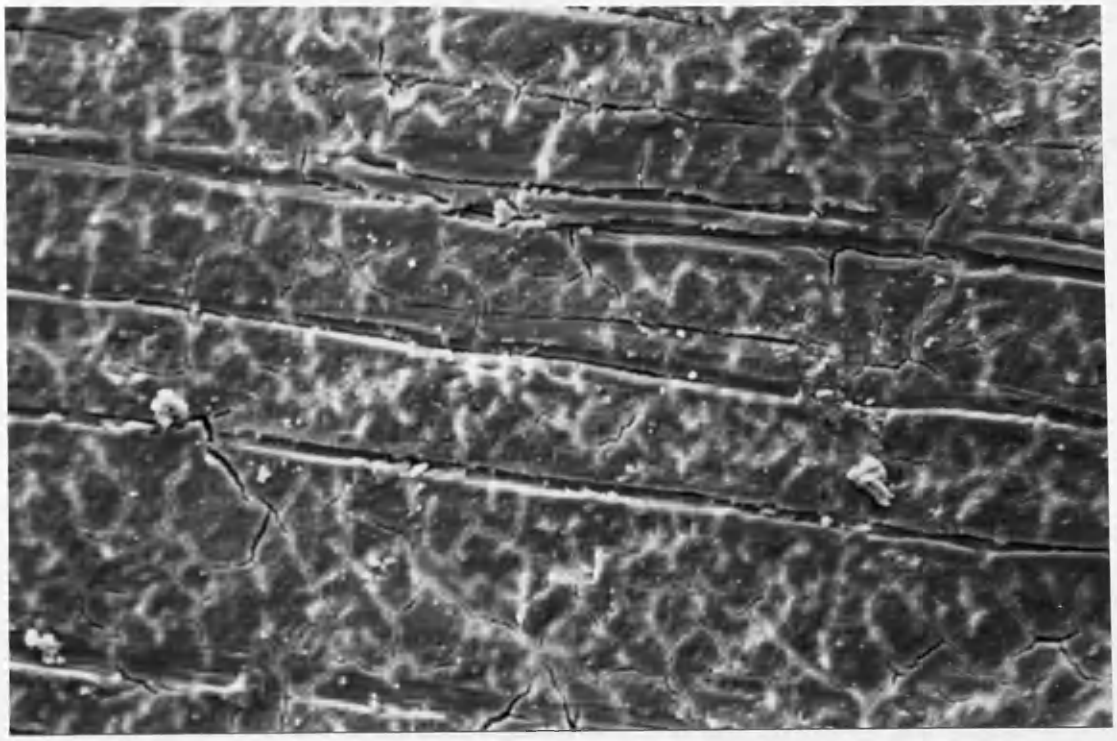


Figure 61. Wire mark x 720

Figure 62. Brush mark - Broad deep gouge - Beginning of lay x 180

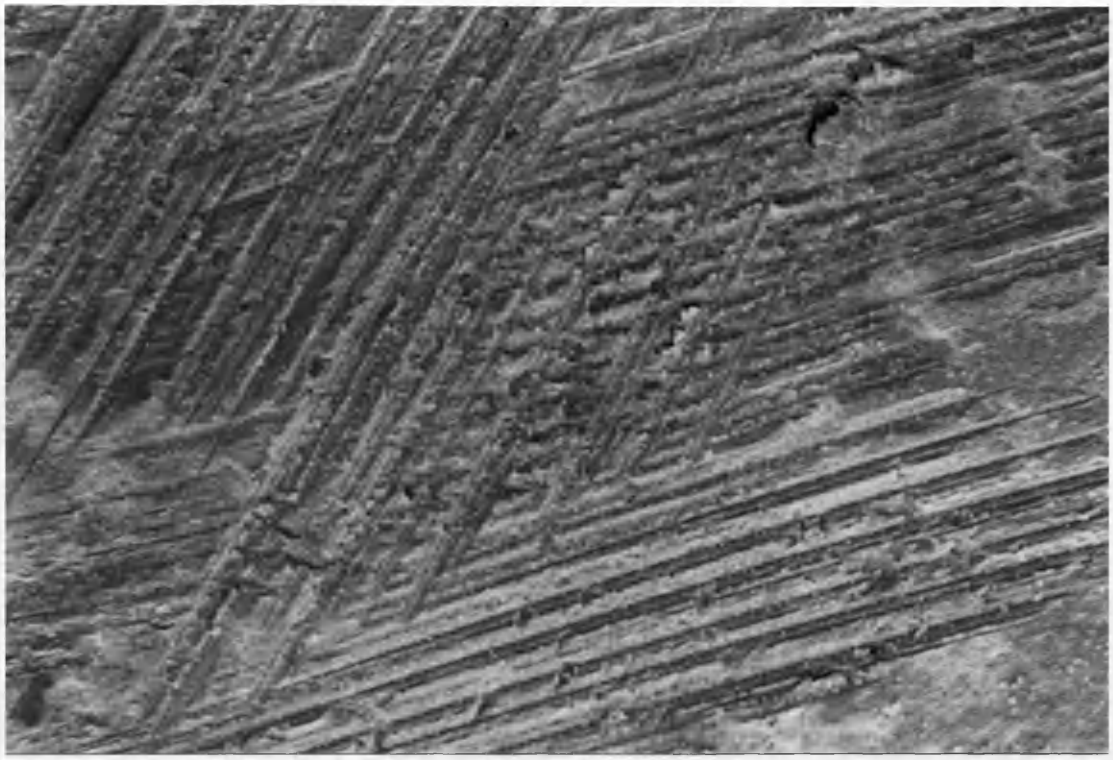


Figure 63. Brush mark - Herring bone striations - Beginning of lay x 90

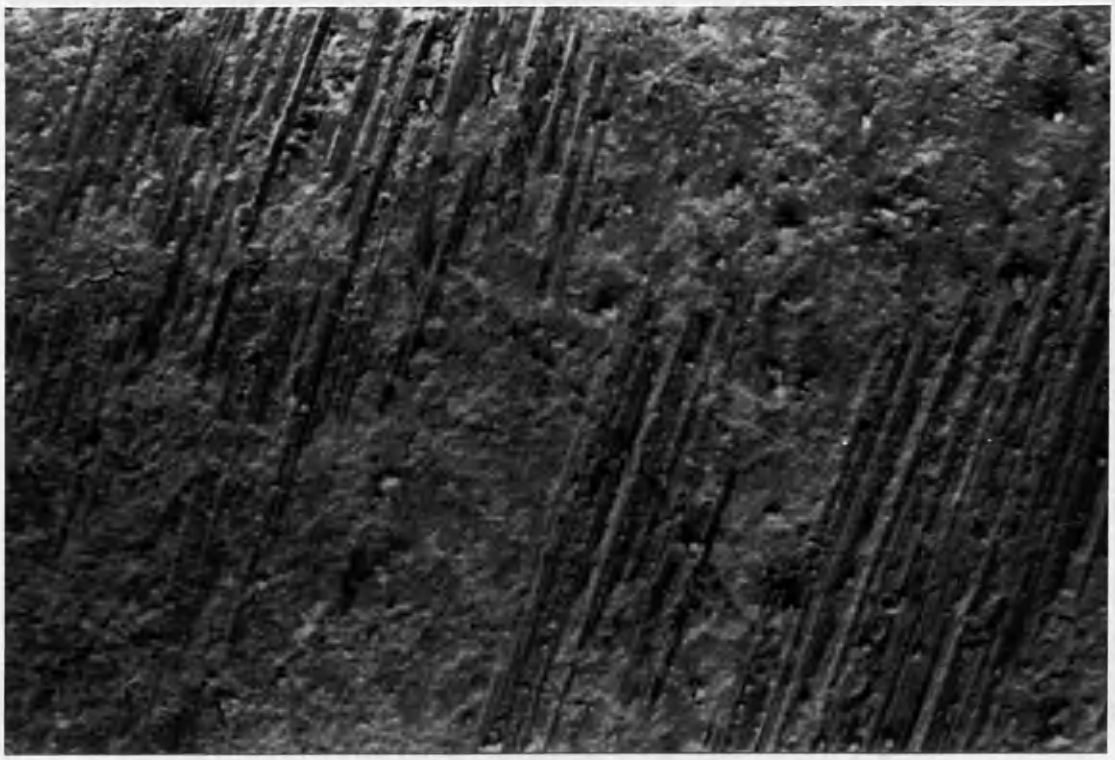


Figure 64. Brush mark - Beginning of lay x 90

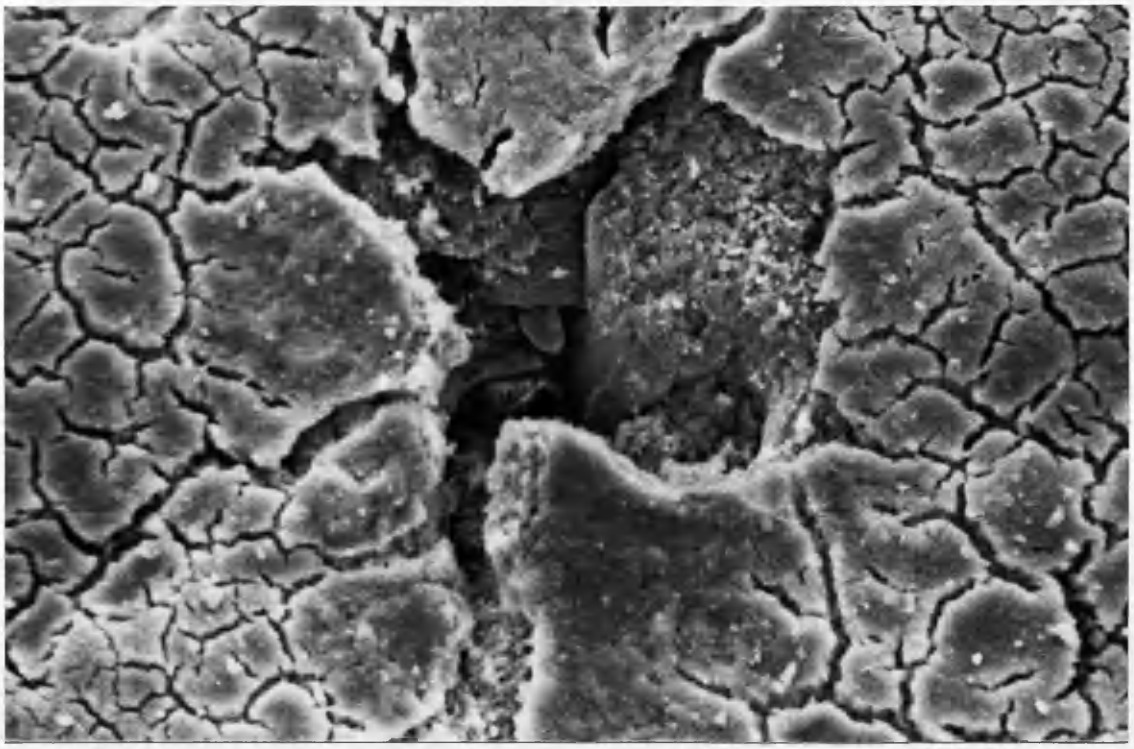


Figure 65. Brush wash - Exposed palisade layer - Beginning of lay x 1,440

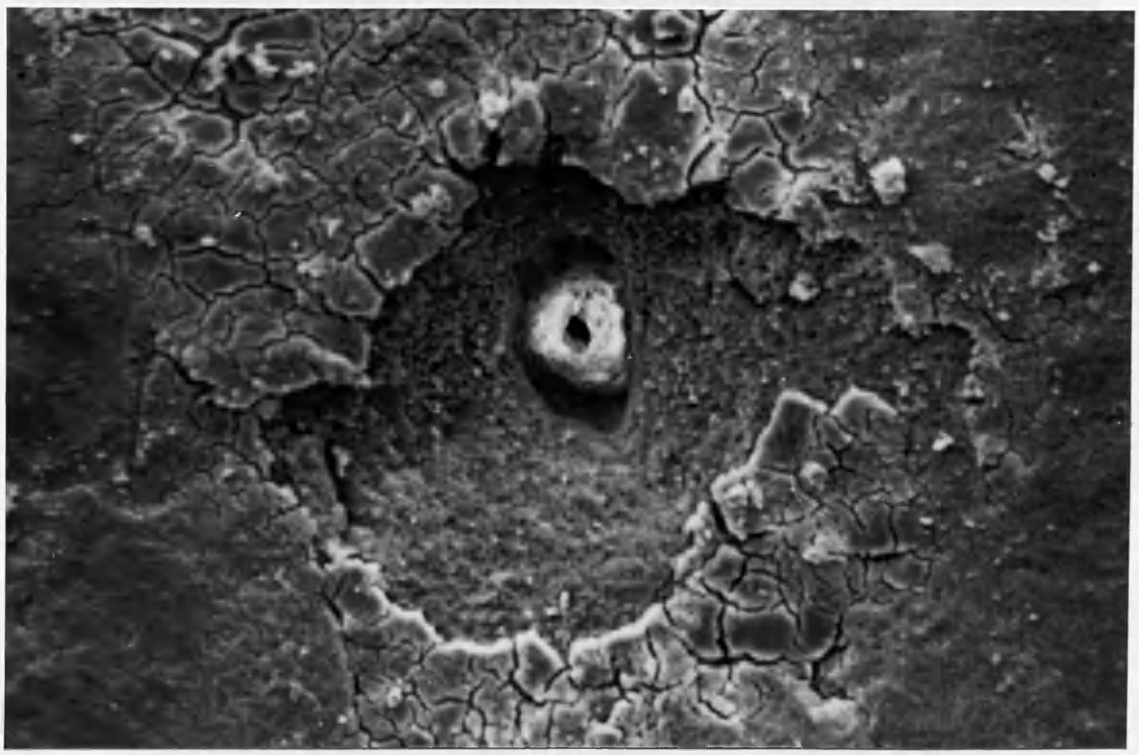


Figure 66. Brush wash - Unplugged patent pore - Beginning of lay x 720

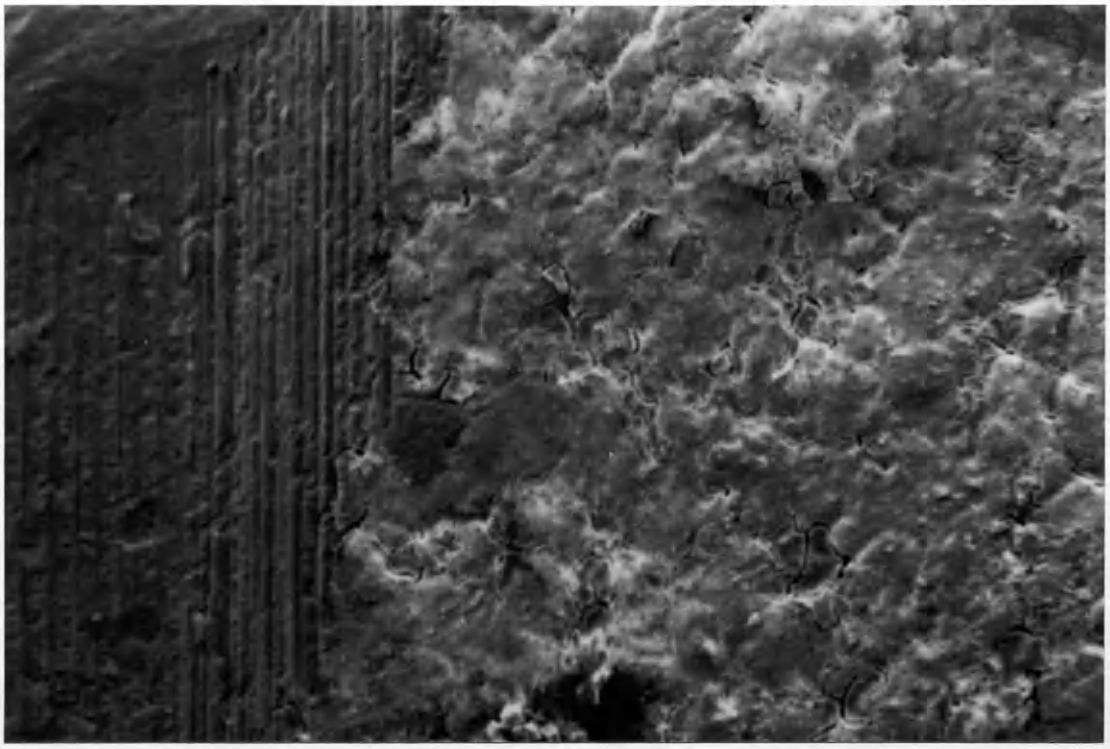


Figure 67. Brush wash - Deep striations - Middle of lay x 180

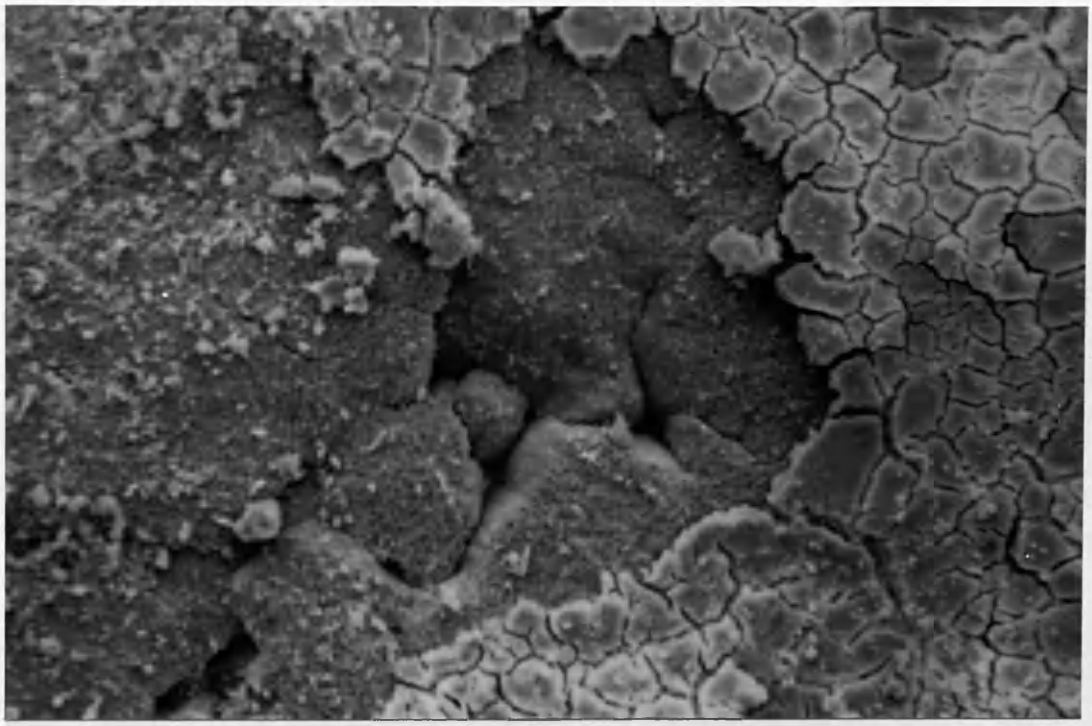


Figure 68. Brush wash - Exposed palisade layer and remains of cuticle - Middle of lay x 720

