STUDIES ON THE REPRODUCTIVE TRACT OF THE DOMESTIC FOWL (Gallus domesticus)

by

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INTRODUCTION

Contemporary knowledge of the unilateral avian oviduct derives in great part from twentieth century advances in technology. Yet long before this time, the oviduct was identified, its structure described and its role in egg formation defined.

One of the earliest accounts of the functional duct is provided by Aldrovandi (1600) who uses the term "uterus" for the entire oviduct:

> "The substance of the uterus is thick and membranous. It receives the egg when it has grown and become almost complete in itself. Its shape is concave, oblong, broader where it stretches to its exit and three fingers in length. For the rest it is narrow and somewhat round exhibiting the form of a slender intestine." (Lind 1963).

This description although somewhat fanciful is not inaccurate. Latter day biologists have merely formalised these observations by providing anatomical terms for each portion of the oviduct.

The early 20th century witnessed great interest in

in/
the histology of the oviduct. Curtis (1910), Surface (1912), Giersberg (1922), Bradley (1928) and Richardson (1935). Of these Richardson's treatise is the most authoritative since he was the first to correlate cellular activity with egg position. He subdivides the oviduct into five distinct regions, viz. infundibulum, albumen secreting portion (magnum), isthmus, uterus (shell gland) and vagina, attributing to each region, with the exception of the vagina, functional responsibility for the production of a particular fraction of the egg.

Concurrent physical and chemical studies on the egg and its surroundings were conducted by Pearl & Curtis (1912), Moran & Hale (1936), Scott, Hughes & Warren (1937), Burmester (1940), including analysis of oviducal secretions (Beadle, Conrad & Scott 1938). Fluctuating calcium levels were investigated by Buckner, Martin & Hull (1930) and the function of medullary bone as a calcium store was established (Heller, Paul & Thomson 1934, Kyes & Potter 1934, Bloom, Bloom & McLean 1941; 1942).

These hypotheses remained unchallenged until the

the/
1960's when it was thought necessary to investigate at cellular level - using more sophisticated histochemical and ultrastructural techniques - the functions of the various portions of the oviduct.

As a result of Burmester & Card's (1939) resection experiments on Richardson's chalaziferous region of the infundibulum which had no deleterious effect on chalazae formation, interest was renewed in the infundibular glands. According to Baker & Stadelman (1958) chalazae are formed mechanically from the egg white by condensation of ovomucin fibres caused by the rotation of the egg and are in no way dependent upon glandular secretions from the infundibulum. Aitken & Johnston (1963) hypothesised that chalazae formation might involve enzymes secreted by the infundibular glands. More recently Bain & Hall (1969) implicated the infundibulum in the formation of the perivitelline membrane. The latter consists of an inner part which is formed intrafollicularly (Bellairs, Harkness & Harkness 1963; Wyburn, Aitken & Johnston 1965) and an outer oviducal part which appears to correspond to the chalaziferous membrane which in turn appears to be continuous

continuous/
with the chalazae (Fromm, 1964).

The infundibulum has been implicated also in sperm retention (Olsen & Neher 1948).

To the naked eye the infundibulum and magnum are easily distinguished on account of their different colour and the deeply folded mucosa of the latter. At the cellular level the transition is less abrupt with infundibular glands continuing for 2 - 3mms before being replaced by the typical granule packed cells characteristic of the magnum.

Studies on egg white have shown it to contain about 40 individual proteins. (Parkinson 1966; Feeney & Allison 1969). Wyburn, Johnston, Draper & Davidson (1970) investigating the ultrastructural changes which take place in the magnum throughout the laying cycle, reported the presence of several gland cell types in the lamina propria, viz. A, B, C cells. The C cells which are found immediately after the passage of the egg are regarded as the recovery phase of the A cells which have discharged their granules on the passage of the egg. The A cells are thought to be the source of ovalbumin (Kohler, Grimley & O'Malley 1969) and the B cells the source of lyzozyme.

A review of the literature on the structure and function of the isthmus and shell gland, the subject of this thesis, suggests that in this region of the oviduct in particular there is considerable variance of opinion.

The magnum and isthmus are sharply separated by a narrow aglandular zone measuring 1 - 2mm in width appearing as a thin translucent line. The significance of this is not understood but it is a useful guide to the upper limit of the isthmus as in general appearance this region and the magnum are similar and even with the optical microscope are not readily distinguished in routine (H&E) preparations. The lower limit of the isthmus on the other hand is the subject of controversy. Thus it is sometimes considered to extend to the point at which the duct becomes expanded to form a pouch-like dilatation (Leonard 1968, Draper, Davidson, Wyburn & Johnston 1972); while Johnston, Aitken & Wyburn (1963) suggested that the lower limit of the isthmus was indicated by a colour change from the dull white characteristic of the latter to the brown colour characteristic of the shell gland and reported that a transitional zone containing glands with some of the features of glands of the pouch region occurred at the junction between isthmus and shell gland. Their ultrastructural studies showed them to contain complex secretory

secretory/
granules consisting of smaller membrane bound granules of different density together enclosed in a common membrane.

Khairallah (1966) in her ultrastructural investigation reported the presence of numerous granules of variable electron density in the isthmian gland cells. Her photomicrographs suggest a similarity between these granules and those described by Johnston et al (1963), adding further to the controversial features of reported isthmian structure. Unfortunately Khairallah (1966) failed to specify precisely the exact location from which her specimens were taken and it is still open to question whether her findings are the result of accidental choice of the limited locality where Johnston et al (1963) reported these granules to occur.