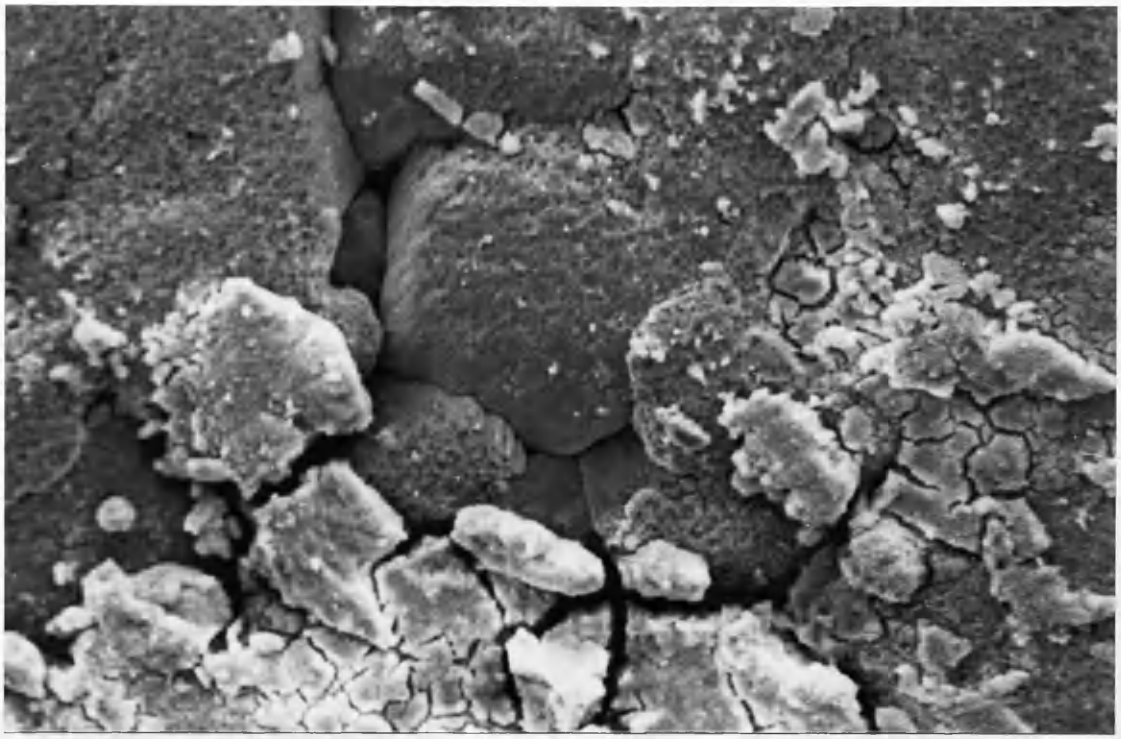


Figure 69. Brush wash - Exposed palisade layer and disrupted cuticle - Middle of lay x 720

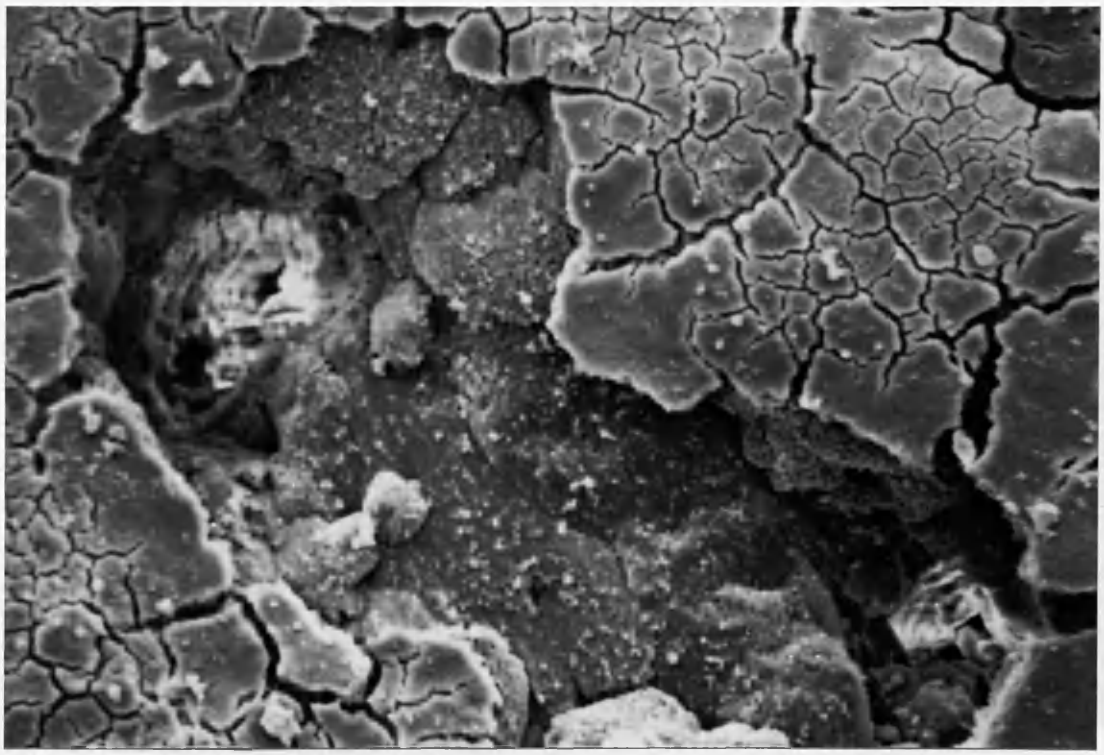


Figure 70. Brush wash - Exposed patent pore - Middle of lay x 720

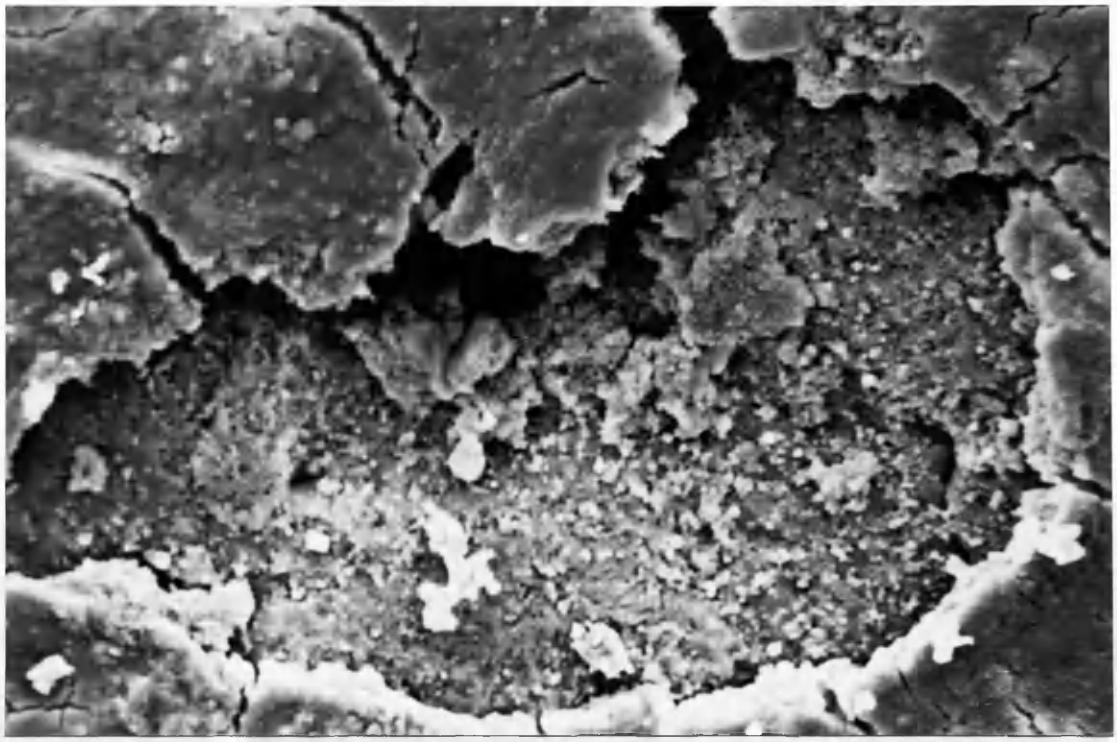


Figure 71. Brush wash - Exposed palisade layer - End of lay x 1,440

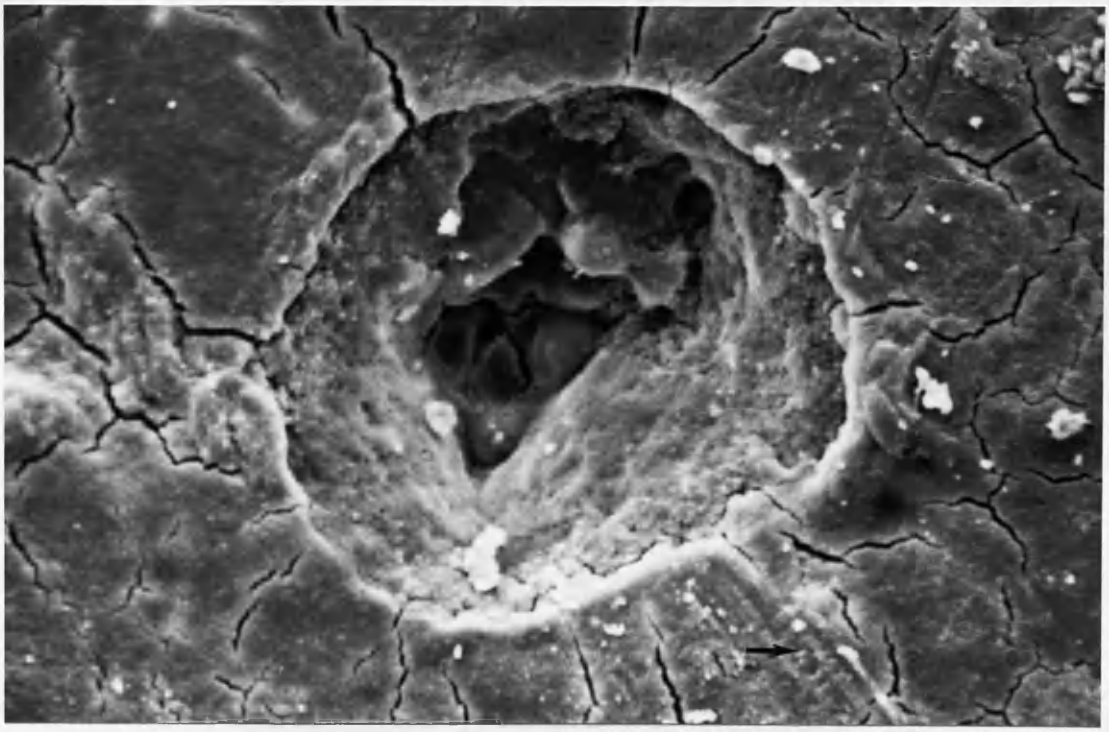


Figure 72. Brush wash - Exposed palisade layer - End of lay
Note evidence of brush damage. Striations (arrow) x 1,440

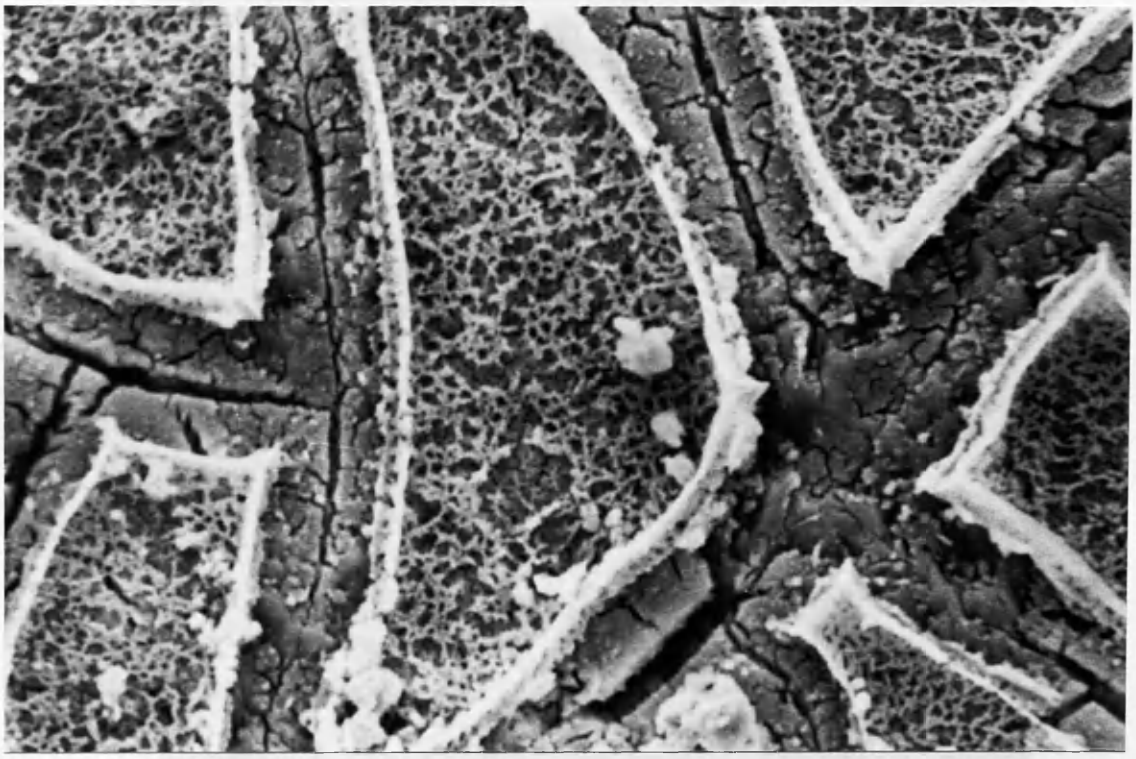


Figure 73. Phosphorus rich mesh like deposit on cuticle of brush washed egg x 2,800

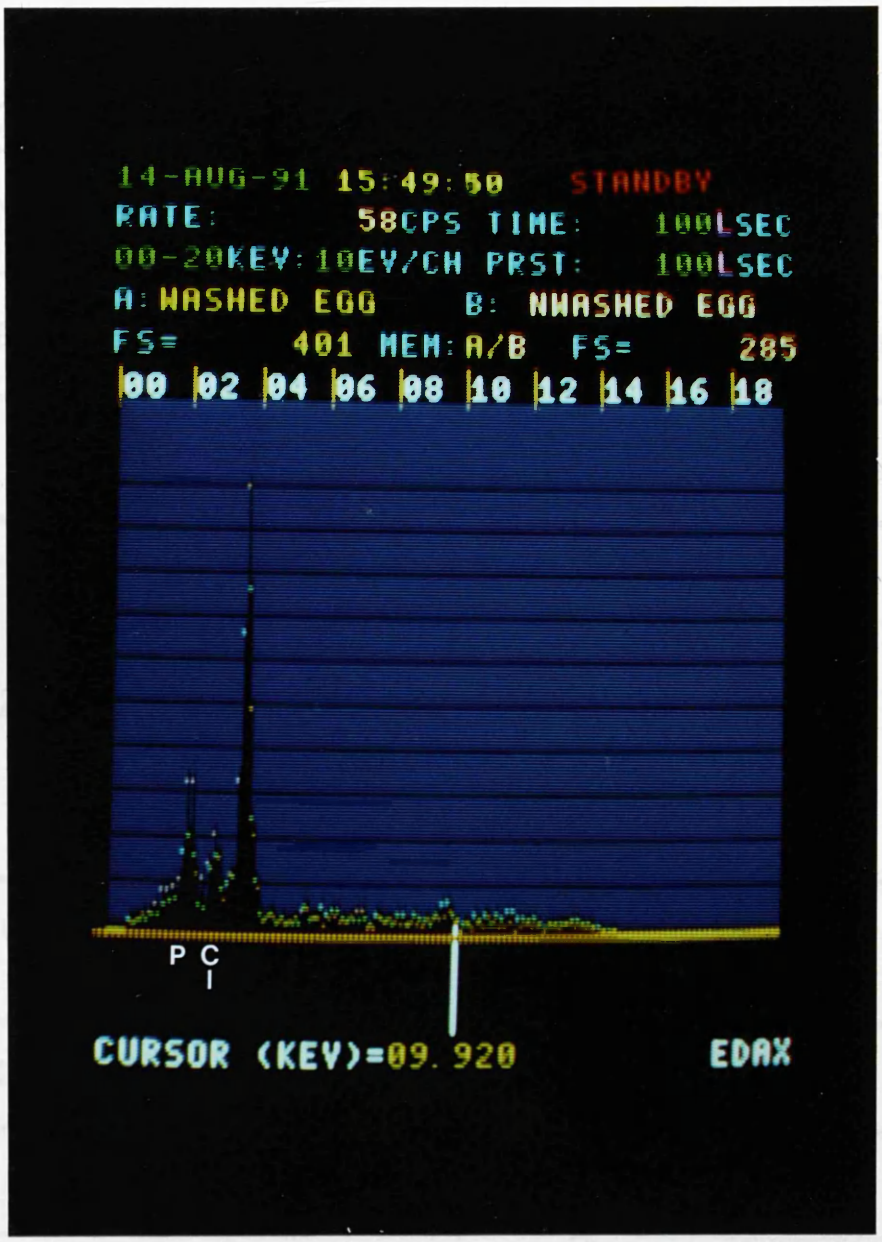


Figure 74. X - ray analysis of unwashed / washed mammillary surface. The green dots relate to the unwashed sample and the yellow to the washed. Chlorine and phosphorus peaks indicate that the levels are higher in the washed sample.

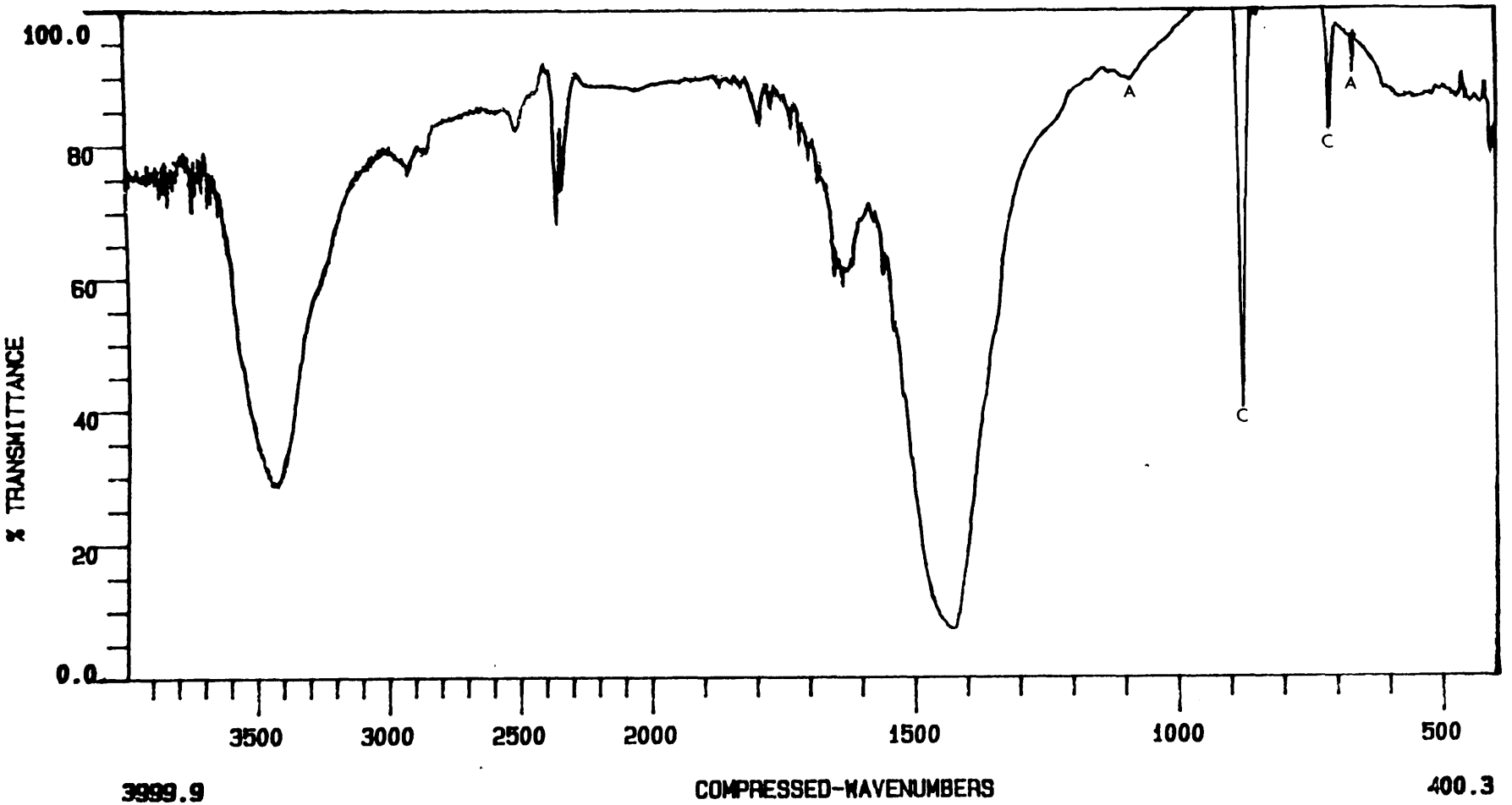
TABLE I. BACTERIOLOGICAL ANALYSIS.

BRUSH WASH	BEFORE WASHING	AFTER WASHING
BEGINNING	PSEUDOMONAS spp.	PSEUDOMONAS spp.
MIDDLE	PSEUDOMONAS spp.	PSEUDOMONAS spp.
END	PSEUDOMONAS spp.	PSEUDOMONAS spp.

TABLE 2. PROTEIN ANALYSIS.