It has been commonly accepted that the soft shell membranes are formed in the isthmus (Pearl & Curtis 1914; Richardson 1935) but such reports failed to define accurately the limits of this segment. Subsequently Leonard (1968) who considered the isthmus to extend to the shell gland pouch reported membrane formation to occur only in the upper isthmus, whilst the lower isthmus functioned in ion transfer. Draper (1966), Draper et al (1972)

Draper et al (1972)/
concurred with the latter finding and reported in addition that glucose was also added here (Davidson & Draper 1969). (Draper et al 1972 - paper published during the termination of this thesis).

Draper et al (1972) subsequently applied the term "granular isthmus" to designate the part of the isthmus concerned with membrane production and "red region" to designate the terminal part.

The picture is further complicated by the finding of calcium in isthmian mucosa (Taylor & Hertelendy 1960), suggesting that this section of the duct is implicated to some extent in calcareous shell formation.

The outer shell membrane is firmly bound to the shell by projections called mamillae which have a core of proteinaceous material continuous with the fibres of the membrane and these appear to act as nucleation centres for the initiation of deposition of shell calcium. (Hertelendy & Taylor 1964). It is not clear however whether these authors are referring to the entire isthmus or merely to the terminal part (red region / tubular shell gland) and to date there has been no attempt to

to/
discern the cells involved or the ultrastructural features of the process, also there are no reports of location in the isthmus of the enzymes, carbonic anhydrase (Common 1941, Benesch, Barron & Mawson 1944) and acid phosphatase (Burstone 1959, Stringer 1962) commonly regarded as ancillary to calcium secretion.

In view of the above it seemed reasonable to conduct a further study of the isthmus and in particular to extend a projected study of calcium secretion and the various cytological manifestations associated therewith to this region.

A diversity of processes occur in the shell gland including the addition of plumping fluid, the elaboration of the calcified shell, pigment deposition and cuticle production.

In view of these varied and cyclical phenomena it is surprising to find that in reports on the ultrastructure of the shell gland mucosa, Johnston et al (1963), Makita & Nishida (1966), Breen (1966) and Breen & de Bruyn (1969) the only major structural variation observed in the cells lining this region is the blebbing of the microvilli lining the tubular gland cells during shell formation. Breen & de Bruyn (1969) equated this

this/
with the release of a thin watery fluid which they supposed to contain calcium although they did not actually attempt to demonstrate this ion. It is feasible that they were discussing plumping fluid which according to Simkiss & Taylor (1971) is added to the egg in this region. Draper (1966) suggested that plumping was initiated in the isthmus and completed in the shell gland. There is however no evidence in the literature to date as regards the specific cells involved in the transfer of this fluid.

The shell consists of calcium carbonate in the form of calcite crystals of hexagonal structure embedded in a spongy matrix (Simkiss 1967), The carbonate fraction of the shell is provided by the enzymic action of carbonic anhydrase, which has been localised in the apices of the tubular gland cells of the shell gland (Diamanstein & Schluens 1964) on metabolic CO_2.

Calcium for shell formation is derived from the diet or, when required, from medullary bone. Its release from the latter has been correlated with an increase in blood acid phosphatase and it is interesting that this enzyme has been localised in the surface epithelial cells of the shell gland by Diculesco (1961)

Diculesco (1961)/ who however did not determine whether it was a feature of both ciliated and non ciliated cells and whether it existed in a bound form, i.e. lysosomal or not. Nevalainen (1969) demonstrated the presence of lysosomal acid phosphatase in both the surface cells and tubular gland cells in calcium deficient hens. The author does not comment however on the possibility of these being a pertinent feature of laying birds - a surprising point since he does remark on their presence in control birds on a normal diet.

Neither Diculesco (1961) nor Nevalainen (1969) reported cyclic variations in acid phosphatase concentration which seems highly probable if the enzyme is concerned in shell formation. It would appear therefore that the studies on acid phosphatase activity and its association with shell formation are by no means definitive and that further studies, to determine the ultrastructural localisation of this enzyme (whether lysosomal or otherwise) if any variations with the laying cycle occur and if possible to observe its secretion, are warranted.

Calcium has been demonstrated by microincineration studies by Turchini (1924) and Richardson (1935) in the surface

surface/
epithelium of the shell gland pouch. However as Richardson (1935) says "to take an individual cell and to hope to define the exact region of the cytoplasm where certain inorganic elements are located after incineration is not to be expected."

More recently Hohman & Schraer (1966), using homogenised shell gland mucosa, showed that the mitochondrial fraction sequestered calcium at a greater rate than the nuclear, microsomal or supernatant fraction and hypothesised that mitochondria may play a significant role in calcium transfer. However the uptake of calcium by mitochondria is a fairly universal feature essential for the prevention of cytoplasmic saturation and not necessarily indicative of a transport phenomenon. Nevertheless this requires investigation and justifies a particular study of mitochondria in the shell gland pouch from the point of view of cyclic variation in numbers and position associated with active shell formation.

Gay & Schraer (1967) placed tissue from the shell gland in a medium of Ca^{45} and observed the accumulation of radioactive label in the surface epithelial cells, thereby implicating these cells in calcium transfer; however using this technique these authors are witnessing adsorption of Ca^{45} rather than secretion

secretion/
of radioactive calcium, their findings are thus open to criticism.

According to Simkiss & Taylor (1971) "the shell may contain pigments but these are usually restricted to its exterior or to the cuticle". The latter is usually about 10μ thick and is composed of 90% peptide with galactose, mannose, fucose and hexosamine (Baker & Balch 1962): Tyler & Simkiss (1959), Baker & Balch (1962) believe that the basal cells of the shell gland surface epithelium are responsible for its formation.