BRUSH WASH	BEFORE WASHING Protein (µg./ml.)	AFTER WASHING Protein (µg./ml.)
BEGINNING	172	349
MIDDLE	82	148
END	65	153

GRAPH 2. INFRA-RED ANALYSIS.



Spectra illustrates calcite peaks (C) at 879 & 715 and Aragonite peaks (A) at 1100 & 675

5. ROTARY ACTION MACHINE.

5-1 CUTICLE DAMAGE - VISUAL.

The eggs used in the Rotary and Jet wash machines were both from Strain B. Figure 75 illustrates the unwashed controls for both machines. At the beginning of lay the cuticle was fairly evenly distributed. As evidenced by the decrease in staining reaction, the rotary wash removed much of the cuticular layer (Figure 76).

5-1 a CUTICLE DAMAGE - SCANNING MICROSCOPY.

The cuticular damage following rotary washing although less dramatic than the brush action was nevertheless as damaging, with the eggs positioned around the periphery of the bucket being subjected to the greatest gravitational force (Figures 77-82). The images obtained were consistent with a rubbing movement.

5-2 SANITISER.

Three structurally different crystal deposits were found on the cuticular surface:- cubic, rounded and rod shaped. Figures 83-85 illustrate the cubic and round deposits lying on the surface of undamaged and damaged cuticular layers. Figures 86, 87 show them lying within the "open" palisade layer of a cuticleless egg. The rod shaped deposits shown in figures 88, 89 look like budding bacteria (unconfirmed), nevertheless it should be noted that it was in the post-wash sample from this machine that rod shaped bacteria were cultured. Edax analysis proved that the cubic and rounded shaped crystals from the sanitiser were rich in phosphorus and chlorine (Figure 90).

5-3 BACTERIOLOGICAL ANALYSIS.

The prewash samples were all clear of micro-organisms but the cultured post wash samples from the three periods of lay had Bacillus spp., Staphylococcus aureus, Aeromonas hydrophilia, and Gram -ve rods which were neither identified as Yersinia spp. nor as Salmonella spp. in the middle of lay wash, but as Yersinia spp. at the end of lay (Table 3). These are all potential food poisoning organisms.

5-4 PROTEIN ANALYSIS.

The protein concentration at all three points of lay showed an increase in the postwashed sample (Table 4).

Figure 75. Eggs stained with edicol supra green - Rotary and jet controls - Beginning of lay.

Figure 76. Eggs stained with edicol supra green - Rotary washed eggs - Beginning of lay.

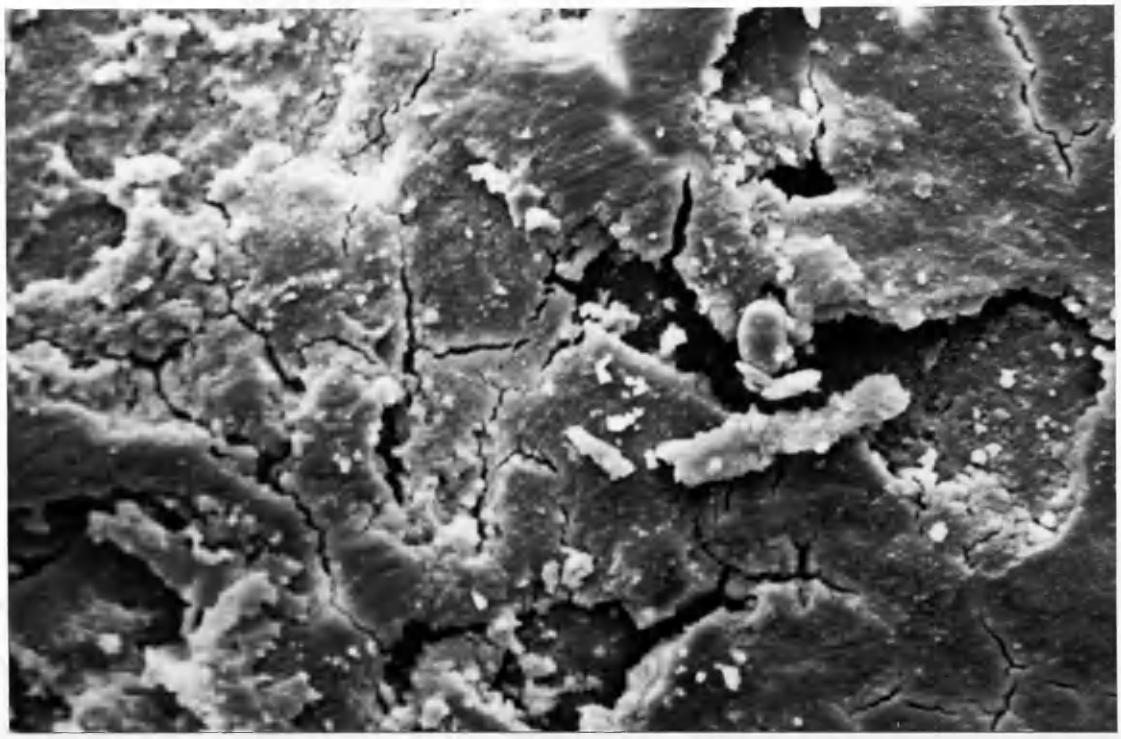


Figure 77. Rotary wash - Disrupted cuticle - Beginning of lay x 1,440

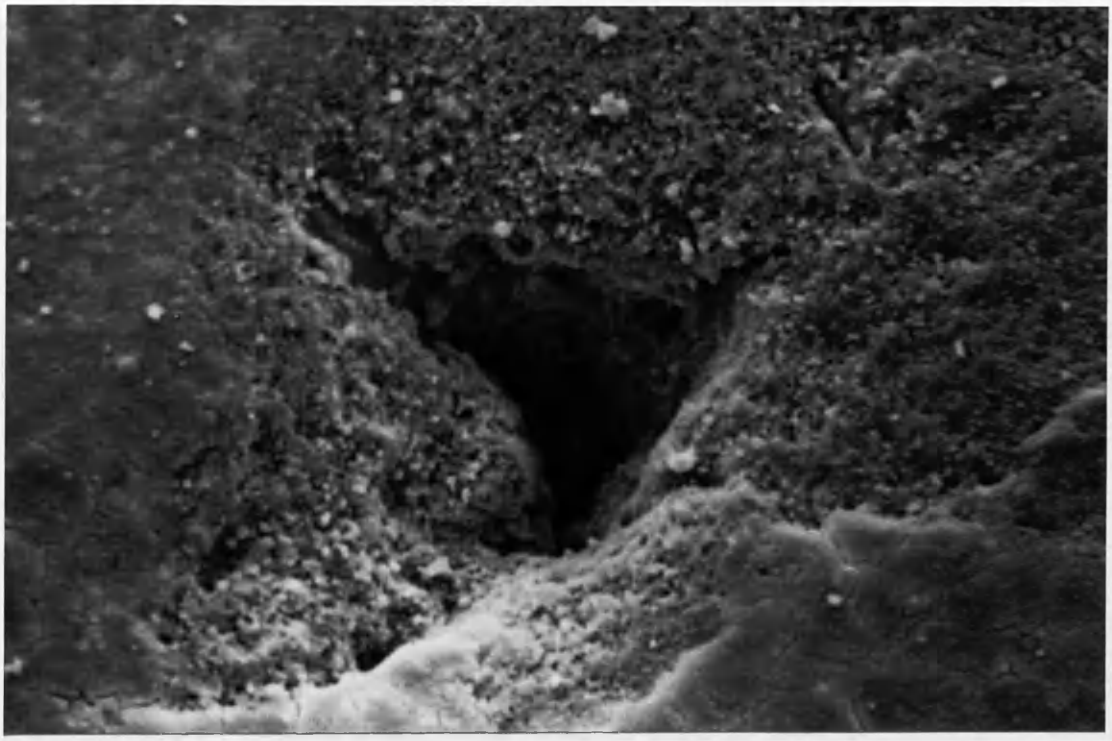


Figure 78. Rotary wash - Exposed palisade layer - Beginning of lay x 1,440

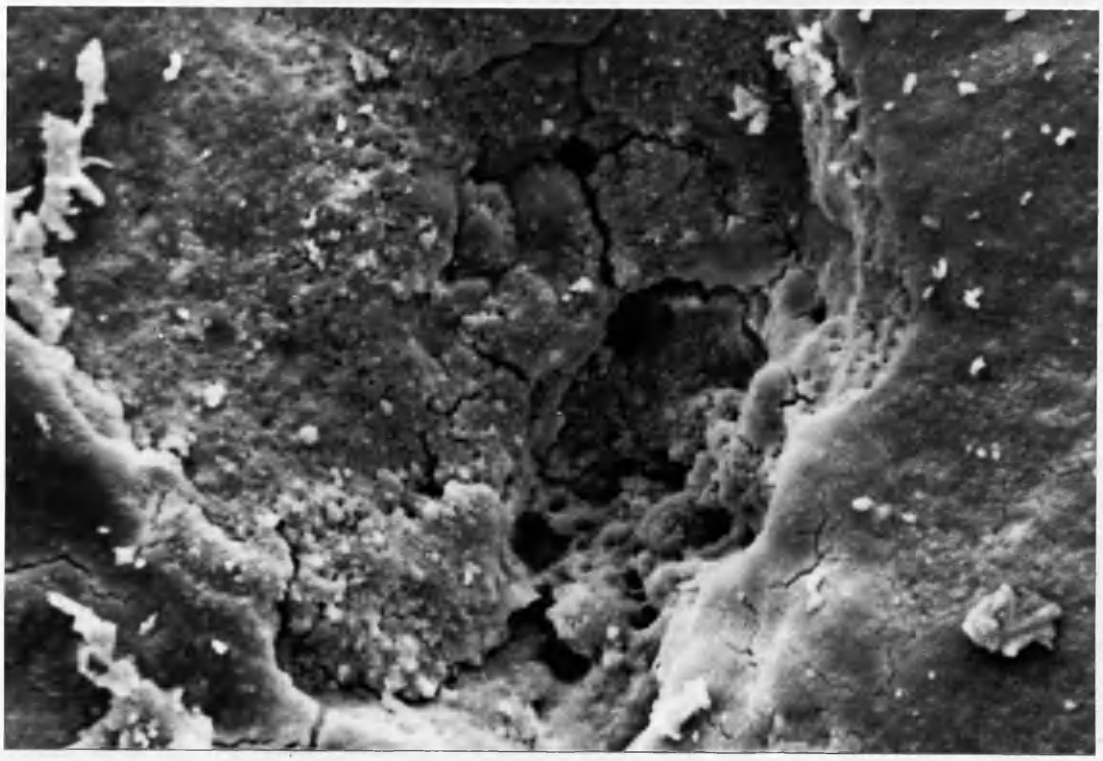


Figure 79. Rotary wash - Disrupted cuticle - Beginning of lay x 1,440

Figure 80. Rotary wash - Exposed patent pore - Beginning of lay x 1,440

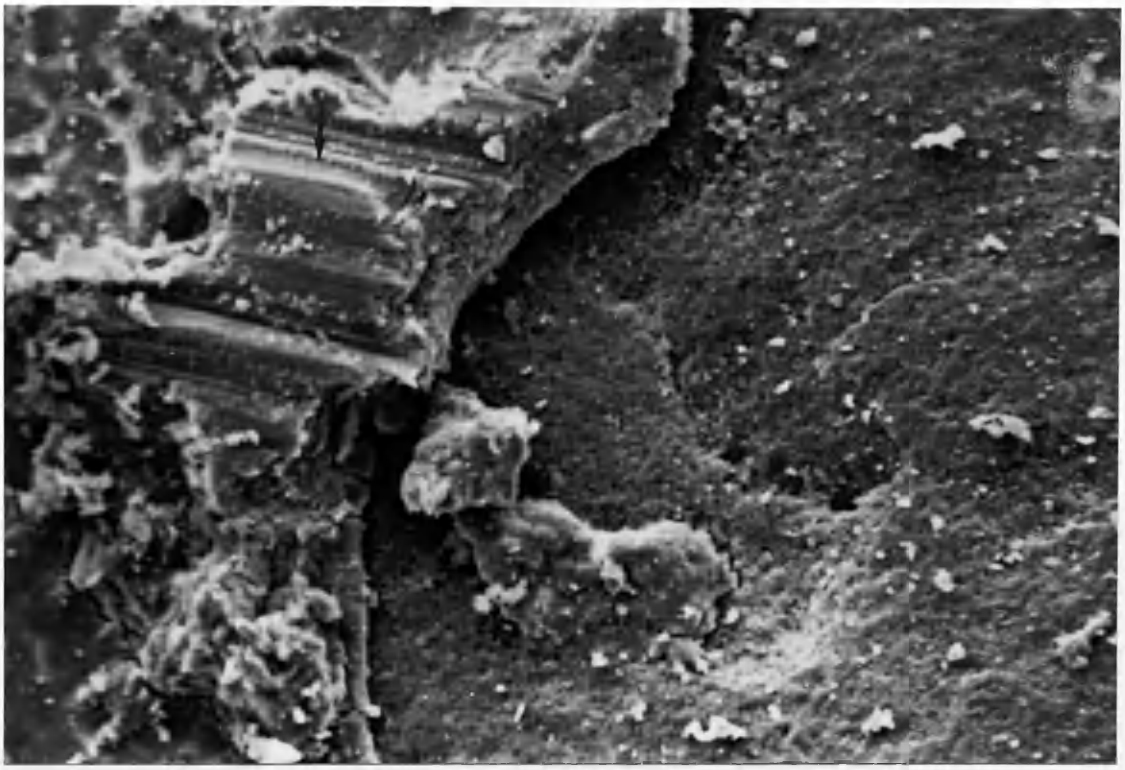


Figure 81. Rotary wash - Disrupted cuticle - Middle of lay. The ridges (arrow) may reflect pressure of the egg against the edge of the bucket x 720

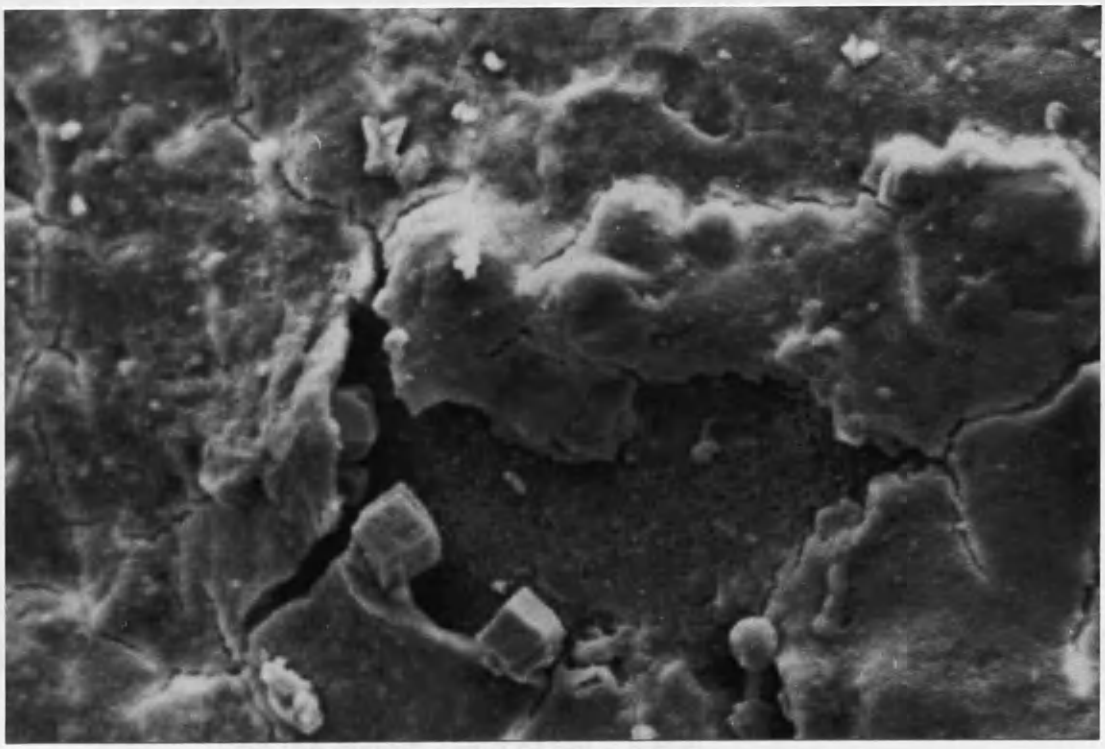


Figure 82. Rotary wash - Exposed palisade layer and cubic calcite - End of lay x 1440

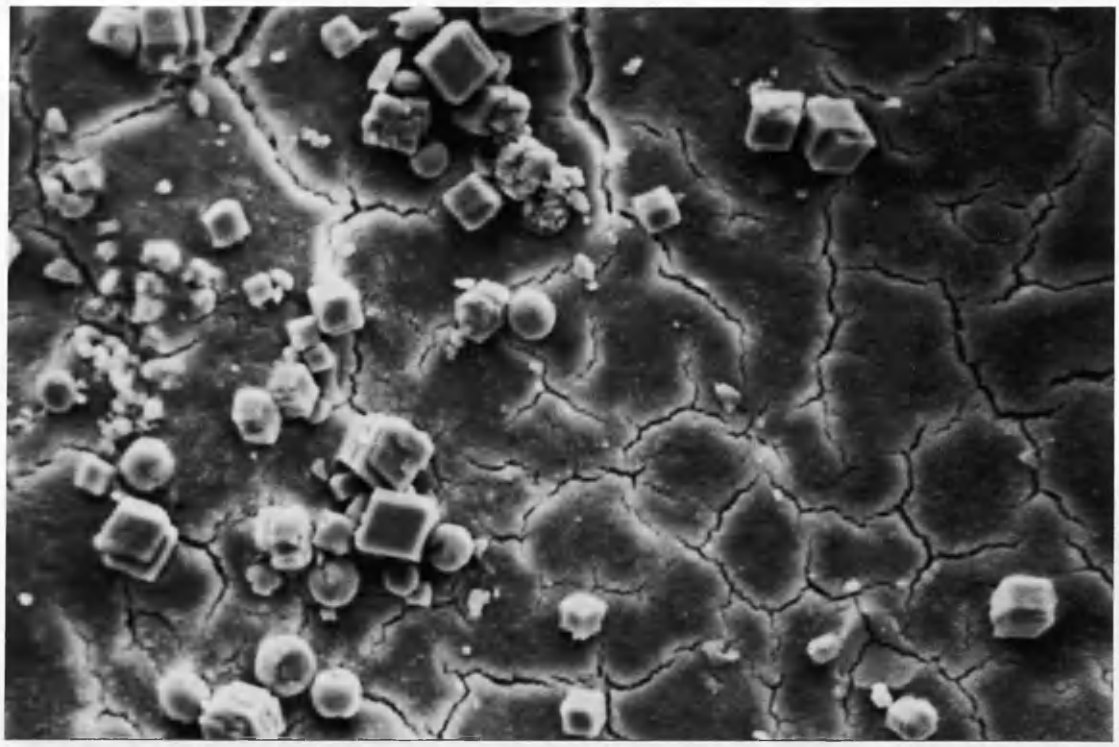


Figure 83. Rotary wash - Deposits on undamaged cuticle - End of lay x 1,440

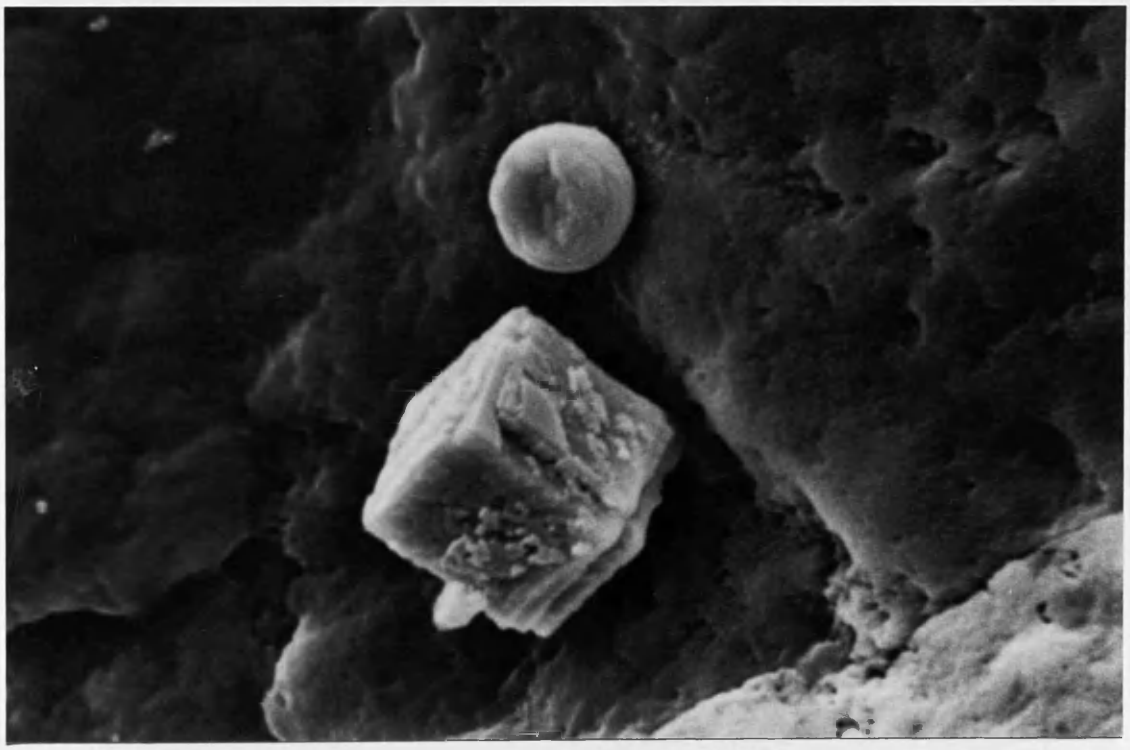


Figure 84. Rotary wash - Cubic and rounded phosphorus rich deposits on the cuticle - End of lay x 5,600

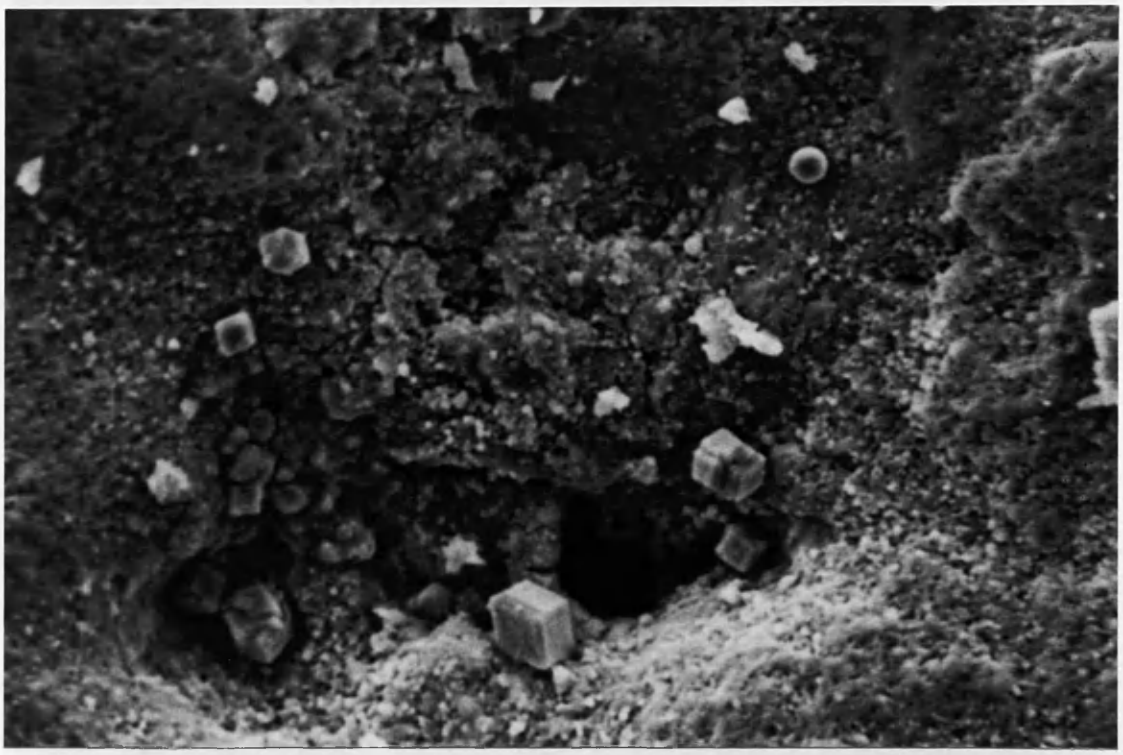


Figure 85. Rotary wash - Deposits on disrupted cuticle and palisade layer x 1440

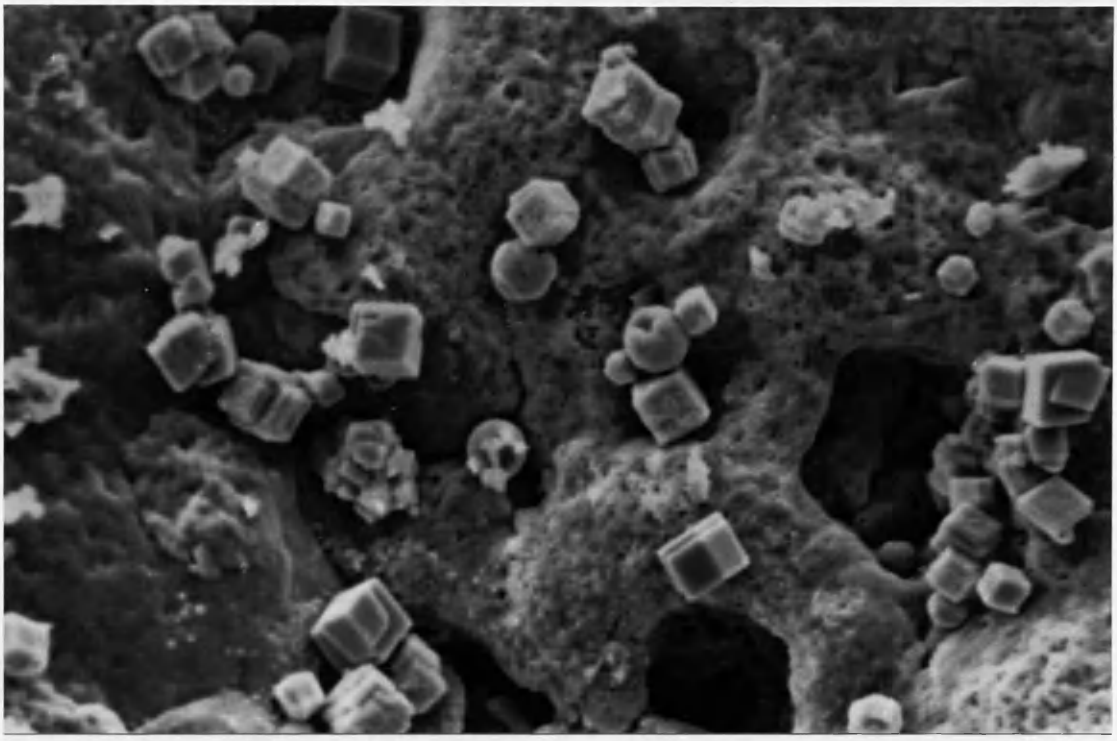


Figure 86. Deposits on cuticleless egg - End of lay x 1,440

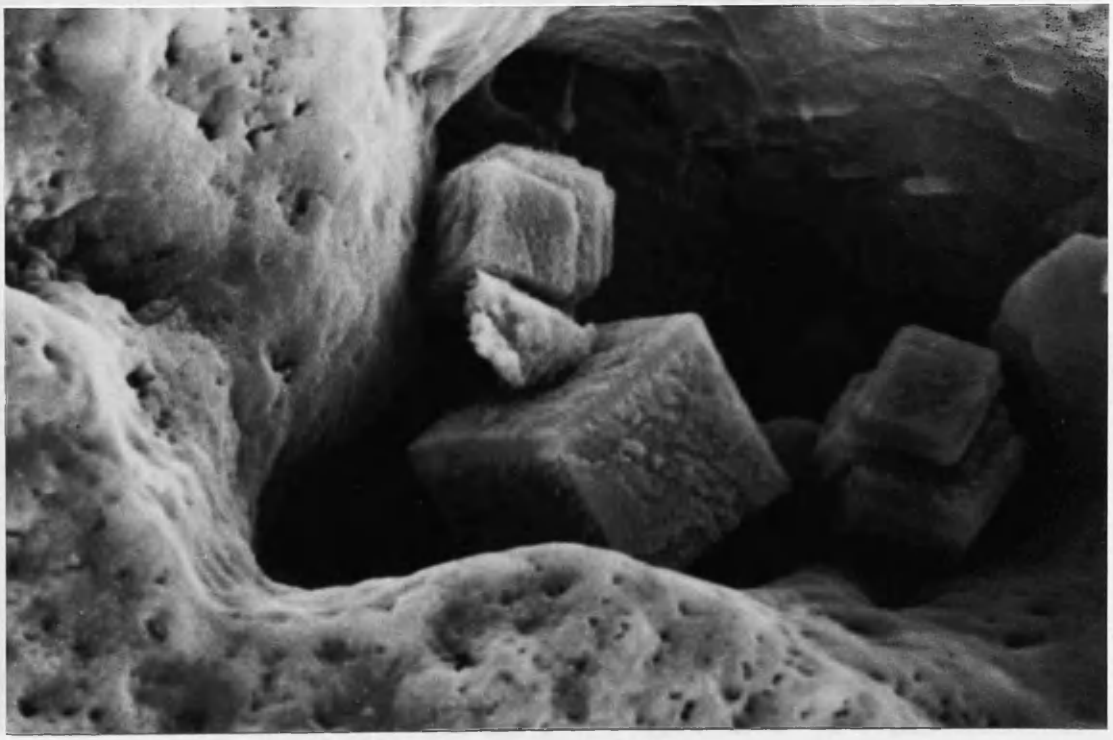


Figure 87. Deposits within the palisade layer of the cuticleless egg - End of lay x 5,600

Figure 88. Rotary wash - Rod shaped deposits on the cuticular surface - End of lay x 5,600

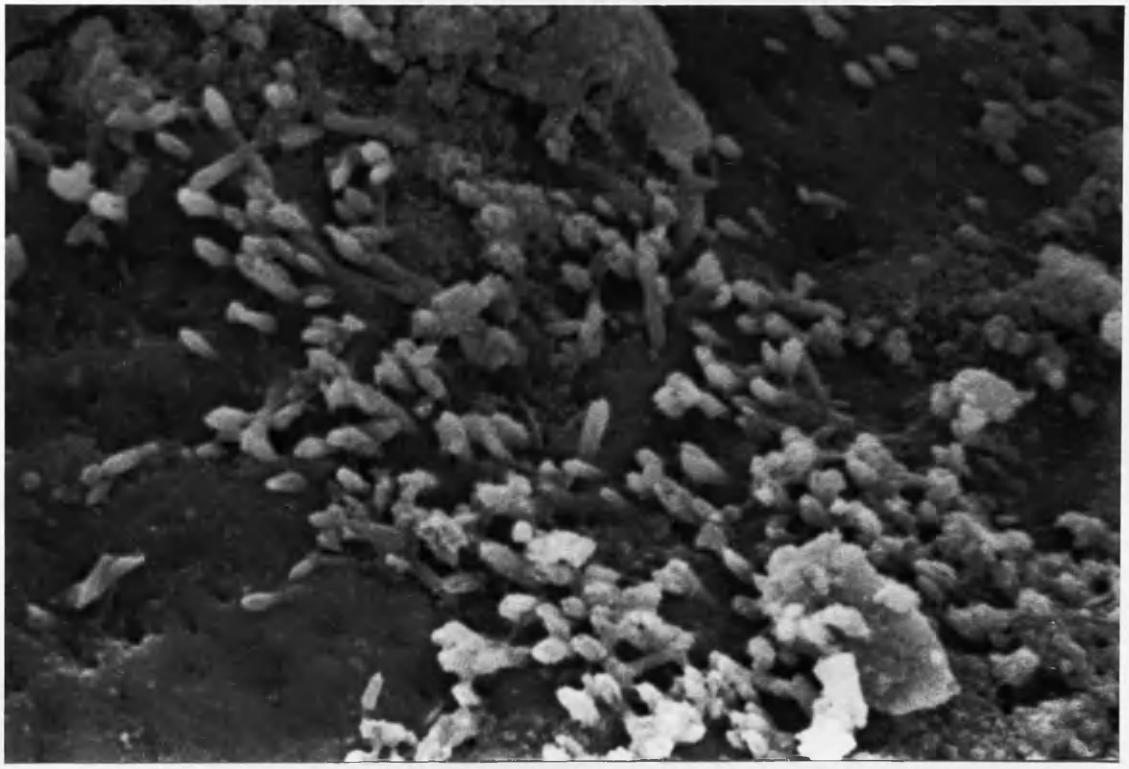


Figure 89. Rotary wash - Rod shaped deposits on the palisade layer where the cuticle has been disrupted - End of lay x 5,600

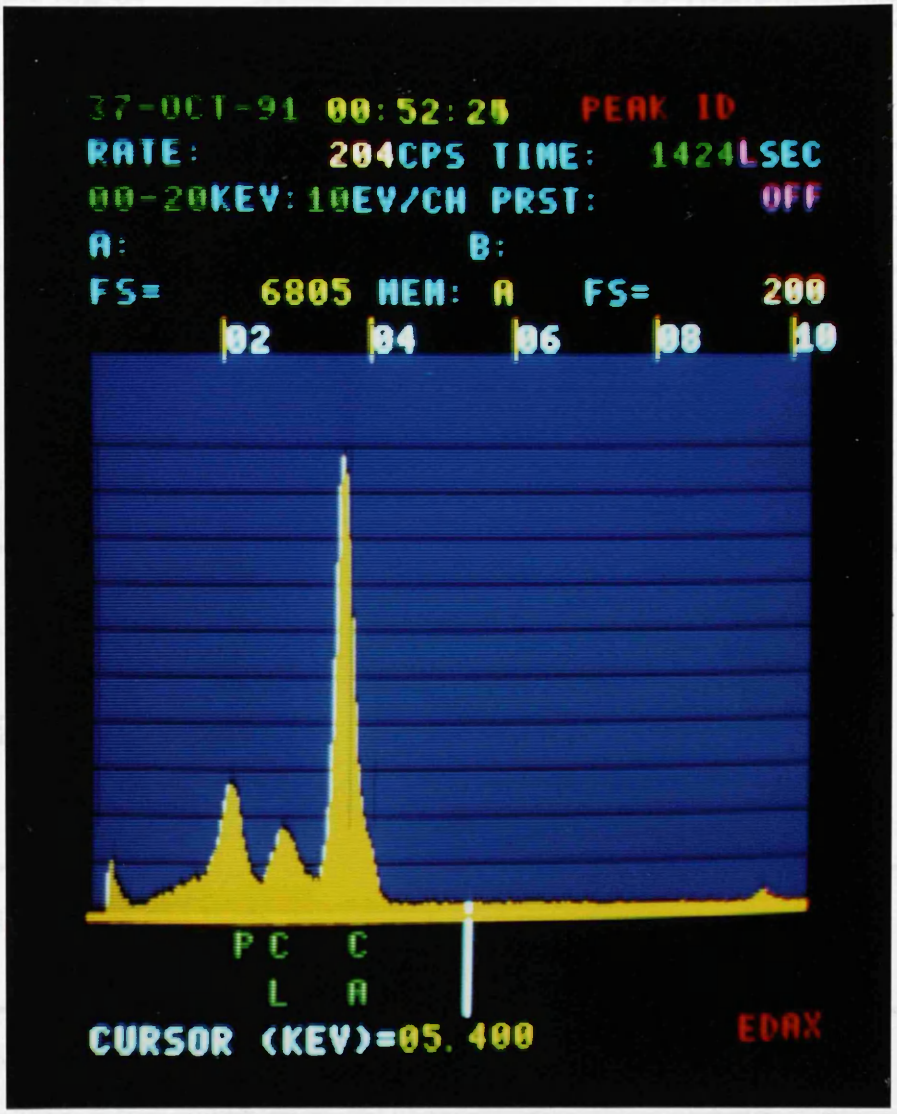


Figure 90. X - ray analysis of the deposits on the cuticle after rotary wash

TABLE 3. BACTERIOLOGICAL ANALYSIS.

ROTARY WASH	BEFORE WASHING	AFTER WASHING
BEGINNING	NONE	BACILLUS spp.
MIDDLE	NONE	GRAM -VE RODS*
END	NONE	STAPHYLOCOCCUS aureus AEROMONAS hydrophila YERSINIA

TABLE 4. PROTEIN ANALYSIS.

ROTARY WASH	BEFORE WASHING Protein (µg./ml.)	AFTER WASHING Protein (µg./ml.)
BEGINNING	41	54
MIDDLE	17	26
END	15	30

* NEITHER SALMONELLA NOR YERSINIA

6. JET ACTION MACHINE.

6-1 CUTICLE DAMAGE - VISUAL.

Figure 91 shows the cuticle intact after this washing action. 4 out of the 25 eggs from the end of lay still had adherent faeces and blood after washing (Figure 92).

6-1a CUTICLE DAMAGE - SCANNING MICROSCOPY.

Jet action washing did cause some damage to the cuticle as illustrated in Figure 93. Figure 94 demonstrates droplets of albumen, fragments of feed and a hair sitting on the cuticle.

6-2 SANITISER.

No deposits were found with this sanitiser.

6-3 BACTERIOLOGICAL ANALYSIS.

Only the prewash sample from the beginning of lay was clear. All subsequent samples had micro-organisms present which were identified as Acinetobacter lwoffii, Aeromonas hydrophilia, Chromobacteria spp., ∂-haemolytic Streptococcus, ß-haemolytic Streptococcus, E. coli ß-haemolytic and E. coli non haemolytic (Table 5). These are relatively harmless with the exception of Aeromonas hydrophilia which is a potential food poisoning organism.

6-4 PROTEIN ANALYSIS.

The protein concentration in the postwash sample was greater than the prewash sample from the middle and end of lay but neither of the samples from the beginning of lay contained protein (Table 6).

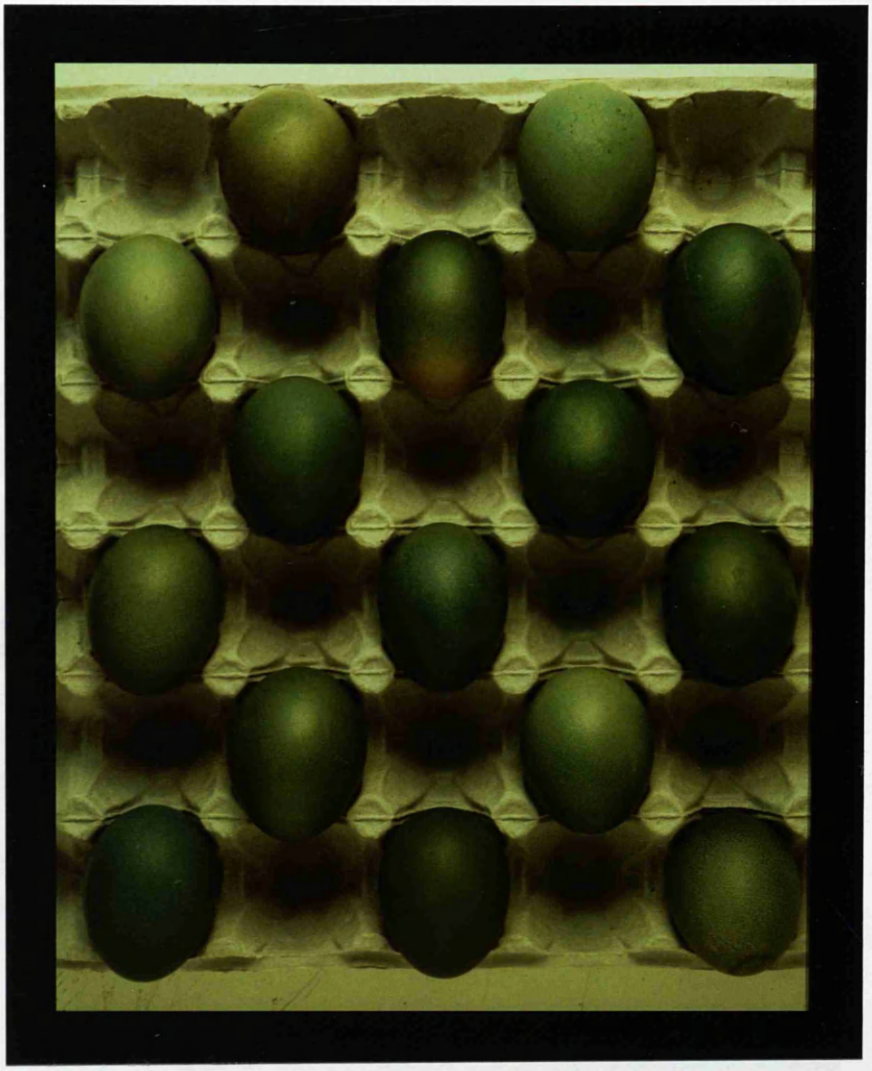


Figure 91. Eggs stained with edicol supra green - Jet washed eggs - Beginning of lay.