The presence of pigment in eggshells was first investigated by Fischer & Kögl (1923), Fischer & Müller (1925) and Bierry & Gouzon (1939). It was termed "ooporphyrin" and was identified in several species including the domestic fowl, duck, goose and owl. Woodard & Mather (1964) observed that pigmentation in the Japanese quail (Coturnix coturnix japonica) occurred approximately 3½ hours before oviposition. This finding was corroborated by Tamura, Fujii, Kunisaki & Yamane (1965) who reported the presence of pigment granules in the apical cells of the shell gland of this species.

species./

It is not clear from their description, however, whether these granules are the basophilic granules reported by Richardson (1935) and Johnston et al (1963), or whether they are morphologically and chemically distinct. Johnston et al (1963) were of the opinion that these basophil granules were associated with production of the proteinaceous framework of the shell.

The term porphyrin is a collective name for a chemical group of substances all of which fluoresce in ultraviolet light, viz. coproporphyrin, uroporphyrin and protoporphyrin. The distinction between these three groups cannot be made using straightforward histological techniques and so recourse to biochemical and chemical methods must be made in an attempt to provide the definitive answer.

MATERIALS AND METHODS

1. Experimental Animals - General Remarks

All the hens, apart from those used in the porphyrin investigation, were of the White Leghorn variety. The birds approximately 1 year old were kept in individual wire cages and fed a diet of layers pellets and water ad libitum. They were subjected to 12 hours artificial daylight and 12 hours darkness. The birds were observed for several weeks to establish times of oviposition. It was thus possible to predict with reasonable accuracy the location of the ovum prior to experimentation.

2. Histology

2.1 Light Microscopy

2.1.1 Materials

The material came from 14 laying White Leghorns, subdivided into 6 groups.

Group	Position of egg	No. in group
1	Oviduct empty	2
2	Mid magnum	1
3	Isthmus (with membranes)	4
4	Shell gland tubular portion	2
5	Soft membraned egg in shell gland pouch	3
6	Hard shell egg in shell gland pouch	2

2.1.1 (Cont'd.) The birds were restrained in the supine position and given a lethal dose of nembutal intravenously. The abdominal cavity was opened and the oviduct dissected out into a bath of 10% B.N.F. The oviduct was opened, pinned out, the position of the egg was noted and pieces of material were removed from the areas shown in Fig.1.

2.1.2. Fixation and Embedding

Tissues were fixed overnight in 10% B.N.F. (Lillie 1965) then dehydrated in an alcohol/ chloroform series and embedded in paraffin wax.

2.1.3. Staining

Routine Haematoxylin-Eosin (H&E) sections were prepared for the purposes of orientation for electron microscopy.

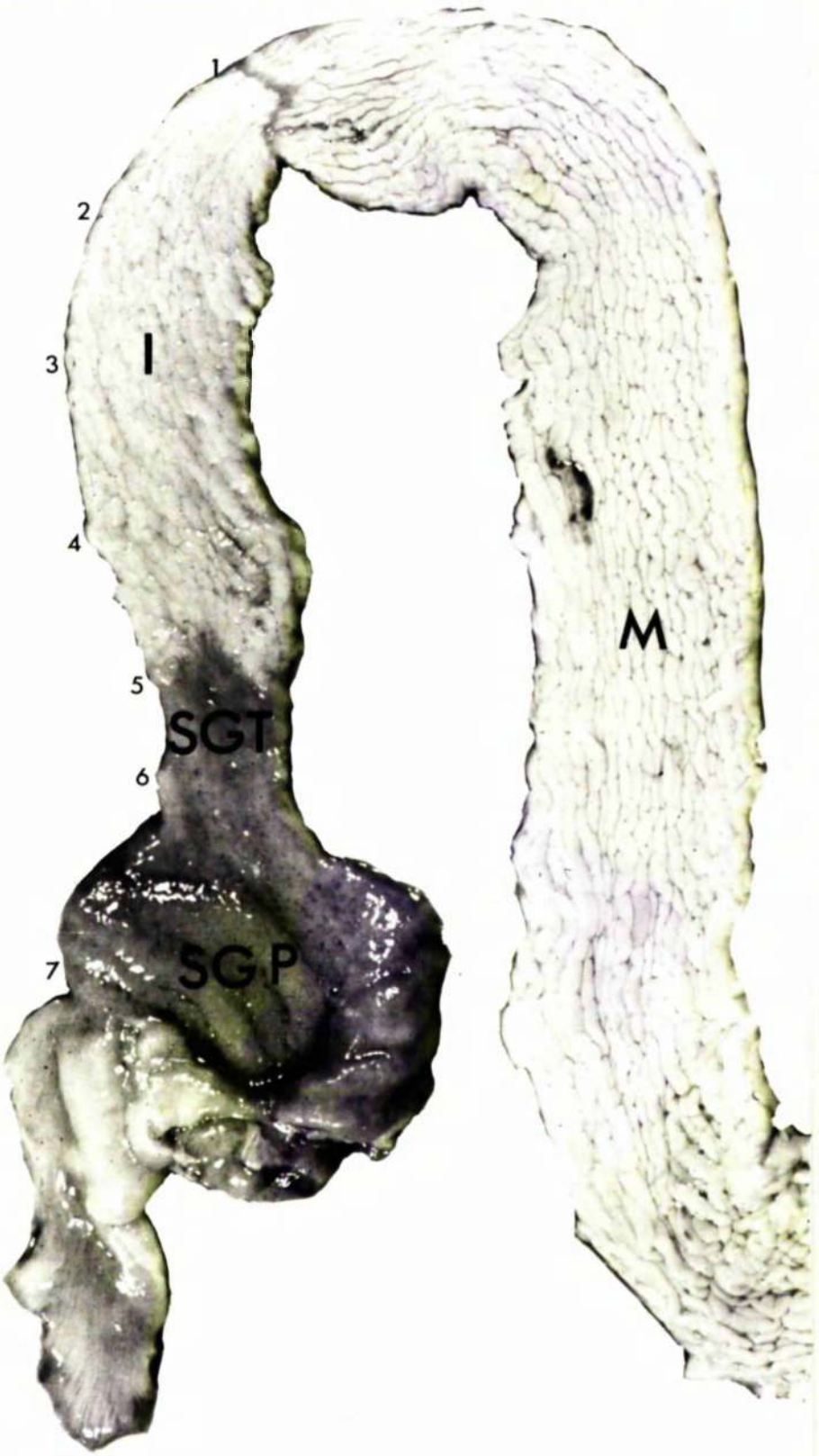


Fig 1. Part of the oviduct of a laying White Leghorn. Note colour change from isthmus to shell gland.