Figure 92. Jet washed eggs - End of lay. Four out of twenty five washed eggs had faecal deposits or blood spots attached after washing.

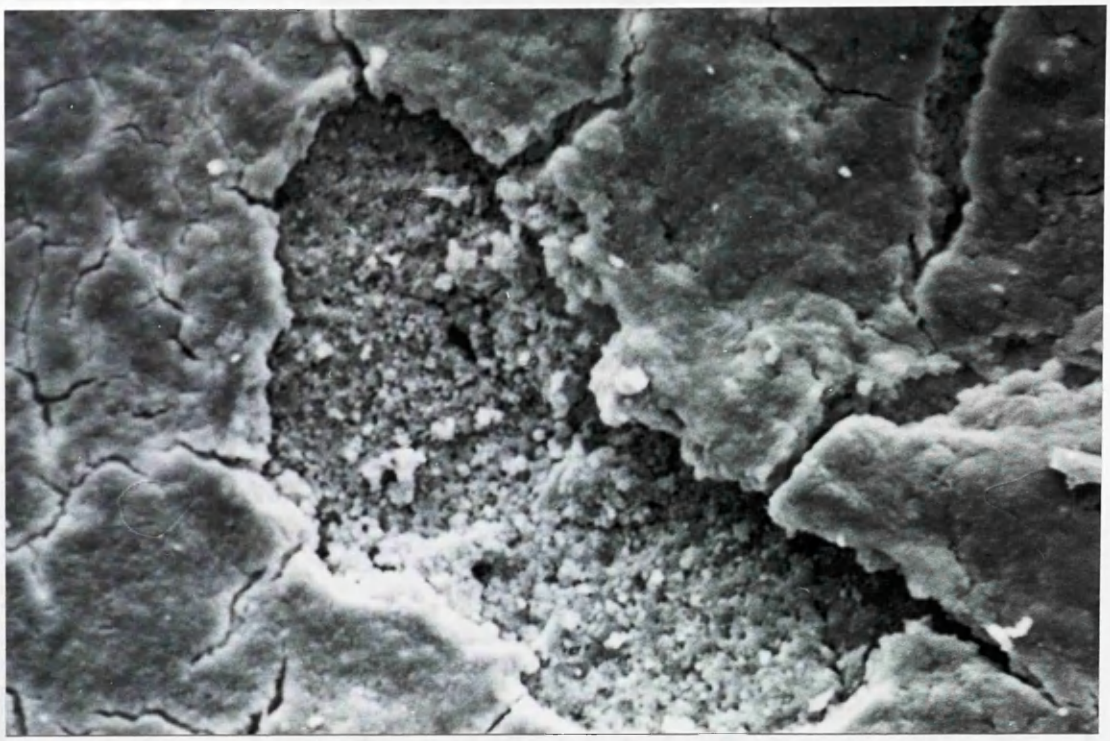


Figure 93. Jet wash - Disrupted cuticle. This micrograph is typical of the damage to the cuticle at all three periods of lay x 1,440

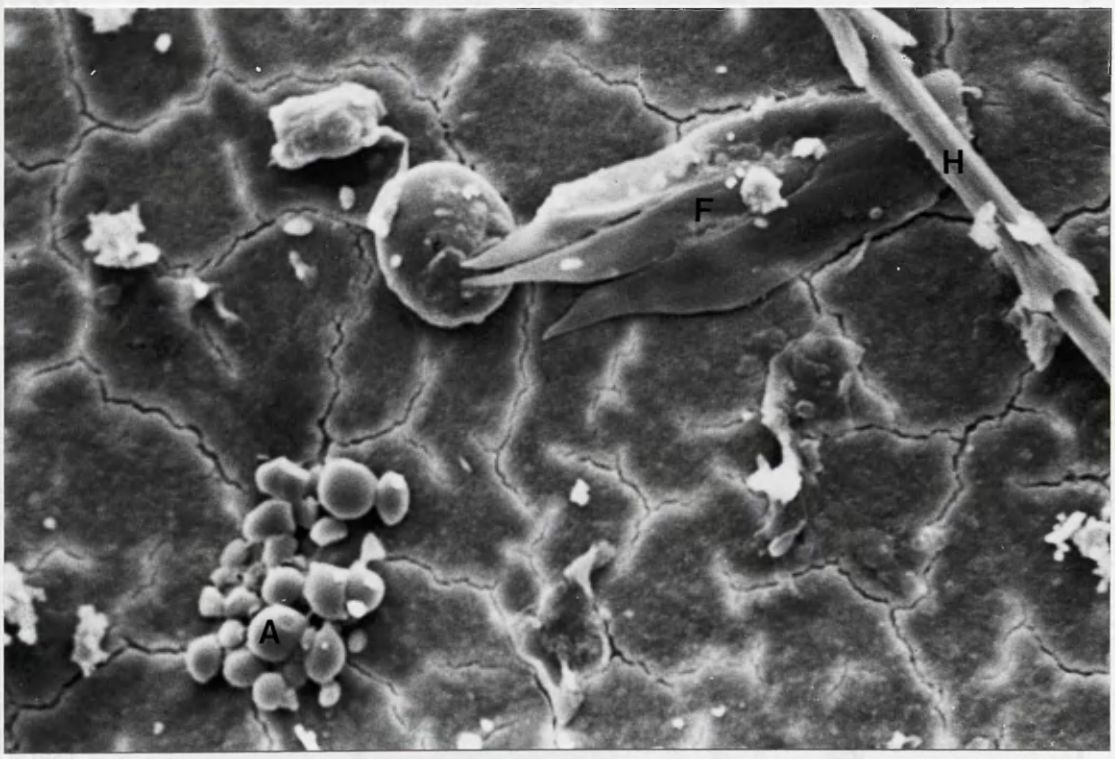


Figure 94. Debris on cuticle. This micrograph displays a variety of debris. Droplets of albumen (A), fragments of feed (F) and Hair (H) x 1,440

TABLE 5. BACTERIOLOGICAL ANALYSIS.

JET WASH	BEFORE WASHING	AFTER WASHING
BEGINNING	NONE	ACINETOBACTER lwoffii
MIDDLE	AEROMONAS hydrophila CHROMOBACTERIA spp. ∂-haemolytic STREPTOCOCCUS ß-haemolytic STREPTOCOCCUS	AEROMONAS hydrophilia CHROMOBACTERIA spp. ∂-haemolytic STREPTOCOCCUS ß-haemolytic STREPTOCOCCUS
END	E. COLI ∂-haemolytic E. COLI non haemolytic ACINETOBACTERIA spp.	E. COLI ∂-haemolytic E. COLI non haemolytic

TABLE 6. PROTEIN ANALYSIS.

JET WASH	BEFORE WASHING Protein (µg./ml.)	AFTER WASHING Protein (µg./ml.)
BEGINNING	NONE	NONE
MIDDLE	6	10
END	25	50

7. SALMONELLA enteritidis.

Figures 95-98 illustrate the morphology of Salmonella enteritidis Phage type 4 at light and ultrastructural level.

8. TRANSFER OF SALMONELLA enteritidis ACROSS THE SHELL.

8-1 The transfer of Salmonella enteritidis appears to be encouraged by certain washing actions (Graph 3).

8-2 TABLE 7. (Appendix 1).

The eggs washed by the brush action showed a significant difference in microbial transfer at the end of lay. It is worth noting that of the 25 unwashed eggs challenged at the beginning of lay only 1 allowed bacterial transfer (100%) while of the 25 washed eggs challenged 10 permitted bacterial transfer ranging from10%-72%. Rotary and jet systems did not appear to influence bacterial transfer at any point of the laying year. The level of microbial transfer (unwashed and washed) ranged between 2%-40%.

8-3 TABLES 8, 9. (Appendices 2, 3).

Only the eggs washed in the brush action machine and their controls, i.e. Strain A eggs showed a significant difference in bacterial penetration with respect to age.

T

8-4 TABLES 10, 11.(Appendices 4, 5).

Bacterial penetration was at its highest in the control group of the brush wash system at mid lay and peaked in the washed eggs from the same system at the middle of lay also.

8-5 TABLES 12, 13.

Shell structure varies with bird age irrespective of strain, although genetically linked differences exist.

8-6 TABLE 14.

Strain differences were obvious at the beginning and end of lay. Strain A displayed a statistically significant increase ($P \leq 0.05$) in the incidence of cap imperfections, fewer type B bodies and more of the phenomenon described as cuffing ($P \leq 0.01$). At the end of lay Strain B eggs displayed less confluence ($P \leq 0.001$) and less changed membrane.

8-7 The Regression Analysis carried out to investigate if there was a correlation between the structural variation and the microbial penetration at all three periods of lay was inconclusive.

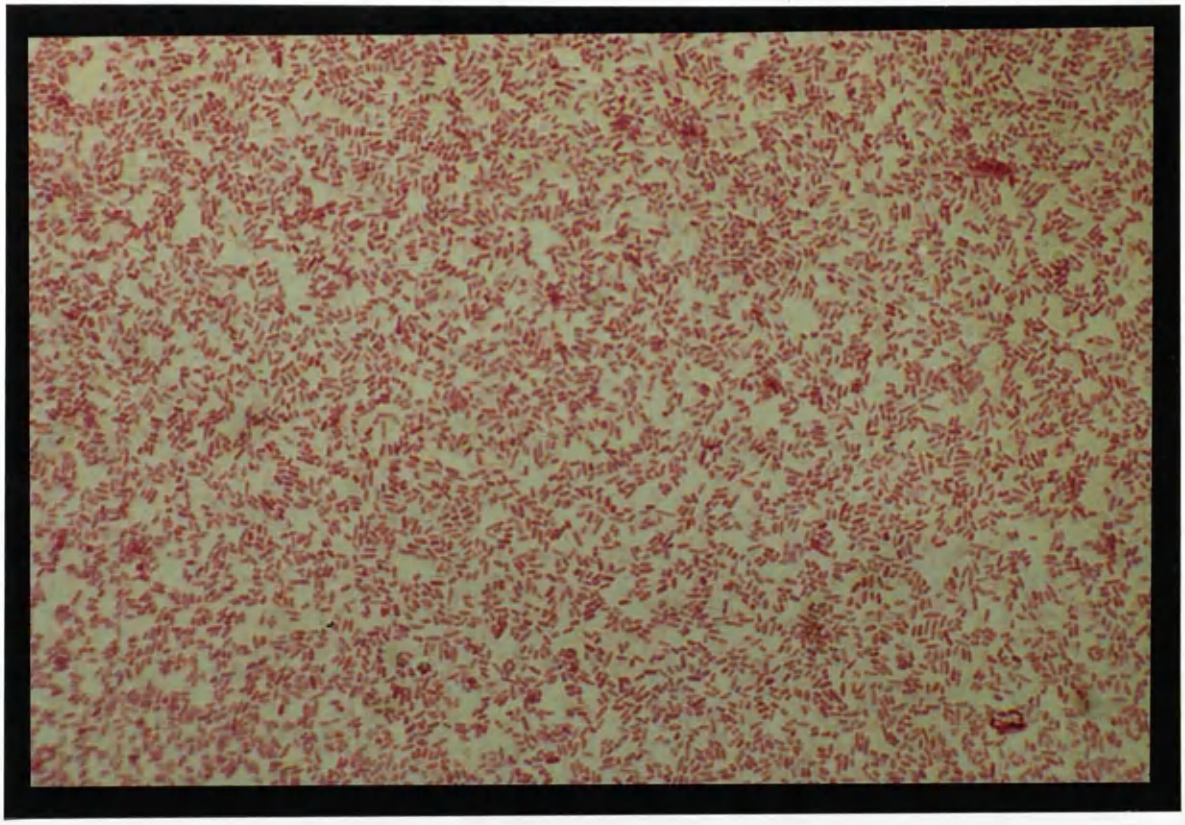


Figure 95. Salmonella enteritidis (Gram -ve rods) stained with Gram Jensen stain x 1,000

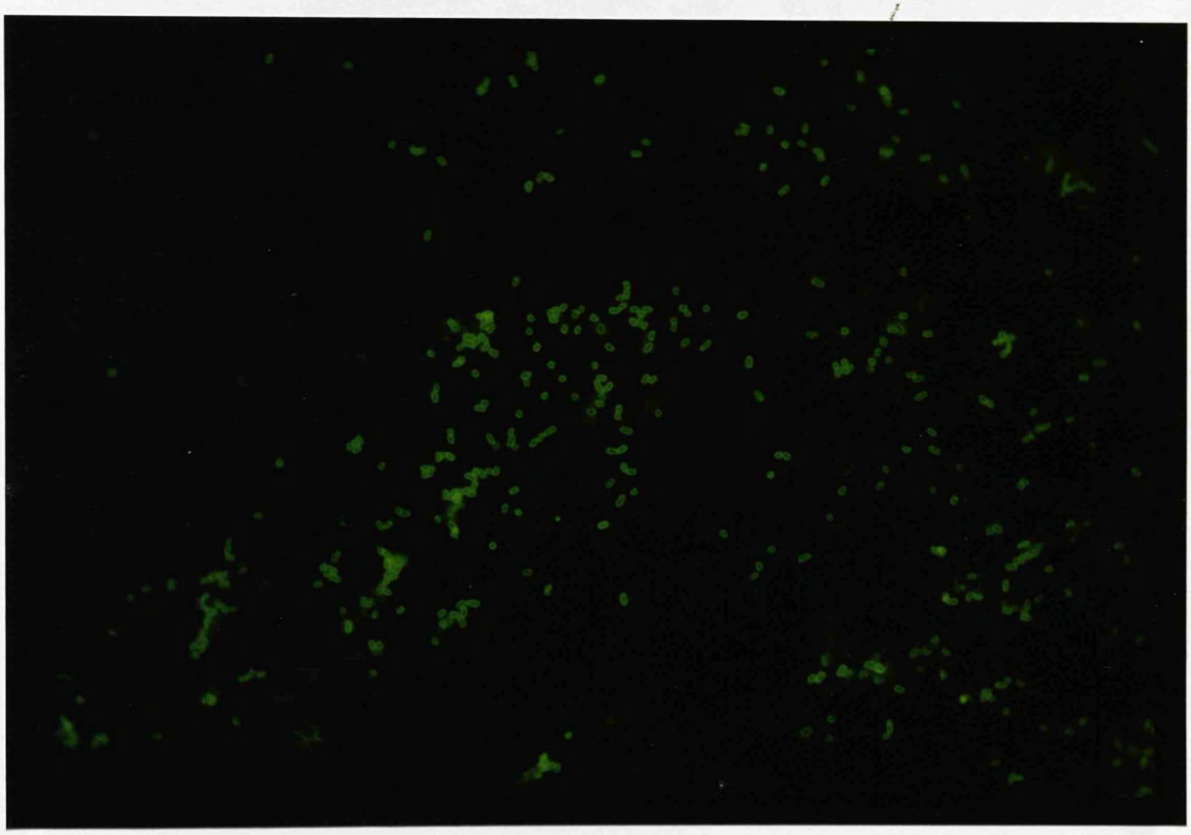


Figure 96. Salmonella enteritidis as a fluorescent antibody x 1,000

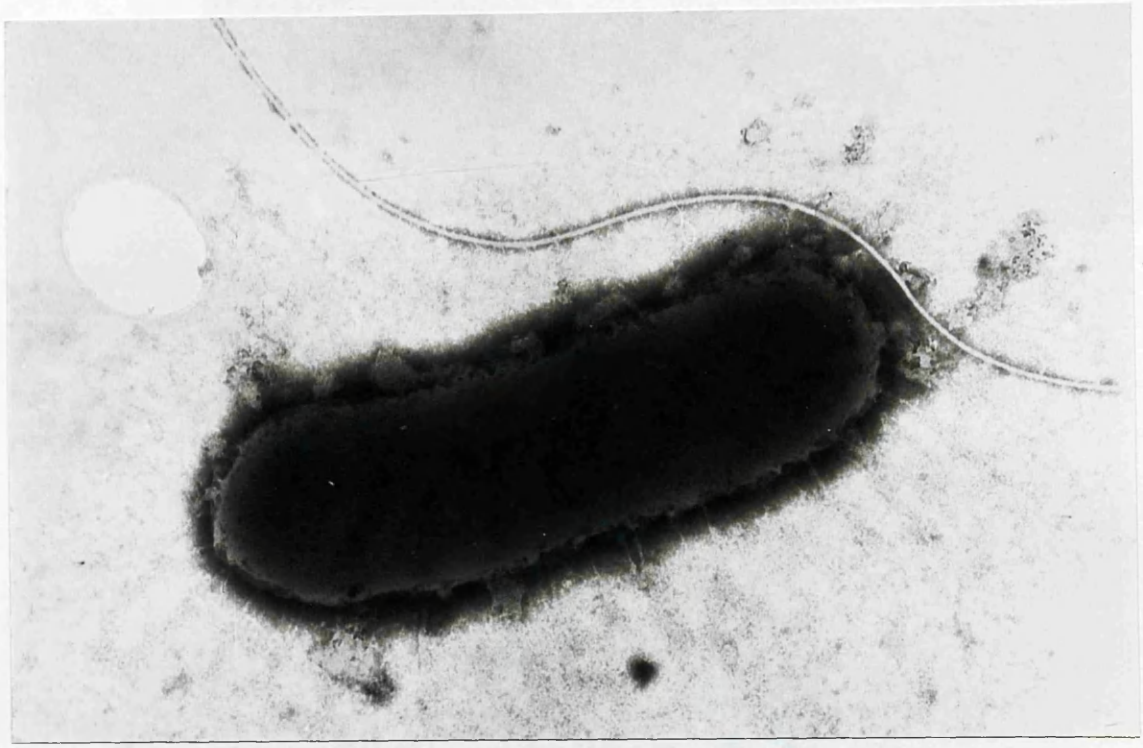


Figure 97. Transmission electron micrograph of Salmonella enteritidis, negatively stained x 51,000