M: magnum I: isthmus SGT: shell gland tubular portion SGP: shell gland pouch.

Tissue taken from points 1 to 7.

2.2 Electron Microscopy

2.2.1. Materials

The material for ultrastructural investigation came from 30 laying White Leghorns which were subdivided into 6 groups according to the position of the egg in transit.

Group	Position of Egg	No. in Group
1.	Oviduct empty	3
2.	Upper magnum	4
3.	Lower magnum	5
4.	Isthmus (with membranes)	6
5.	Soft membraned egg in shell gland	5
6.	Hard shelled egg in shell gland	7

In each case the bird was given a lethal dose of nembutal via the wing vein; before death supervened the chestwall and abdomen were opened to expose the aorta and oviduct respectively.

2.2.2 Fixation

Glutaraldehyde on its own is a slow penetrant, however once inside the cell it will bind efficiently and

2.2.2 (Cont'd.) and/ irreversibly to membranes so preserving structural relationships. Paraformaldehyde on the other hand penetrates cells rapidly although its ability on its own as a fixative for ultrastructural studies is questionable. A preliminary investigation was carried out during which the oviduct was perfused through the aorta with either 30ml buffered glutaraldehyde or 30ml Karnovsky's fluid, a mixture of glutaraldehyde and paraformaldehyde, in order to ensure that the features observed were not an artefact of one fixation method. Glutaraldehyde on its own caused some dilation of mitochondria and rough endoplasmic reticulum (R.E.R.) possibly the result of prolonged anoxia. It was decided therefore to complete this study using Karnovsky's fluid alone.

Successful fixation was indicated by blanching of the entire oviduct, which was removed from the body cavity and opened out in a bath of Karnovsky's fluid, the position of the egg was noted and small

small/
pieces of tissue 1mm^2 were removed from the areas depicted in Fig. 1.

Buffered Glutaraldehyde

Prepared by the addition of 10ml 25% stock glutaraldehyde p^H3.5 to 90ml, 0.1 - 0.2m Sorensens phosphate buffer p^H7.4. The p^H of the stock glutaraldehyde was maintained above 3.5 by the addition of barium carbonate. This resulted in a precipitate which was removed by centrifugation leaving a clear supernatant. After 24 hours fixation the tissues were washed for 2 - 4 hours in phosphate buffer, post-fixed in 1% buffered isotonic osmic acid for 1 hour (Zetterqvist 1956), rinsed in distilled water and dehydrated through a graded series of acetones.

Karnovsky's Fixative (1965)

25ml of .2m cacodylate buffer and 0.05ml 1M $CaCl_2$ were made up to 45ml with distilled water. The resulting mixture was heated to 60^{o}C. 0.67g paraformaldehyde were then stirred in when dissolved

dissolved/
and cooled, 2ml of 25% glutaraldehyde was added and the solution made up to 50ml with distilled water. p^H was adjusted to 7.4 using $\frac{N}{10}$ HCl. The tonicity of this fluid is 820. However the tonicity of avian plasma is 320. Taking this variation into account, the 50ml volume was made up to 128ml with distilled water. Tissues were left for 5 - 12 hours, postfixed in 1% buffered osmic and dehydrated as before.

2.2.3 Embedding Material

The embedding material used was the epoxy resin Epon.

The stock solution was prepared as follows:

EPIKOTE RESIN (Epon 812)	106.75ml
D.D.S.A. (Dodecenyl Succinic anhydride)	108.00ml
M.N.A. (Methyl nadic anhydride)	35.5 ml
	250.00ml

To make the embedding resin 2ml D.M.P. 30 (2, 4, 6, tri (dimethylamino methyl) phenol was added to 100ml

2.2.3 (Cont'd.) 100ml/ stock solution. The resultant mixture was left for five minutes on a magnetic stirrer to ensure even mixing.

2.2.4 Sectioning and Staining

Fine sections 70 mμ thick were cut on the L.K.B. Mark III ultratome, mounted on uncoated copper grids, and stained with uranyl acetate, except where otherwise stated.

3. Histochemistry

Analyses of the two soft shell membranes and the calcified shell have shown them to contain carbohydrate, protein and fat (Simkiss & Taylor 1971). In order to identify the source of these various components a number of stains specific for these substances were applied to paraffin embedded sections of isthmus and shell gland prepared as in 2.1.

3.1 Periodic Acid Schiff (P.A.S.)

Paraffin embedded sections were brought to water before being treated with 0.5% periodic acid for 10 minutes. The sections were washed in running water for 5 minutes and then stained with Schiff reagent for 15 minutes. They were washed in running water for a further 2 minutes and placed in 2% sodium metabisulphite. They were then washed again for 10 minutes, counterstained with haematoxylin, dehydrated, cleared and mounted in D.P.X.