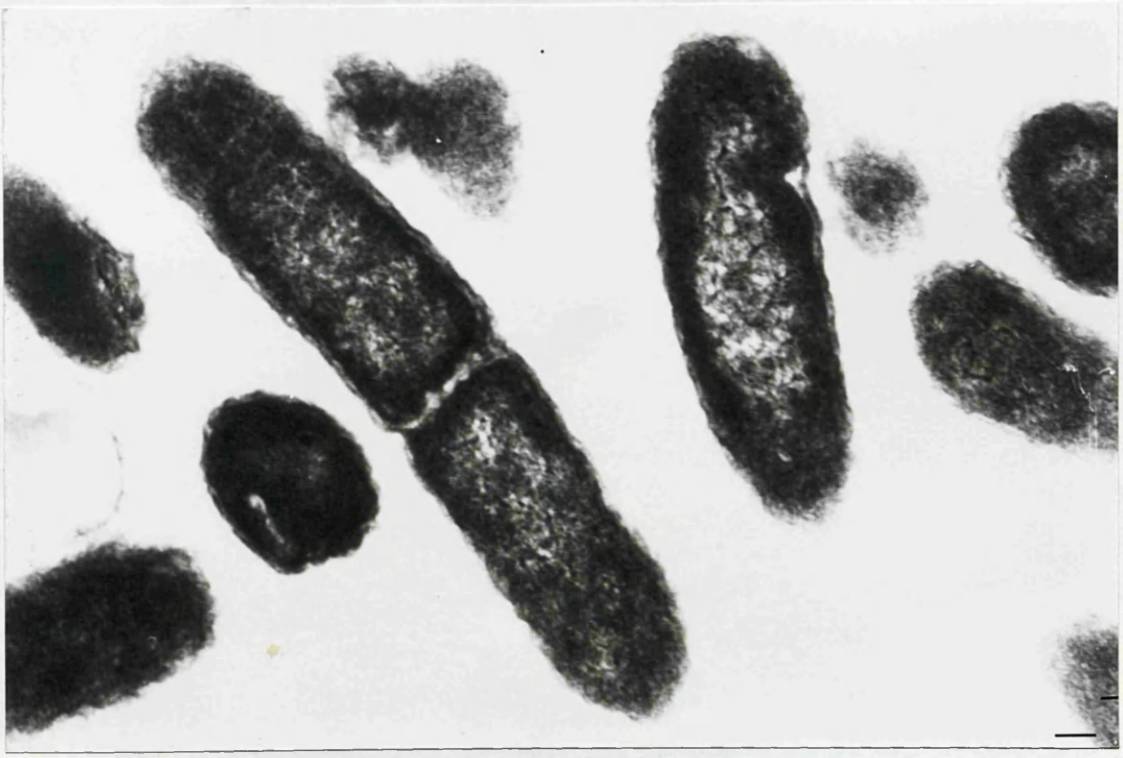
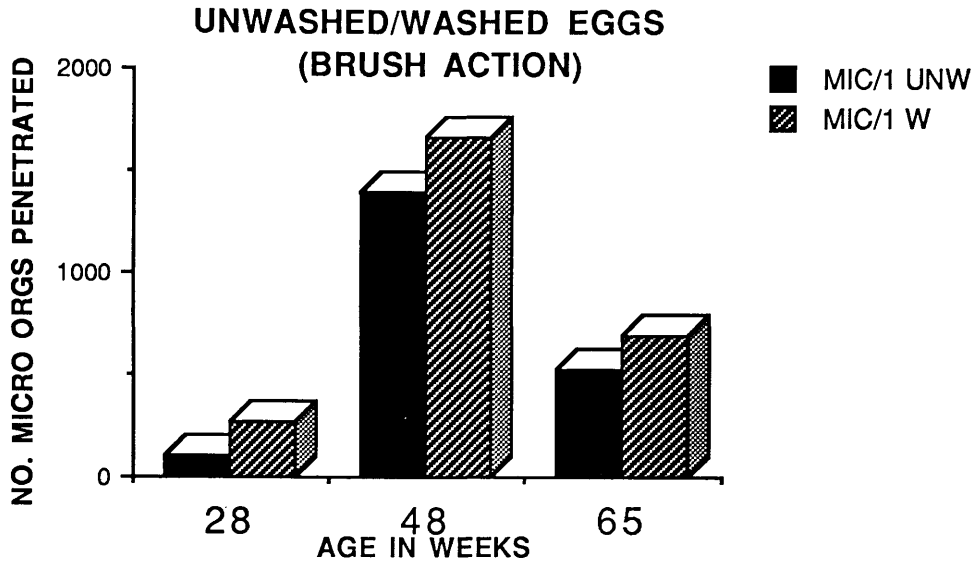
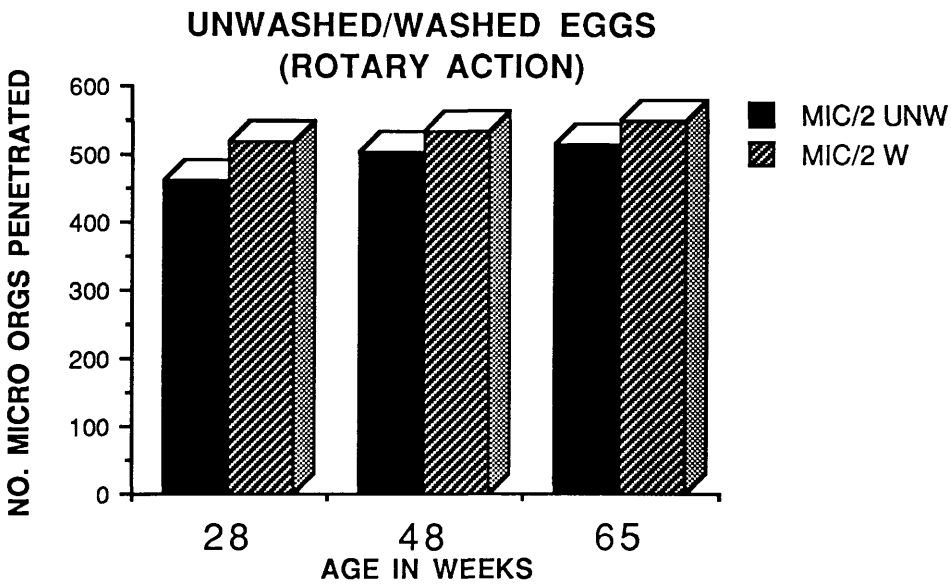


Figure 98. Transmission electron micrograph of Salmonella enteritidis x 51,000

GRAPH 3. MICROBIAL PENETRATION AT THE DIFFERENT PERIODS OF LAY.





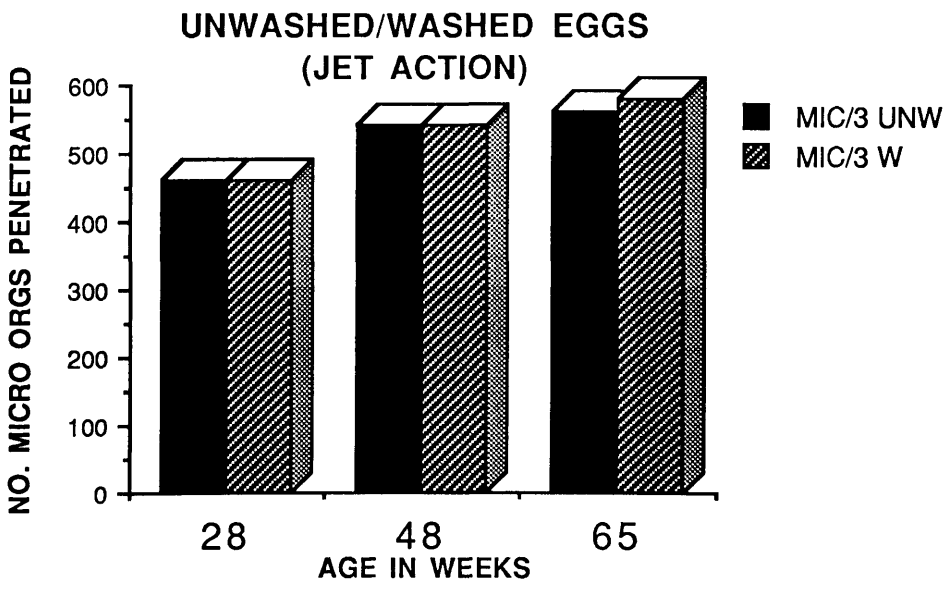


TABLE 7.

COMPARISON OF MICROBIAL TRANSFER OF UNWASHED AND WASHED EGGS AT DIFFERENT PERIODS OF LAY.

	BEGINNING		MIDDLE		END	
	UNW.	W.	UNW.	W.	UNW.	W.
	% PEN.	%PEN.	% PEN.	% PEN.	% PEN.	% PEN.
BRUSH	4.00	10.56	56.12	66.44	20.76	27.68 **
ROTARY	18.40	18.76	22.92	21.48	20.64	19.16
JET	18.40	20.80	22.92	21.32	20.64	19.56

*** = Very highly significant at a 0.1% level ($p < 0.001$)
** = highly significant at a 1% level ($p < 0.01$)
* = Significant at a 5% level ($p < 0.05$)

TABLE 8.

COMPARISON OF MICROBIAL TRANSFER 0F UNWASHED EGGS AT DIFFERENT PERIODS OF LAY.

	BEGINNING	MIDDLE	END	B/E
	%PENETRATION	%PENETRATION	%PENETRATION	
BRUSH	4.00±20.00	56.12±17.93 ***	20.76±7.51 ***	* *
ROTARY	18.40±9.42	22.92±9.30	20.64±4.77	
JET	18.40±9.42	22.92±9.30	20.64±4.77	

*** = Very highly significant at a 0.1% level ($p < 0.001$)
** = highly significant at a 1% level ($p < 0.01$)
* = Significant at a 5% level ($p < 0.05$)

TABLE 9.

COMPARISON OF MICROBIAL TRANSFER 0F WASHED EGGS AT DIFFERENT PERIODS OF LAY.

	BEGINNING	MIDDLE	END	B/E
	%PENETRATION	%PENETRATION	%PENETRATION	
BRUSH	10.56±19.29	66.44±22.77***	27.68±6.52***	* *
ROTARY	18.76±8.89	21.48±4.08	19.16±5.47	
JET	20.80±7.51	21.32±6.00	19.56±4.66	

*** = Very highly significant at a 0.1% level ($p < 0.001$)
** = highly significant at a 1% level ($p < 0.01$)
* = Significant at a 5% level ($p < 0.05$)

TABLE 10.

CORRELATION 0F MICROBIAL TRANSFER OF THE UNWASHED EGGS OF THE BRUSH, ROTARY AND JET ACTION MACHINES AT THE DIFFERENT PERIODS OF LAY.

	BRUSH	ROTARY	JET	B / J	B / R / J
	% PEN.	% PEN.	% PEN.		
BEGINNING	4.00±20.00	18.40±9.42 **	18.40±9.42	* *	* *
MIDDLE	56.12±17.93	22.92±9.30***	22.92±9.30	* * *	* * *
END	20.76±7.51	20.64±4.77	20.64±4.77		

*** = Very highly significant at a 0.1% level ($p < 0.001$)
** = highly significant at a 1% level ($p < 0.01$)
* = Significant at a 5% level ($p < 0.05$)

TABLE 11.

CORRELATION OF MICROBIAL TRANSFER OF THE WASHED EGGS FROM THE BRUSH, ROTARY AND JET ACTION MACHINES AT THE DIFFERENT PERIODS OF LAY.

	BRUSH	ROTARY	JET	B / J	B / R / J
	% PEN.	%PEN.	% PEN.		
BEGINNING	10.56±19.29	18.76±8.89	20.80±7.51	*	*
MIDDLE	66.44±22.77	21.48±9.30***	21.32±6.00	* * *	* * *
END	27.68±6.52	19.16±5.47***	19.56±4.66	* * *	* * *

*** = Very highly significant at a 0.1% level ($p < 0.001$)
** = highly significant at a 1% level ($p < 0.01$)
* = Significant at a 5% level ($p < 0.05$)

TABLE 12.

AGE ASSOCIATED VARIATIONS IN THE MAMMILLARY LAYER & CUTICLE

STRAIN A.

VARIATION	STRAIN A			
	BEGINNING	MIDDLE	END	B/E
CONFLUENCE	5.52±0.87	4.76±1.05 **	5.24±1.16	
CAPS	1.08±0.40	2.24±1.27 ***	2.36±1.85	* * *
EARLY FUSION	2.12±0.60	2.08±0.40	2.56+0.92 *	*
LATE FUSION	3.40±1.22	3.12±0.60	3.84±1.37 *	
MAM. ORG.	2.08±0.40	2.16±0.55	2.12±0.60	
TYPE B's	1.64±0.49	2.00±1.22	2.88±1.74 *	* * *
PITTING	1.48±1.66	1.48±1.33	3.40±2.83 **	* *
ARAGONITE	1.12±0.33	1.36±0.86	1.72±1.31	*
TYPE A's	1.28±0.46	1.16±0.37	1.20±0.41	
CUBICS	1.40±0.50	1.40±0.50	1.28±0.46	
CUFFING	4.48±0.51	4.64±0.86	4.88±0.33	* *
CH. MEM.	3.16±1.37	5.20±4.72 *	2.24±1.85 **	*
CUTICLE	1.36±0.70	1.64±0.91	1.76±0.83	
TOTAL SCORE	28.76±2.99	31.76±4.41**	33.72±6.78	* *

*** = Very highly significant at a 0.1% level ($p < 0.001$)
** = highly significant at a 1% level ($p < 0.01$)
* = Significant at a 5% level ($p < 0.05$)

TABLE 13.

AGE ASSOCIATED VARIATIONS IN THE MAMMILLARY LAYER & CUTICLE

STRAIN B.

VARIATION	STRAIN B			
	BEGINNING	MIDDLE	END	B / E
CONFLUENCE	5.04±1.02	4.72±1.40	3.88±1.54	* *
CAPS	1.60±1.22	2.04±1.51	2.20±1.50	
EARLY FUSION	2.00±0.00	2.16±1.55	2.28±0.79	
LATE FUSION	3.00±0.00	3.24±0.83	3.40±1.22	
MAM. ORG.	2.08±0.40	2.16±0.55	2.00±0.00	
TYPE B's	1.32±0.48	2.08±1.98	2.72±2.05	* *
PITTING	1.00±0.00	1.64±1.50 *	3.88±2.32 ***	* * *
ARAGONITE	1.08±0.28	1.56±1.12 *	1.60±1.12	*
TYPE A's	1.04±0.20	1.16±0.37	1.16±0.37	
CUBICS	1.60±0.87	1.44±0.51	1.48±0.87	
CUFFING	4.84±0.37	4.84±0.37	4.84±0.37	
CH.MEM.	3.52±1.12	3.84±0.94	1.36±0.99 ***	* * *
CUTICLE	1.56±0.82	1.48±0.65	1.48±0.51	
TOTAL SCORE	28.08±2.10	30.88±5.63 *	30.80±5.52	*

*** = Very highly significant at a 0.1% level ($p < 0.001$)
** = highly significant at a 1% level ($p < 0.01$)
* = Significant at a 5% level ($p < 0.05$)

TABLE 14.

STRUCTURAL VARIATIONS IN THE MAMMILLARY LAYER & CUTICLE: STRAIN COMPARISONS.

VARIATION	BEGINNING		MIDDLE		END	
	STRAIN A	STRAIN B	STRAIN A	STRAIN B	STRAIN A	STRAIN B
CONFLUENCE	5.52±0.87	5.04±1.02	4.76±1.05	4.72±1.40	5.24±1.16	3.88±1.54***
CAPS	1.08±0.40	1.60±1.22 *	2.24±1.27	2.04±1.51	2.36±1.85	2.20±1.50
EARLY FUSION	2.12±0.6	2.00±0.00	2.08±0.40	2.16±1.55	2.56±0.92	2.28±0.79
LATE FUSION	3.40±1.22	3.00±0.00	3.12±0.60	3.24±0.83	3.84±1.37	3.40±1.22
MAM. ORG.	2.08±0.40	2.08±0.40	2.16±0.55	2.16±0.55	2.12±0.60	2.00±0.00
TYPE B's	1.64±0.49	1.32±0.48 *	2.00±1.22	2.08±1.98	2.88±1.74	2.72±2.05
PITTING	1.48±1.66	1.00±0.00	1.48±1.33	1.64±1.50	3.40±2.83	3.88±2.32
ARAGONITE	1.12±0.33	1.08±0.28	1.36±0.86	1.56±1.12	1.72±1.31	1.60±1.12
TYPE A's	1.28±0.46	1.04±0.20*	1.16±0.37	1.16±0.37	1.20±0.41	1.16±0.37
CUBICS	1.40±0.50	1.60±0.87	1.40±0.50	1.44±0.51	1.28±0.46	1.48±0.87
CUFFING	4.48±0.51	4.84±0.37 **	4.64±0.86	4.84±0.37	4.88±0.33	4.84±0.37
CHANGED MEMB.	3.16±1.37	3.52±1.12	5.20±4.72	3.84±0.94	2.24±1.85	1.36±0.99*
CUTICLE	1.36±0.70	1.56±0.82	1.64±0.91	1.48±0.65	1.76±0.83	1.48±0.51
TOTAL SCORE	28.76±2.99	28.08±2.10	31.76±4.41	30.88±5.63	33.72±6.78	30.80±5.52

*** = Very highly significant at a 0.1% level ($p < 0.001$)
** = highly significant at a 1% level ($p < 0.01$)
* = Significant at a 5% level ($p < 0.05$)

DISCUSSION.

An industry which is responsible for the throughput of 500,000 cases of table eggs per week cannot afford itself the luxury of a critical evaluation of individual product quality and so blanket measures are applied, which in the case of the egg are unable to take account of the diversity of structure which exists therein.

Quality is essentially subjective, with cleanliness, size and colour all reflected in the final price, the fact that none of these parameters gives a true indication of shell quality or the nutritional value of the egg's contents is seemingly irrelevant.

It is natural, considering its provenance that the egg will come into contact with faecal material. In the battery system, cage design minimises this situation and in general terms, the eggs from such systems are relatively clean. Public antipathy towards this system of intensive rearing has in recent years, in this country, seen a move towards alternative systems of housing in which birds have greater freedom of movement. Leaving aside the reported increases in feather pecking and leg disorders, these systems also increase the incidence of floor egg laying and so a greater likelihood of faecal contact. Dirty eggs do not achieve a price premium and it is hypothesised that under these circumstances egg washing or cleaning will be practised.

The egg washing debate has been aired for many years. In 1919, Jenkins and Pennington concluded that egg spoilage during storage was the result of moisture on the shells subsequent to washing. In a later paper Jenkins et al. (1920) demonstrated increased spoilage under experimental conditions using either water or dilute sulphuric acid. Grzimek (1936) reported that both dry cleaned and untreated dirty eggs survived storage better than

their washed counterparts. Haines (1938) developed the argument to suggest that washing assisted bacterial penetration particularly when the cuticle was damaged. At oviposition the egg emerges from a warm, humid environment into an atmosphere appreciably cooler. As the egg dries, the cuticle, that theoretical first line of defence, dries and shrinks and it has been hypothesised that the process can drag bacteria into the shell either via patent pore sites or through the fissures created in the shell by the drying process (Simons and Wiertz 1970).

The eggshell rarely conforms to its textbook image as illustrated by Solomon (1991) and underlined in this thesis. Cuticular diversity is the norm and the seemingly tough impenetrable barrier is easily abraded during the washing action.

With reference to the brush action machine, this part of the whole exercise was most edifying in so far as the serious damage first reported to the company concerned was subsequently reduced by altering brush pressure. It must be noted however, that the damage was invisible to the naked eye and the process would have progressed unchecked without the interim report.

None of the machines tested left the cuticular layer intact, although the degree of damage did vary, with the jet action wash emerging at the top of the league table in terms of minimal physical damage to the product. Sanitiser induced cuticular damage has been reported (Simons and Wiertz 1966). The authors observed changes in the appearance of the cuticle which had been subjected to Nusan. This particular sanitiser was used in the rotary action machine and so the observed changes must also be interpretated with due recognition to its presence. In the leaflet supplied with the machine, the recommended concentration of

sanitiser is three level measures per load but the packing station had reduced this level to one measure per load. The supplier also recommended changing the wash water when it was "dirty".

This subjective appraisal of hygiene levels gives cause for concern and it is perhaps significant to note that the post wash water from this machine harboured several potentialy dangerous micro-organisms. Whether the packing station was cognisant of the effect of Nusan on the cuticle and so was taking avoiding reaction by reducing the concentration of sanitiser is unknown, but their flaunting of the rules only served to increase the danger of contamination to eggs being damaged by the pressure of packing. The manufacturers of this machine also recommend no after "rinse" and the end result, as illustrated, was the precipitation of salts from the wash water on to the outer surface of the shell.

Rotary cleaners have received criticism from a variety of sources, yet they are still in use, reflecting the theory voiced by some sectors of the industry that if an egg can be rendered clean then the method by which it is effected is immaterial so long as it is cheap. The observation that in many instances the wash water contained a bacterial suspension prior to the washing procedure draws attention to the inherent problems associated with the maintenance of a high standard of cleanliness in the plant. Bacteria are brought into the wash water via adherent faecal material and other soiling agents and their response to the temperature and pH of the wash water is not uniform, thus Laird et al. (1990) observed that Listeria monocytogenes survives the normal pH and temperature of washing machines (pH 10.5, temperature 45^{o}C) while Salmonella spp. requires pH>10 to prevent its survival (Holley and Proulx 1986). According to Southam et al. (1987) alkaline conditions permit the survival of Yersinia

enterocolitica.

According to Williams and Dillard (1973) the partial or complete removal of the cuticular layer by washing permits rapid penetration of the remaining true shell by bacteria such as Salmonella, with moisture facilitating the process (Tung et al. 1979). These results corroborate the earlier findings of Grzimek (1936) and Lorenz and Starr (1952) who reported the more protective nature of a "dry" shell. En route from the cloaca, the shell is moist and at this point it is most vulnerable to bacterial penetration from faecal material (Sparks 1985). As the cuticle matures, it in theory assumes a more protective function. In the present trials, irrespective of the type of wash action cuticular damage was sustained with a trend to increased bacterial penetration after washing. That, this was not due solely to cuticular damage will be discussed subsequently.

The egg, with its nutritious yolk mass intended for embryo development, is designed to withstand a certain degree of mechanical trauma and to minimise bacterial transfer. Thus, in addition to the cuticular layer, the shell, in its own right provides a physical barrier to ingress. The structural integrity of the shell is variable and diet, age and strain have all been shown to influence its formation (Solomon 1991). The paired shell membranes which surround the yolk and albumen and support the growth of the shell, also afford some protection with the inner membrane providing the more effective barrier to the translocation of bacteria (Vadehra and Baker 1972). According to Lifshitz et al. (1964) the inner membrane is even more effective than the shell as an impediment to bacterial movement. Nevertheless when large innocula are used membrane resistance is quickly breached (Board et al. 1968) underlining the temporary nature of their protective function.