3.2 P.A.S./Diastase

Paraffin embedded sections were taken to water and

3.2 (Cont'd.) and/ digested for 45 minutes at 37°C in the following before being treated with P.A.S. as described above:

0.1% solution of malt diastase in 0.02M phosphate buffer p^H 6.0

Buffer:

90ml	0.28g/100ml	$NaH_2PO_4H_2O$
10ml	0.76g/100ml	$Na_2HPO_4\ 12H_2O$

3.3 P.A.S./Alcian Blue

Paraffin embedded sections were brought to water then rinsed briefly in 3% aqueous acetic acid before being stained for 30 minutes in 1% Alcian Blue in 3% acetic acid. Upon completion of this process, the sections were rinsed briefly in water and then in 3% acetic acid, running water and distilled water. They were then treated as described above, i.e. oxidised in 0.5% periodic acid, etc.

3.4 Alcian Blue P^H2.6

Paraffin embedded sections of isthmus and shell gland, tubular and pouch regions were brought to water through a graded series of alcohols before being stained for 30 minutes in 1% alcian blue in 3% acetic acid P^H2.6. The sections were then washed in running water for 5 minutes, dehydrated, cleared and mounted in D.P.X.

3.5 Alcian Blue P^H1

Paraffin embedded sections were brought to water as described above and stained for 30 minutes in 1% alcian blue in 0.1 N-HCl (P^H1). The sections were blotted dry with fine filter paper (Whatman's No. 50), dehydrated, cleared and mounted in D.P.X.

3.6 Sudan Black

Frozen sections 10μ thick were cut on the Slee cryostat and dipped in 70% alcohol. They were then stained in a 0.37% alcoholic 70% solution of Sudan Black B for 20 minutes before differentiation in 70% alcohol. Sections were washed in water and mounted in D.P.X.

3.7 Biebrich Scarlet (Spicer & Lillie 1961)

For the demonstration of basic protein; the stain was used as a 0.04% solution in glycine buffers at p^H8.9, 10. Staining time was 20 minutes.

3.8 Light Microscopic Localisation of Acid Phosphatase (Gomöri 1950, 1952)

3.8.1 Materials

14 White Leghorns were used in this experiment. The birds were given a lethal dose of Nembutal intravenously and the oviduct was dissected out into a bath of Ringer p^H7.4.

Group	Position of Egg	No. of Birds
1	No egg in oviduct	2
2	Egg in lower magnum	3
3	Egg in isthmus with membranes	5
4	Soft shelled egg in shell gland	2
5	Calcified egg in shell gland	2

3.8.2 Method

Pieces of isthmus and shell gland 3mm^2 approximately were frozen on solid CO_2 (dry ice). 10µ sections were cut on the Slee cryostat and incubated according to Gomöri (1950, 1952). The medium was buffered to p^H6.2.

3.9 Ultrastructural Localisation of Acid Phosphatase

In view of the results obtained above, in which acid phosphatase activity was restricted to the shell gland pouch region, it was decided to extend this investigation in an attempt to specify the exact intracellular localisation of this hydrolytic enzyme and to determine whether it exists in the same form in both the surface epithelium and glandular mucosa, since it is known that acid phosphatase can be both lysosomal (De Duve *et al* 1955) and extra lysosomal (Hruban *et al* 1965).

3.9.1 Materials

Group	Position of Egg	No. of Birds
1	Oviduct empty	1
2	Egg in mid magnum	1
3	Egg in isthmus (with membranes)	2
4	Soft shelled egg in shell gland	2
5	Calcified egg in shell gland	3

The birds were restrained in the supine position and killed by intravenous injection of Nembutal. The oviduct was dissected out into Ringer P^{H}7.4.

3.9.2 Method

Small blocks of shell gland pouch mucosa were fixed for 24 hours at 0-2°C in 4% formaldehyde containing 7.5% sucrose, buffered with 0.067m phosphate at P^H7.2.

The blocks were then rinsed three times, five minutes each wash, in 7.5% sucrose at 0-2°C, they were then incubated for 10-15 minutes in a Gomöri staining medium (1950) buffered at P^H6.2 containing 7.5% sucrose and 1% formaldehyde. Control blocks were placed in 4% formaldehyde. The tissue was then washed 3 times at 0-2°C for five minutes in 7.5% sucrose containing 4% formaldehyde and buffered with 0.05M acetate buffer. The blocks were post-fixed for one hour at 0-2°C in Caulfields O_sO_4 sucrose fixative (1957) buffered at P^H7.4, then dehydrated through a graded series of acetones and embedded in epon (Holt & Hicks 1961). Fine sections, cut on the L.K.B. Ultramicrotome and mounted on copper grids, were viewed unstained to eliminate the possibility of artefactual deposit.

3.10 Ultrastructural Localisation of Calcium

3.10.1 Radioautography

A total of 14 White Leghorns were used in these experiments, seven for light microscopy and seven for electron microscopy. The birds were further divided into 3 groups according to the position of the egg in the oviduct:

Group	Position of egg	No. of birds in group
1	Egg in lower magnum	2
2	Egg in isthmus	2
3	Calcifying egg in shell gland	3

In each case 1 ml of calcium chloride containing 100 microcuries of Ca^{45} (Radiochemicals) was injected into the wing vein. After intervals ranging from 2 - 30 minutes an incision was made in the abdominal wall and the oviduct dissected out. Small pieces of material from isthmus and shell gland mucosa, $1mm^2$, were removed and placed in wire baskets which were immediately immersed in fuming iso-pentane cooled in liquid nitrogen.