Whether bacteria physically penetrate the membranes via the interstices between the intersecting fibres or digest their way in through enzyme action is a matter of debate (Hartung and Stadelman 1963).

Egg white affords resistance to bacterial action through its complement of lysozyme and conalbumen and its high pH. Lysozyme inactivates bacteria by attacking the cell wall and conalbumen binds certain ions such as iron which are essential for bacterial multiplication. The latter process is facilitated by the rising pH values of albumen during storage. If bacteria reach the yolk mass then they will multiply unchecked. The yolk is held within the albumen by means of the chalazae (Solomon 1991). These twisted strands which originate from the albumen as it rotates distally, keep the germ cell central. During storage, the table egg should be held with the air cell uppermost, so as to maintain the yolk in its central position. If this condition is not met then with the gradual deterioration of albumen through water loss, the yolk will juxtapose to the membranes and so provide easier access to potentially harmful organisms.

Given that each or all of the barriers can be breached and that the outermost cuticular layer is a questionable first line of defence, does the structural organisation of the shell afford any protection? It is recognised that shell quality declines with bird age (Solomon 1985; Watt 1985; Bain 1990; Nascimento 1990). Add to the age effect, environmental effects such as heat (Izat et al. 1985), lighting (Leeson et al. - unpublished results) and stress (Watt 1989) then structural variation would appear to be the "norm". The amount of shell deposited increases linearly with the time spent by the egg in the pouch region and evidence has been presented to suggest that eggs laid in the afternoon are better than

those laid in the morning, the theory being that during the hours of daylight more calcium is consumed (Roland et al. 1973; Choi et al. 1981). According to Hurwitz (1978) shell quality is a reflection of the interval between individual oviposition times, the time of oviposition, the rate of shell deposition and the uterine environment.

This statement has since been developed by the work of Reid (1985), Watt (1989), Bain (1990), Nascimento (1990) and Solomon (1991). In their respective analyses of eggshells from a variety of strains and under different systems of husbandry, the authors have identified a number of structural variants, some indicative of oviducal malfunction in regions anterior to the pouch and others providing conclusive evidence of a change in the rate of mineralisation, and hence in the form of calcium carbonate deposited. The present results confirm these findings with the respect to the decline in shell quality and structure with bird age.

During the execution of this study, the eggs produced by Strain A birds declined in quality at 48 weeks of age to such an extent that the author queried "stress" effects. The company concerned subsequently verified that Infectious Bronchitis (I.B.) had been diagnosed. According to Garside (1967) in the laying bird, egg production falls 10-14 days after challenge and both internal and external quality decline. Within a four week period an improvement is observed in shell strength, although the texture and the shape of the egg are still inferior and the albumen is still watery. The latter is a classic response to I.B. (Spackman 1985). The recovery phase is difficult to time with intervals of from 4-10 weeks being reported in the literature (Jordan 1990; Cook 1968). According to the former author, after a disease challenge of this type, the expected potential production is never attained.

The morphological condition of the oviduct is crucial to normal shell formation as highlighted by the work of Crinion et al. (1971) and Watt (1989). The former experimentally infected one day chicks with I.B. and observed permanent lesions in the oviduct. Watt (1989) subjected laying birds, individually housed, to a one hour period of four birds per cage and this transient alteration in stocking density was sufficent to cause egg retention and for thirty days thereafter structural changes within the eggshell incompatible with its function as a mechically sound package. Analysis of oviducal tissue from these experimental birds revealed cell breakdown in the surface epithelial lining of the distal oviduct. The membrane deposits observed on the surface of certain eggs in figures 16 and 17 may be corroborative evidence of oviducal damage in response to I.B. It is hypothesised that clumped membrane fibres from the isthmus have either moved distally during the later stages of shell formation and so become incorporated in the forming shell or antiperistalsis during this later phase has forced the egg caudally. Irrespectively of its aetiology, the incorporation of this fibrous material into the framework of the shell is disruptive.

It is not possible from the evidence provided by the company to say when the birds were first challenged. Eggs from young birds characteristically contain a variety of crystal forms including Type B bodies (Solomon 1991). Their presence in the Strain A eggs at the beginning of lay was not therefore questioned at that point in the analysis. These structures are however also a feature of the eggs of stressed birds (Watt 1989) and so it is feasible that their inclusion was indicative of an initial response to the stress of the disease. There is no doubt that the observed breakdown in shell structure observed in both control and experimental eggs midlay

eased bacterial penetration with the attendant cuticular damage only serving to exacerbate the situation.

The eggs from Strain B flock also displayed age related structural changes, although the re-timing of the sampling programme because of the closure of the site, meant that the dramatic reduction in quality at 72 weeks of age could not be taken into account. At the middle of lay the eggs contained a number of inherent undesirable defects in the form of pitting and aragonite. The former, often caused by the accumulation of oviducal debris on the shell membrane prior to calcification inhibits normal shell growth and the space created by its presence represents an area in which stress can accumulate (Bain 1990). These points of weakness are also thinner than adjacent areas. The aragonite modification of calcium carbonate is more commonly associated with the eggs of reptiles. Under stress, birds display the capacity to form aragonite within the shell, primarily at the mammillary surface i.e. during the early phase of shell formation. They do however retain the capacity to deposit this form at any phase of shell growth as illustrated in figure 15. Aragonite, which is less stable than calcite is indicative of a rapid phase of crystal growth and one can say no more at this point other than its presence suggests a temporary imbalance in the oviducal environment.

By end of lay these structural imperfections had increased, although as illustrated in the results section, at any point in the laying year, irrespective of strain, the range of structural variants described by Bain (1990) and Solomon (1991) were present to a greater or lesser extent.

Nascimento (1990) correlated the presence of specific

structural traits with bacterial ingress. The present investigation provides some evidence to support his findings in terms of trends although the whole interpretation has been complicated by the I.B. challenge and the re-scheduling of the collection programme with respect to Strain B.

This investigation has focussed on the transfer of Salmonella enteritidis across the shell wall, but it is only one of the many strains which are of potential risk to the egg contents. According to Board (1969) an egg may contain between 9,500 - 3,100,000 organisms per shell with this number escalating to 289, 000,000 micro-organisms on the surface of extremely soiled hatching eggs. As previously stated, the principle sources of micro-organisms on the outside of shells are faecal material plus dust and dirt from the surrounding environment. While washing and sanitising will reduce the microbial load, the process does not render the egg immune from subsequent attack. The shell must function as a barrier until it parts company from its contents and so its integrity is crucial at every stage.

This thesis has served to highlight a number of issues which should now be developed. The first of these concerns the structural organisation of the product being subjected to yet another handling process. Given that so many external factors can have a detrimental influence on quality, in addition to genetic effects, then if spoilage of the egg contents occurs subsequent to washing the latter process cannot be held solely responsible if structural integrity is suspect. Washing undoubtedly causes damage to the cuticular surface, and occasionally results in egg cracking and therefore leakage of contents into the wash water. The process by its very nature removes the bacterial load, and if the plant is well maintained and the manufacturers recommendations followed there

should be no cross contamination.

In the development of machinery to carry out the washing it is relatively easy to stipulate the exclusion of "double corners" to minimise bacterial build up, to ensure that re-circulated water is passed through filters to remove organic material, to insist on the use of clean rinse water and to design a machine with the ability to discharge detergents and sanitisers at the prescribed rates and hold them at the correct temperature, but the machinery will be operated by a human being and irrespective of the number of fail safe devices incorporated there will always exist the possibility of human error. Given the fallibility of the individual and the vagaries of egg formation, the process of washing will always be open to question.

BIBLIOGRAPHY.

AGER, E.A., NELSON, K.E., GALTON, M.M. & BORING, J.R. (1967). Two Out-Breaks of Egg-Borne Salmonellosis and Implications for their Prevention. J. Amer. Med. Ass. **199:** 372-378

BAIN, M.M. (1990). Eggshell Strength: A Mechanical / Ultrastructural Evaluation. Ph.D. Thesis, University of Glasgow.

BAKER, R.C., GOFF, J.P. & MULIX, (1980). Samonella Recovery following Oral and Intravenous Innoculation of Laying Hens. Poultry Science, **59:** 1067-1072

BOARD, R.G. (1969). The Microbiology of the Hen's Egg. Advances in Applied Microbiology, **11:** 245-281

BOARD, R.G. (1975). The Microstructure of the Cuticleless Shell of the Eggs of the Domestic Hen. British Poultry Science, **16**: 89-91

BOARD, R.G. & AYRES, J.C. (1965). Influence of Temperature on Bacterial Infection of the Hen's Egg. Applied Microbiology, **13**: 358-364

BOARD, R.G. & FULLER, R. (1974). Non-Specific Antimicrobial Defences of the Avian Egg, Embryo and Neonate. Biological review, **49**: 15-49

BOARD, R.G. & HALLS, N.A. (1973). The Cuticle: A Barrier to Liquid and Particle Penetration of the Shell of the Hen's Egg. British Poultry Science, **14**: 69-97

BOARD, R.G., HENDEN, L.P. & BOARD, R.G. (1968). The Influence of Iron on the Course of Bacterial Infection of the Hen's Egg. British Poultry Science, **9**: 211-215

BRANT, A.W. & STARR, P.B. (1962). Some Physical Factors related to Egg Spoilage. Poultry Science, **41:** 1468-1473

CANTOR, A. & M^{C}FARLANE, V.H. (1948). Salmonella Organisms on and in Chicken Eggs. Poultry Science, **27:** 350

CHOI, J.H., MILES, R.D., ARAFA, A.S. & HARMS, R.H. (1981). The Influence of Oviposition Time on Egg Weight, Shell Quality and Blood Phosphorus. Poultry Science, **60:** 824-828

COOK, J.K.A. (1968). Duration of Experimental Infectious Bronchitis in Chickens. Research in Veterinary Science, **9:** 506-514

CRINION, R.A.P., BALL, R.A. & HOFSTAD, M.S. (1971). Abnormalities in Laying Chickens following Exposure to Infectious Bronchitis at One Day Old. Avian Diseases, **15:** 42-48

FORSYTHE, R.H., ROSS, W.J. & AYRES, J.C. (1967). Salmonella Recovery Following Gastro-Intestinal and Ovarian Innoculation in the Domestic Fowl. Poultry Science, **46**: 849-855

FROMM, D. (1960). Permeability of the Egg Shell Influenced by Washing, Ambient Temperature Changes and Environmental Temperature and Humidity. Poultry Science, **39:** 1490-1495

GARIBALDI, J.A. & BAYNE, H.G. (1962). The Effect of Iron on the Pseudomonas Spoilage on Farm Washed Eggs. Poultry Science, **41:** 850-853.

GARIBALDI, J.A., LINEWEAVER, H. & IJICHI, K. (1969). Number of Salmonellae in Commercially Broken Eggs before Pasteurization. Poultry Science **48:** 1096-1101

GARSIDE, J.S. (1967). Avian Infectious Bronchitis. Veterinary Record, **80,** Clinical supplement No. **7**

GILBERT, A.B. (1979). In "Form and Function in Birds." **Vol.1:** 237-345 (A.S. King & J. McLelland, Eds.) Academic Press, London & New York.

GILBERT, A.B. & WOOD-GUSH, D.G.M. (1971). In "Physiology of the Domestic Fowl" 1372-1374

GILLESPIE, J.M., SCOTT, W.J. & VICKERY, J.R. (1950a). Studies in the Preservation of Shell Eggs. 11) The Incidence of Bacterial Rotting in Unwashed Eggs and in Eggs Washed by Hand. Australian Journal of Applied Science, **1**: 215-223

GILLESPIE, J.M., SCOTT, W.J. & VICKERY, J.R. (1950b). Studies in the Preservation of Shell Eggs. 111) The Storage of Machine Washed Eggs. Australian Journal of Applied Science, **1**: 313-329

GRZIMEK, B. (1936). Versuch mit der Mechanis Chen. Reinigung von Schmuzeiern. Tietarztliche Rundschau, **42:** 407-408

HAINES, R.B. (1938). Observations on the Bacterial Flora of the Hen's Egg with a Description of the New Species of Proteus and Pseudomonas Causing Rots in Eggs. Journal of Hygiene, **3 8**: 338-355

HAINES, R.B. & MORAN, T. (1940). Porosity of and Bacterial Invasion Through the Shell of the Hen's Egg. Journal of Hygiene, **4 0**: 453-461

HANSON, B.S. (1968). Disease and Egg Quality. In "Egg Quality - A Study of the Hen's Egg". (T.C. Carter, ed.) British Egg Marketing Board Symposium No.4

HARTUNG, T.E. & STADELMAN, W.J. (1963). Pseudomonas fluorescens Penetration of Egg Shell Membranes as Influenced by Shell Porosity, Age of the Egg and Degree of Bacterial Challenge. Poultry Science, **42:** 147-150

HELLIG, H. (1989). Salmonella enteritidis in the U.K. Poultry International, **Oct., 1989**: 72-76

HODGES, R.D. (1974). "The Histology of the Fowl" Academic Press, London and New York.

HOLLEY, R.A. & PROULX, M. (1986). Use of Egg Washwater pH to Prevent Survival of Salmonella at Moderate Temperatures. Poultry Science, **65:** 922-928

HURWITZ, S. (1978). Calcium Metabolism in Birds. Chemical Zoology, Volume **X,** Aves, 273-306

HURWITZ, S. (1985). Effect of Nutrition on Egg Quality. In "Egg Quality - Current Problems and Recent Advances" R.G. Wells & C.G. Belyavin. Poultry Science Symposium, 20: 235-254

IZAT, A.L., GARDNER, F A. & MELLOR, D.B. (1985). Effects of Age of the Bird and Seasons of the Year. 1. Shell Quality. Poultry Science, **6 4**: 1960-1966

JENKINS, M.K. & PENNINGTON, M.E. (1919). Commercial Preservation of Eggs by Cold Storage. U.S.D.A. Bull. No. **7 7 5**

JENKINS, M.K., HEPBURN, J.S., SWAN, C. & SHERWOOD C.M. (1920). Effect of Cold Storage on Shell Eggs. Ice Refrigeration, **58:** 140- 147

JORDAN, F.T.W. (1990). Infectious Bronchitis in "Poultry Diseases" 3RD Edition Bailliere Tindall: 159-165

KING, A.S. (1975). In Sisson and Grossman's "The Anatomy of the Domestic Animals" (R.Getty ed.) Vol **2**: 1919-1964 Saunders, Philadelphia

KRAFT, A.A., MCNALLY, E.G. & BRANT, A.W. (1958). Shell Quality and Bacterial Infection of Shell Eggs. Poultry Science **3 7**: 638-643

KUHL, H. (1989). E.E.C. Restrictions on Egg Washing are Outdated and Economically Damaging Poultry International, **Jan., 1989:** 20-22

LAIRD, J.M., BARTLETT, F.M. & MCKELLAR, R.C. (1990). Survival of Listeria monocytogenes in Egg Washwater. International Journal of Food Microbiology, **12:** 115-122

LIFSHITZ, A., BAKER, R.C. & NAYLOR, H.B. (1964). The Relative Importance of Chicken Exterior Structures in Resisting Bacterial Penetration. Journal of Food Science, **2 9**: 94-99

LORENZ, F.W. & STARR, P.B. (1952). Spoilage of Washed Eggs. 1. Effect of Sprayed Versus Static Water Under Different Washing Temperatures. Poultry Science, **31,** 204-214

MARCH, B.E. (1969). Bacterial Infection of Washed and Unwashed Eggs with Respect to Salmonellae. Applied Microbiology, **17**: 98-101

MOATS, W.A. (1978). Egg Washing - A Review. Journal of Food Protection, **41**: 919-925

MOHUMED, A.S.A.S. (1986). The Effect of Housing on Eggshell Structure and Quality at the End of the Laying Year. M.Sc. Thesis University of Glasgow.

MOURSY, A.W. & AHMED, A.A. (1971). Penetration Rate of Salmonella Organisms Through Intact Shells of Hen's Eggs. Veterinary Medical Journal, **19**: 89-95

NASCIMENTO, V.P. (1990). The Ease of Translocation of Salmonella enteritidis Through the Eggshell Wall: An Ultrastructure Study. M.Sc. University of Glasgow.

NATHUSIUS, W. VON. (1894). Uber Farben der Vogel-Cier. Zool. Anz., **17**: 440-452

REID, J. (1983). The Use of the Plasma Chemistry Unit as an Aid to the Scanning Microscope Study of the Avian Eggshell Structure. British Poultry Science, **24**: 233-235

REID, J. (1985). The Effect of Diet on Certain Aspects of the Eggshell of the Domestic Fowl (Gallus domesticus). Ph.D. Thesis, University of Glasgow.

ROLAND, D.A. SNR., SLOAN, D.R., & HARMS, R.H. (1973). Calcium Metabolism in the Laying Hen. **6**. Shell Quality in Relation to Oviposition. Poultry Science, **52**: 506-510

ROMANOFF, A.L. & ROMANOFF, A.J. (1949). "The Avian Egg." Chapman & Hall, London.

SAJNER, J. (1955). Uber de Mikroskopischen Veranderungen der Eischale der Vogel im Laufe Inkubationszeit. Acta Anat (Basel), **25**: 141-159

SAUVEUR, B. & PICARD, M. (1985). Environmental Effects on Egg Quality. In "Egg Quality - Current Problems and Recent Advances" R.G. Wells & C.G. Belyavin. Poultry Science Symposium, **20:** 219-234

SCHMIDT, W.J. (1956). Beitrage zur Mikroskopischen Kenntnis der Farbstoffe in der Kalkschale des Vogeleies. Z. Zellforsch. Mikrosk. Anat **44:** 413-426

SCHMIDT, W.J. (1962). Leigt der Eischalenkalk der Vogel als Submikroskopische Kristallite vor ? Z. Zellforsch. Mikrosk. Anat **57:** 848-880

SEDMAK, J.J. & GROSSBERG, S.E. (1977). A Rapid, Sensitive, and Versatile Assay for Protein Using Coomassie Brilliant Blue G 250. Analytical Biochemistry, **79:** 544-552

SIMKISS, K. (1961). Calcium Metabolism and Avian Reproduction. Biological Rev., **36**: 321-367

SIMKISS, K. (1967). Calcium Metabolism in Laying Birds. In Calcium in Reproductive Physiology. Reinhold Publishing Company, New York.

SIMKISS, K. (1968). The Structure and Formation of the Shell Membranes in "Egg Quality - A Study of the Hen's Egg" (T.C.Carter ed.) British Egg Marketing Board Symposium, **4:** 3-25 Oliver & Boyd, Edinburgh

SIMMONS, E.R., AYRES, J.C. & KRAFT, A.A. (1970). Effect of Moisture and Temperature on Ability of Salmonella to Infect Shell Eggs. Poultry Science, **49**: 761-768

SIMONS, P.C.M. & WIERTZ, G. (1966). The Ultrastructure of the Surface of the Cuticle of the Hen's Egg in Relation to Egg Cleaning. Poultry Science, **45**: 1153-1162

SIMONS, P.C.M. & WIERTZ, G. (1970). Notes on the Structure of Shell and Membranes of the Hen's Egg: A Study with the Scanning Electron Microscope. Ann. Biol. Anim. Bioch. Biophys., **10:** 31

SOLOMON. S.E. (1973). Studies of the Reproductive Tract of the Domestic Fowl Gallus domesticus. Ph.D. Thesis. University of Glasgow

SOLOMON, S.E. (1979). The Location of Beta-N-Acetyl-Glucoaminidase in the Oviduct of the Domestic Fowl. British Poultry Science **20**: 139-142

SOLOMON, S.E. (1983). The Oviduct. In "Physiology and Biochemistry of the Domestic Fowl" (B.M. Freeman ed.) Vol. **4**: 379-419 Academic Press, London and New York

SOLOMON, S.E. (1985). Structural Evaluation of Eggshell Quality. The Eggs Authority - Technical Bulletin No.**18**: 1-8

SOLOMON, S.E. (1987). Egg Shell Pigmentation. In "Egg Quality - Current Problems and Recent Advances". (R.G. Wells & C.G. Belavin, eds.) Poultry Science Symposium No.**20**: 147-157 Butterworths, London.