3.10.1.1 Wax embedding

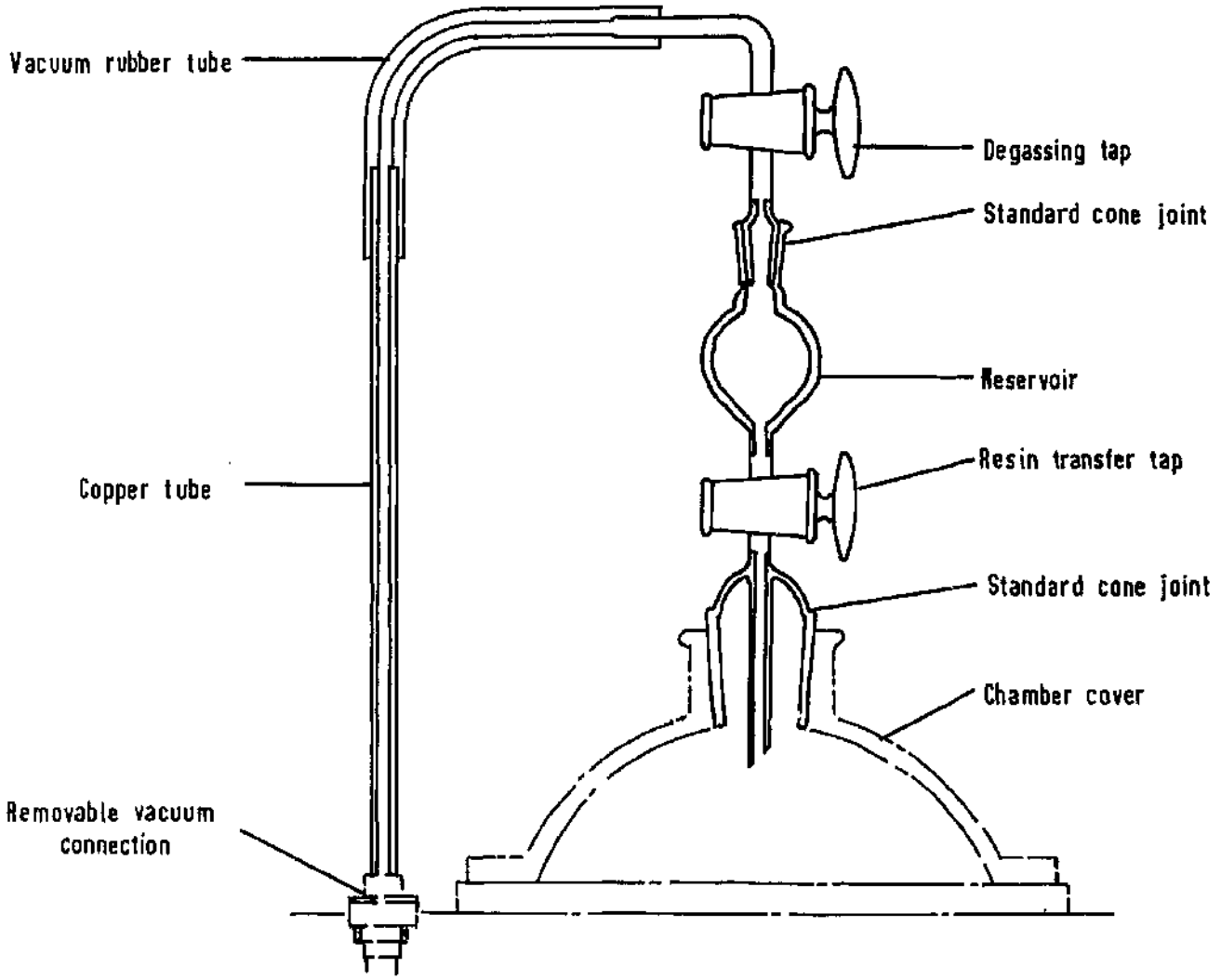
Melted paraffin wax was placed in a planchette machined to give a flat bottom and a small block of perspex added to ensure that after solidification of the paraffin a cavity would be left in it allowing direct contact between tissue and metal, this being essential to ensure adequate cooling of the tissue. The planchette was then placed in an Edwards/Pearse Tissue Drier, Model 1, and degassed overnight under vacuum. When degassing was complete the planchette was cooled to operating temperature of — 30°C and thereafter the tissue was removed from the nitrogen cooled isopentane with forceps also cooled in liquid nitrogen and placed in the space left in the centre of the wax after removal of the plastic block. The tissue was then dried overnight under vacuum; when complete the temperature of the drier was raised to room temperature to sublimate ice in the vapour trap and finally was further raised to melt the wax and allow infiltration of the tissue. The tissue was allowed to remain in the molten wax for 10 minutes

3.10.1.1 (Cont'd.) minutes/ before cooling to room temperature after which it was removed and the embedding processes completed in a further volume of paraffin wax. (Pearse 1968).

3.10.1.2 Resin embedding

For epon embedding an Edwards type resin embedding funnel, Plate 1, was inserted into the cover of the vacuum chamber of the dryer. The frozen tissue was transferred to the planchette as previously described, the chamber cover replaced and the tissue dried under vacuum overnight at -30°C. During drying epon was placed in the resin dropping funnel and degassed by connection to the vacuum pump. When degassing was complete, the dropping funnel tap was opened and the epon allowed to drop on to the tissue until it was completely covered. The resin was allowed to penetrate under vacuum for several hours before air was admitted into the chamber. Thereafter the tissue was transferred to beam capsules and polymerised overnight at 60°C.

Plate 1



RESIN EMBEDDING HEAD

3.10.1.3 Examination of material by light microscopy

5μ sections of wax embedded material cut on the B.T.L. Rotary Microtome were prepared for autoradiography according to the method of Maraldi and di Caterino (1968) using Ilford nuclear research emulsion L4. The sections were left in the dark for periods of 3, 6 and 9 weeks before processing and viewed with the Zeiss photomicroscope.

3.10.1.4 Examination of material by electron microscopy

Sections 70-80 mμ thick were cut on the L.K.B. Ultratome Mark III, and processed for autoradiography according to Maraldi and di Caterino (1968). They were left in the darkfor 4, 8 and 12 weeks before examination with the Hitachi HS8.

Thick sections of untreated epon embedded material were cut on the LKB pyramatome and stained with toluidine blue.

3.10.2 Precipitation of ions as pyroantimonate

3.10.2.1 Materials

Twelve laying White Leghorns, divided into 5 groups, were used in these experiments:

Group	Position of Egg	No. of Birds
1	Egg in mid magnum	2
2	Egg in isthmus (with membranes)	3
3	Soft membraned egg in shell gland	3
4	Calcified egg in shell gland	2
5	Oviduct empty	2

3.10.2.2 Method (Legato & Langer, 1969)

The birds were given a lethal dose of nembutal intravenously. Under deep anaesthesia the oviduct was exposed and perfused through the aorta with a solution containing equal parts of a 5% aqueous solution of potassium pyroantimonate and a 2% solution of osmium tetroxide, adjusted to p^H7.4 with acetic acid, equivalent to the p^H of the blood supplying the shell gland. The criterion for successful fixation was the change in colour of the shell gland from reddish brown to black.

3.10.2.2 (Cont'd.) black./

Pieces of tissue were removed from the isthmus and shell gland tubular and pouch regions, post-fixed in osmic for 1 hour and processed as in 2.2. Staining was omitted to eliminate the possibility of artefactual deposit.

3.11 Localisation of Tissue Porphyrins

Pieces of tissue were taken from the isthmus and shell gland tubular and pouch regions of the quail, White Leghorn and Brown Ranger. The birds were divided into 2 groups, with and without calcifying eggs in the shell gland. The tissue was immersed in liquid nitrogen and once frozen, transferred to the Slee Cryostat. 10µ frozen sections were cut and examined with the Zeiss microscope using barrier filters 53, 0, 44.

4. Chelation of calcium by E.G.T.A. (etnylene glycol-bis- (Bamino ethyl ether) N, N'-tetra acetic acid). This experiment was designed with reference to the results obtained in 3.10.2. Since the precipitate was maximal in those birds with soft membraned eggs

4. (Cont'd.) eggs/ in situ, six hens were chosen at this stage of the cycle. In each case 10ml of a solution of sodium EGTA, prepared by mixing equal quantities of NaOH and EGTA (Sigma NOE-3251), was injected into the aorta before perfusion with 30ml potassium pyroantimonate and osmic. The tissue was then processed as normal for electron microscopy.

5. Electron Diffraction

5.1 Theory

In this technique the magnification is altered by variation of the lens strength.

In diffraction electrons behave similarly to X-rays and the condition for diffraction may be expressed by Bragg's Law.

$n\lambda = 2d \sin\theta$ where λ = wavelength of electrons

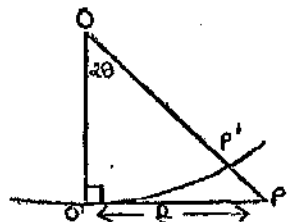
d = interplanar spacing

θ = angle of incidence of beam on plane.

For a first order reflection (n = 1)

$$\frac{1}{d} = 2 \left(\frac{1}{\lambda}\right) \sin\theta$$

5.1 (Cont'd.) The formation of the diffraction can be described in terms of the figure below:



A sphere called the Ewald sphere of radius $\frac{1}{\lambda}$ may be drawn and the distance O'P is the chord subtended by an angle 2θ at the centre of the circle of length $\frac{1}{d}$

Now if OO' is the effective camera length L, O'P' will be proportional to $\frac{1}{d}$ and P will be the diffraction spot on the final screen. The camera length L is not a real one and must be calculated.

Since O'P' = O'P and

$\frac{D}{2}$ = L tan 2θ from the figure above

tan 2θ = 2 Sin θ where θ is small

and λ = 2d Sin θ

$\frac{D}{2} = \frac{L\lambda}{d}$

5.1. (Cont'd.) D = diameter of circle and $2\lambda L$ = camera constant. The diffraction pattern so obtained is representative of the reciprocal lattice of the crystal lattice and the distance $\frac{1}{d}$ is known as the reciprocal lattice spacing. Thus where (h, k, L) are Miller indices of a set of parallel planes, these make intercepts $(\frac{1}{h}, \frac{1}{k}, \frac{1}{L})$ on the axes (a, b, c). Thus P is a reciprocal lattice point which represents a whole stack of crystal lattice nets in real space. Where the Ewald sphere intersects a net at a point, a diffraction spot is obtained.

The camera constant is usually calculated by standardisation with thallous chloride, whose lattice spacings are known. If the plane on which the crystal is lying and the crystal structure is known, a reciprocal lattice array can be constructed geometrically and hence the spots corresponding to particular lattice planes can be identified on the diffraction pattern.

In a polycrystalline sample, a pattern equivalent to rotating a single crystal through all directions is

5.1 (Cont'd.) is/ obtained and hence rings and not spots are shown. (Hirsch et al, 1965)

5.2 Method

Unstained sections of the material prepared as in 3.10.2 above were mounted on uncoated copper grids and viewed with a Siemens Elmiskop 1A aligned for electron diffraction, Fig. 2. The selected area diffraction technique was calibrated using a standard thallous chloride specimen. For comparative purposes two films were prepared from a pure solution of calcium pyroantimonate, made by dissolving equal quantities of potassium pyroantimonate and calcium carbonate in distilled water. The first fine film was achieved by spraying the solution on to a carbon coated grid. A thicker film was obtained by evaporating a drop of calcium pyro-antimonate on to a carbon coated grid. The lattice spacings obtained by electron diffraction were compared with the standard X-ray powder spacings as given by the A.S.T.M. index (1965).