SOLOMON, S.E. (1988). The Value of Eggshell Structure as a Measure of Eggshell Quality. World's Poultry Congress Proceedings XV111 Japan: 262-265

SOLOMON, S.E. (1991). Egg and Eggshell Quality. Wolfe Publishing Ltd. 149 pp

SOUTHAM, G., PEARSON, J. & HOLLEY, R.A. (1987). Survival and Growth of Yersinia enterocolitica in Egg Wash Water. Journal of Food Protection, **50:** 103-107

SPACKMAN, D. (1985). The Effect of Disease on Egg Quality. In "Egg Quality - Current Problems and Recent Advances" R.G, Wells & C.G. Belyavin. Poultry Science Symposium, **20:** 255-282

SPARKS, N.H.C. (1985). The Hen's Eggshell: A Resistance Network Ph.D. Thesis, University of Bath

STOKES, J.L., OSBORNE, W.W. & BAYNE, H.G. (1956). Penetration and Growth of Salmonella in Shell Eggs. Food Research, **2 1**: 510-518

TAMURA, T. & FUJII, S. (1967). Comparative Observations on the Distribution of Fluorescent Pigments (Porphyrins) in the Egg Coverings of Chickens and Quail. Journal of the Faculty of Fish and Animal Husbandry. Hiroshima University, **7**: 35-41

TULLETT, S.G., BOARD, R.G., LOVE, G., PERROTT, H.R. & SCOTT, V.D. (1976). Vaterite Deposition During Eggshell Formation in the Cormorant, Gannet and Shag, and in "Shell-less" Eggs of the Domestic Fowl. Acta zool. (Stockh.) **57:** 79-87

TUNG, M.A., GARLAND, M.R. & GILL, P.K. (1979). A Scanning Electron Microscope Study of Bacterial Invasion in Hen's Egg Shell. Canadian Institute of Food Science and Technology, **12:** 16-22

TYLER, C. (1955). Studies on Eggshells. VI: The Distribution of Pores in Eggshells. Journal of the Science of Food and Agriculture, **6:** 170-176

TYLER, C. (1956). Studies on Eggshells. VII: Some Aspects of Structure as Shown by Plastics Models. Journal of theScience of Food and Agriculture, **7:** 483-493

TYLER, C. (1969). The Distribution of Pores in the Eggshells of the Domestic Fowl. A Further Study. British Poultry Science **10:** 357-380

TYLER, C. AND SIMKISS, K. (1959). Studies on Eggshells. X1.- Some Abnormalities. Journal of Scientific Food and Agriculture, **July, 1959:** 362-366

VADEHRA, D.V., BAKER, R.C. & NAYLOR, H.B. (1970). Infection Routes of Bacteria into Chicken Eggs. Journal of Food Science, **3 5**: 61-62

VADEHRA, D.V. & BAKER, R.C. (1972). Bacterial Penetration of Eggs. New York's Food and Life Sciences, **5:** 9-11

WALDEN, C.C., ALLEN, I.V.F., & TRUSSELL, P.C. (1956). The Role of the Eggshell and Shell Membranes in Restraining the Entry of Microorganisms. Poultry Science, **35:** 1190-1196

WATT, J.M. (1985). An Ultrastructural Evaluation of Eggshell Quality in the Domestic Fowl. M.Sc. Thesis, University of Glasgow.

WATT, J.M. (1989). The Effect of Stress on the Reproductive Tract of the Domestic Fowl. Ph.D. Thesis, University of Glasgow.

WEDRAL, E.M., VADEHRA, D.V. & BAKER, R.C. (1974). Chemical Composition of the Cuticle and the Inner Membranes from the Eggs of Gallus gallus. Comparative Biochemistry and Physiology, **47B**: 631-640

WILLIAMS, J.E. & WHITTEMORE, A.D. (1967). A Method of Studying Microbial Penetration through the Outer Structures of the Avian Egg. Avian Diseases, **11**: 467-490

WILLIAMS, J.E. & DILLARD, L.H. (1973). The Effect of External Shell Treatments on Salmonella Penetration of Chicken Eggs. Poultry Science, **52**: 1084-1089

WYBURN, G.M., JOHNSTON, H.S., DRAPER, M.H. & DAVIDSON, M.F. (1973). The Ultrastructure of the Shell Forming Region of the Oviduct of Gallus domesticus. Quarterly Journal of Experimental Physiology, **58**: 143-151

APPENDIX.

APPENDIX 1a. (BRUSH MACHINE) ANOVA TEST 1.

% MICROBIAL PENETRATION BETWEEN UNWASHED AND WASHED EGGS AT DIFFERENT PERIODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	537.92	537.92	1.393
Wihin groups	48	18532.16	386.087	$.10 < p \leq .25$
Total	49	19070.08		

Model II estimate of between component variance = 6.073

Group:	Count:	Mean:
B/B/UN	25	4
B/B/W	25	10.56

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	1331.28	1331.28	3.171
Wihin groups	48	20152.8	419.85	$.05 < p \leq .10$
Total	49	21484.08		

Model II estimate of between component variance = 36.457

Group:	Count:	Mean:
B/M/UN	25	56.12
B/M/W	25	66.44

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	598.58	598.58	12.175
Wihin groups	48	2360	49.167	$.0001 < p \leq .005$
Total	49	2958.58		

Model II estimate of between component variance = 21.977

Group:	Count:	Mean:
B/E/UN	25	20.76
B/E/W	25	27.68

APPENDIX 1b. (ROTARY ACTION)

% MICROBIAL PENETRATION BETWEEN UNWASHED AND WASHED EGGS AT THE DIFFERENT PERODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	1.62	1.62	.019
Wihin groups	48	4158.56	86.637	p > .25
Total	49	4160.18		

Model II estimate of between component variance = -3.401

Group:	Count:	Mean:
R/B/UN	25	18.4
R/B/W	25	18.76

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	25.92	25.92	.51
Wihin groups	48	2440.08	50.835	p > .25
Total	49	2466		

Model II estimate of between component variance = -.997

Group:	Count:	Mean:
R/M/W	25	21.48
R/M/UN	25	22.92

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	27.38	27.38	1.039
Wihin groups	48	1265.12	26.357	p > .25
Total	49	1292.5		

Model II estimate of between component variance = .041

Group:	Count:	Mean:
R/E/UN	25	20.64
R/E/W	25	19.16

APPENDIX 1c. (JET ACTION)

% MICROBIAL PENETRATION BETWEEN UNWASHED AND WASHED EGGS AT THE DIFFERENT PERODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	72	72	1.177
Wihin groups	48	2936	61.167	p > .25
Total	49	3008		

Model II estimate of between component variance = .433

Group:	Count:	Mean:
J/B/W	25	20.8
J/B/UN	25	18.4

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	32	32	.529
Wihin groups	48	2903.28	60.485	p > .25
Total	49	2935.28		

Model II estimate of between component variance = -1.139

Group:	Count:	Mean:
J/M/W	25	21.32
J/M/UN	25	22.92

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	14.58	14.58	.657
Wihin groups	48	1065.92	22.207	p > .25
Total	49	1080.5		

Model II estimate of between component variance = -.305

Group:	Count:	Mean:
J/E/W	25	19.56
J/E/UN	25	20.64

APPENDIX 2a. (BRUSH MACHINE) ANOVA TEST 2.

% MICROBIAL PENETRATION OF UNWASHED EGGS AT THE DIFFERENT PERIODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	33956.18	33956.18	94.145
Wihin groups	48	17312.64	360.68	$p \leq .0001$
Total	49	51268.82		

Model II estimate of between component variance = 1343.82

Group:	Count:	Mean:
B/B/UN	25	4
B/M/UN	25	56.12

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	3511.22	3511.22	15.408
Wihin groups	48	10938.56	227.887	$.0001 < p \leq .005$
Total	49	14449.78		

Model II estimate of between component variance = 131.333

Group:	Count:	Mean:
B/B/UN	25	4
B/E/UN	25	20.76

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	15629.12	15629.12	82.884
Wihin groups	48	9051.2	188.567	$p \leq .0001$
Total	49	24680.32		

Model II estimate of between component variance = 617.622

Group:	Count:	Mean:
B/M/UN	25	56.12
B/E/UN	25	20.76

APPENDIX 2b. (ROTARY MACHINE)

% MICROBIAL PENETRATION OF UNWASHED EGGS AT DIFFERENT PERIODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	255.38	255.38	2.887
Wihin groups	48	4245.84	88.455	$.05 < p \leq .10$
Total	49	4501.22		

Model II estimate of between component variance = 6.677

Group:	Count:	Mean:
R/B/UN	25	18.4
R/M/UN	25	22.92

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	62.72	62.72	1.094
Wihin groups	48	2751.76	57.328	$p > .25$
Total	49	2814.48		

Model II estimate of between component variance = .216

Group:	Count:	Mean:
R/B/UN	25	18.4
R/E/UN	25	20.64

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	64.98	64.98	1.206
Wihin groups	48	2585.6	53.867	p > .25
Total	49	2650.58		

Model II estimate of between component variance = .445

Group:	Count:	Mean:
R/M/UN	25	22.92
R/E/UN	25	20.64

APPENDIX 2c. (JET MACHINE)

% MICROBIAL PENETRATION OF UNWASHED EGGS AT DIFFERENT PERIODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	255.38	255.38	2.887
Wihin groups	48	4245.84	88.455	$.05 < p \leq .10$
Total	49	4501.22		

Model II estimate of between component variance = 6.677

Group:	Count:	Mean:
J/B/UN	25	18.4
J/M/UN	25	22.92

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	62.72	62.72	1.094
Wihin groups	48	2751.76	57.328	$p > .25$
Total	49	2814.48		

Model II estimate of between component variance = .216

Group:	Count:	Mean:
J/B/UN	25	18.4
J/E/UN	25	20.64

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	64.98	64.98	1.206
Wihin groups	48	2585.6	53.867	p > .25
Total	49	2650.58		

Model II estimate of between component variance = .445

Group:	Count:	Mean:
J/M/UN	25	22.92
J/E/UN	25	20.64

APPENDIX 3a. (BRUSH MACHINE) ANOVA TEST 3.

% MICROBIAL PENETRATION OF WASHED EGGS AT DIFFERENT PERIODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	39032.18	39032.18	87.662
Wihin groups	48	21372.32	445.257	$p \leq .0001$
Total	49	60404.5		

Model II estimate of between component variance = 1543.477

Group:	Count:	Mean:
B/B/W	25	10.56
B/M/W	25	66.44

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	3663.68	3663.68	17.668
Wihin groups	48	9953.6	207.367	$.0001 < p \leq .005$
Total	49	13617.28		

Model II estimate of between component variance = 138.253

Group:	Count:	Mean:
B/B/W	25	10.56
B/E/W	25	27.68

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	18779.22	18779.22	66.961
Wihin groups	48	13461.6	280.45	$p \leq .0001$
Total	49	32240.82		

Model II estimate of between component variance = 739.951

Group:	Count:	Mean:
B/M/W	25	66.44
B/E/W	25	27.68

APPENDIX 3b. (ROTARY MACHINE)

% MICROBIAL PENETRATION OF WASHED EGGS AT DIFFERENT PERIODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	92.48	92.48	1.887
Wihin groups	48	2352.8	49.017	$.10 < p \leq .25$
Total	49	2445.28		

Model II estimate of between component variance = 1.739

Group:	Count:	Mean:
R/M/W	25	21.48
R/B/W	25	18.76

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	2	2	.036
Wihin groups	48	2671.92	55.665	$p > .25$
Total	49	2673.92		

Model II estimate of between component variance = -2.147

Group:	Count:	Mean:
R/E/W	25	19.16
R/B/W	25	18.76

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	67.28	67.28	2.884
Wihin groups	48	1119.6	23.325	$.05 < p \leq .10$
Total	49	1186.88		

Model II estimate of between component variance = 1.758

Group:	Count:	Mean:
R/M/W	25	21.48
R/E/W	25	19.16

APPENDIX 3c. (JET MACHINE)

% MICROBIAL PENETRATION OF WASHED EGGS AT DIFFERENT PERIODS OF LAY.

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	3.38	3.38	.102
Wihin groups	48	1593.44	33.197	p > .25
Total	49	1596.82		

Model II estimate of between component variance = -1.193

Group:	Count:	Mean:
J/B/W	25	20.8
J/M/W	25	21.32

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	19.22	19.22	.738
Wihin groups	48	1250.16	26.045	p > .25
Total	49	1269.38		

Model II estimate of between component variance = -.273

Group:	Count:	Mean:
J/B/W	25	20.8
J/E/W	25	19.56

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	38.72	38.72	1.343
Wihin groups	48	1383.6	28.825	p > .25
Total	49	1422.32		

Model II estimate of between component variance = .396

Group:	Count:	Mean:
J/M/W	25	21.32
J/E/W	25	19.56

APPENDIX 4a. ANOVA TEST 4.

COMPARISON OF MICROBIAL PENETRATION OF THE UNWASHED EGGS OF THE BRUSH, ROTARY AND JET ACTION MACHINES AT THE BEGINNING OF LAY.

One Way ANOVA 3 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	3456	1728	8.879
Wihin groups	72	14012	194.611	.0001 < p ≤ .005
Total	74	17468		

Model II estimate of between component variance = 61.336

Group:	Count:	Mean:
B/B/UN	25	4
R/B/UN	25	18.4
J/B/UN	25	18.4

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	2592	2592	10.538
Wihin groups	48	11806	245.958	.0001 < p ≤ .005
Total	49	14398		

Model II estimate of between component variance = 93.842

Group:	Count:	Mean:
B/B/UN	25	4
R/B/UN	25	18.4

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	2592	2592	10.538
Wihin groups	48	11806	245.958	.0001 < p ≤ .005
Total	49	14398		

Model II estimate of between component variance = 93.842

Group:	Count:	Mean:
B/B/UN	25	4
J/B/UN	25	18.4

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	0	0	0
Wihin groups	48	4412	91.917	p > .25
Total	49	4412		

Model II estimate of between component variance = -3.677

Group:	Count:	Mean:
R/B/UN	25	18.4
J/B/UN	25	18.4

APPENDIX 4b.

COMPARISON OF MICROBIAL PENETRATION OF THE UNWASHED EGGS OF THE BRUSH, ROTARY AND JET ACTION MACHINES AT THE MIDDLE OF LAY.

One Way ANOVA 3 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	18370.667	9185.333	56.083
Wihin groups	72	11792.32	163.782	$p \leq .0001$
Total	74	30162.987		

Model II estimate of between component variance = 360.862

Group:	Count:	Mean:
B/M/UN	25	56.12
R/M/UN	25	22.92
J/M/UN	25	22.92

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	13778	13778	67.813
Wihin groups	48	9752.48	203.177	$p \leq .0001$
Total	49	23530.48		

Model II estimate of between component variance = 542.993

Group:	Count:	Mean:
B/M/UN	25	56.12
R/M/UN	25	22.92

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	13778	13778	67.813
Wihin groups	48	9752.48	203.177	$p \leq .0001$
Total	49	23530.48		

Model II estimate of between component variance = 542.993

Group:	Count:	Mean:
B/M/UN	25	56.12
J/M/UN	25	22.92

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	0	0	0
Wihin groups	48	4079.68	84.993	$p > .25$
Total	49	4079.68		

Model II estimate of between component variance = -3.4

Group:	Count:	Mean:
R/M/UN	25	22.92
J/M/UN	25	22.92

APPENDIX 4c.

COMPARISON OF MICROBIAL PENETRATION OF THE UNWASHED EGGS OF THE BRUSH, ROTARY AND JET ACTION MACHINES AT THE END OF LAY.

One Way ANOVA 3 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	.24	.12	.004
Wihin groups	72	2430.08	33.751	p > .25
Total	74	2430.32		

Model II estimate of between component variance = -1.345

Group:	Count:	Mean:
B/E/UN	25	20.76
R/E/UN	25	20.64
J/E/UN	25	20.64

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	.18	.18	.005
Wihin groups	48	1884.32	39.257	p > .25
Total	49	1884.5		

Model II estimate of between component variance = -1.563

Group:	Count:	Mean:
B/E/UN	25	20.76
R/E/UN	25	20.64

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	.18	.18	.005
Wihin groups	48	1884.32	39.257	p > .25
Total	49	1884.5		

Model II estimate of between component variance = -1.563

Group:	Count:	Mean:
B/E/UN	25	20.76
J/E/UN	25	20.64

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	0	0	0
Wihin groups	48	1091.52	22.74	p > .25
Total	49	1091.52		

Model II estimate of between component variance = -.91

Group:	Count:	Mean:
R/E/UN	25	20.64
J/E/UN	25	20.64

APPENDIX 5a. ANOVA TEST 5.

COMPARISON OF MICROBIAL PENETRATION OF THE WASHED EGGS OF THE BRUSH, ROTARY AND JET ACTION MACHINES AT THE BEGINNING OF LAY.

One Way ANOVA 3 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	1468.827	734.413	4.553
Wihin groups	72	11614.72	161.316	$.01 < p \leq .025$
Total	74	13083.547		

Model II estimate of between component variance = 22.924

Group:	Count:	Mean:
B/B/W	25	10.56
J/B/W	25	20.8
R/B/W	25	18.76

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	840.5	840.5	3.706
Wihin groups	48	10884.72	226.765	$.05 < p \leq .10$
Total	49	11725.22		

Model II estimate of between component variance = 24.549

Group:	Count:	Mean:
B/B/W	25	10.56
R/B/W	25	18.76

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	1310.72	1310.72	6.511
Wihin groups	48	9662.16	201.295	$.01 < p \leq .025$
Total	49	10972.88		

Model II estimate of between component variance = 44.377

Group:	Count:	Mean:
B/B/W	25	10.56
J/B/W	25	20.8

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	52.02	52.02	.931
Wihin groups	48	2682.56	55.887	$p > .25$
Total	49	2734.58		

Model II estimate of between component variance = -.155

Group:	Count:	Mean:
J/B/W	25	20.8
R/B/W	25	18.76

APPENDIX 5b.

COMPARISON OF MICROBIAL PENETRATION OF THE WASHED EGGS OF THE BRUSH, ROTARY AND JET ACTION MACHINES AT THE MIDDLE OF LAY.

One Way ANOVA 3 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	33810.347	16905.173	88.82
Wihin groups	72	13703.84	190.331	$p \leq .0001$
Total	74	47514.187		

Model II estimate of between component variance = 668.594

Group:	Count:	Mean:
B/M/W	25	66.44
R/M/W	25	21.48
J/M/W	25	21.32

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	25267.52	25267.52	94.455
Wihin groups	48	12840.4	267.508	$p \leq .0001$
Total	49	38107.92		

Model II estimate of between component variance = 1000

Group:	Count:	Mean:
B/M/W	25	66.44
R/M/W	25	21.48

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	25447.68	25447.68	91.816
Wihin groups	48	13303.6	277.158	$p \leq .0001$
Total	49	38751.28		

Model II estimate of between component variance = 1006.821

Group:	Count:	Mean:
B/M/W	25	66.44
J/M/W	25	21.32

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	.32	.32	.012
Wihin groups	48	1263.68	26.327	$p > .25$
Total	49	1264		

Model II estimate of between component variance = -1.04

Group:	Count:	Mean:
R/M/W	25	21.48
J/M/W	25	21.32

APPENDIX 5c.

COMPARISON OF MICROBIAL PENETRATION OF THE WASHED EGGS OF THE BRUSH, ROTARY AND JET ACTION MACHINES AT THE END OF LAY.

One Way ANOVA 3 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	2	1155.707	577.853	18.402
Wihin groups	72	2260.96	31.402	$p \leq .0001$
Total	74	3416.667		

Model II estimate of between component variance = 21.858

Group:	Count:	Mean:
B/E/W	25	27.68
R/E/W	25	19.16
J/E/W	25	19.56

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	907.38	907.38	25.02
Wihin groups	48	1740.8	36.267	$p \leq .0001$
Total	49	2648.18		

Model II estimate of between component variance = 34.845

Group:	Count:	Mean:
B/E/W	25	27.68
R/E/W	25	19.16

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	824.18	824.18	25.662
Wihin groups	48	1541.6	32.117	$p \leq .0001$
Total	49	2365.78		

Model II estimate of between component variance = 31.683

Group:	Count:	Mean:
B/E/W	25	27.68
J/E/W	25	19.56

One Way ANOVA 2 Groups

Analysis of Variance Table

Source	DF:	Sum Squares:	Mean Square:	F-test:
Between groups	1	2	2	.077
Wihin groups	48	1239.52	25.823	$p > .25$
Total	49	1241.52		

Model II estimate of between component variance = -.953

Group:	Count:	Mean:
R/E/W	25	19.16
J/E/W	25	19.56