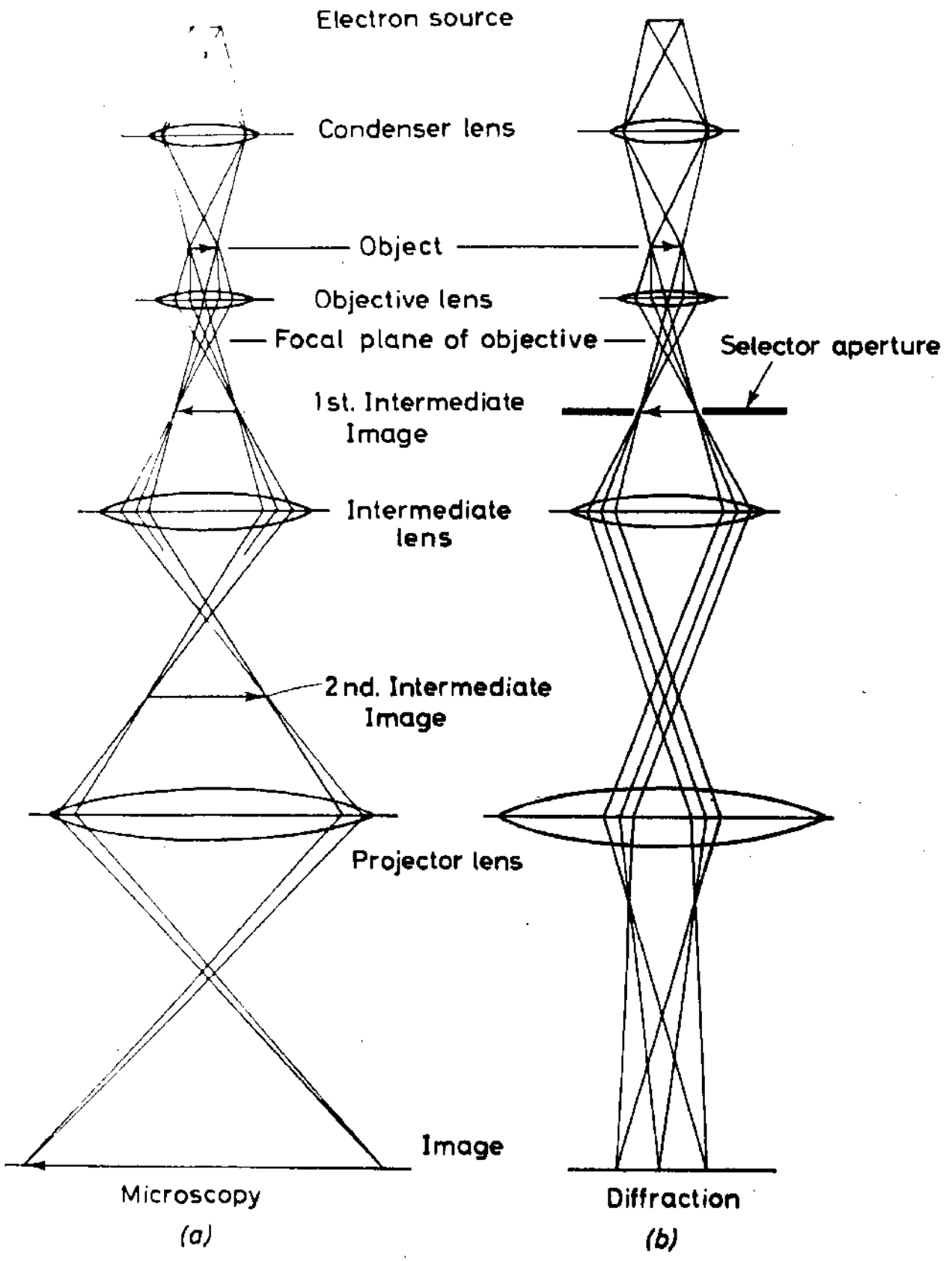


Fig 2. Ray diagrams for (a) transmission microscopy and (b) selected area diffraction. (from Hirsch et al, 1965).

6. Neutron Activation Analysis of Antimony in Eggshells

6.1 Theory

In neutron activation analysis the amount of an element present in a sample is determined by irradiating the sample with neutrons, and then measuring the intensity of the characteristic radioactivity induced in the element. The intensity of this induced radioactivity is directly proportional, other factors remaining constant, to the amount of the element, irrespective of its state of chemical combination.

This experiment was designed to test the efficiency of potassium pyroantimonate as a precipitant and localising agent and therefore to ascertain whether the precipitate found in 3.10.2 and subsequently identified as calcium in 5. was coincidental or whether it was a true reflection of the site of calcium transfer.

6.2 Method

Pieces of shell were removed from eggs which had been in the shell gland at the time of injection of potassium

6.2 (Cont'd.) potassium/ pyroantimonate. The samples were ground in an agate mortar and pestle and weighed before being transferred to polythene ampoules. The latter were sealed by melting in a flame and were then wrapped in foil and sent for irradiation to the nuclear reactor at Harwell. A piece of eggshell taken from a normal oviposited egg was treated similarly. After irradiation the samples were counted on a Gamma-ray multichannel analyser standard substraction procedures were applied to provide the corrected activities for substitution in the following formula where x = antimony

$$\frac{\text{Mass of x in sample}}{\text{Mass of x in standard}} = \frac{\text{Total corrected activity produced by radionuclide in sample}}{\text{Total corrected activity produced by radionuclide in standard}}$$

7. Depth of Pigment Layer

Pieces of shell were taken from quail, White Leghorn, Brown Ranger and Black Headed Gull, Fig. 3 and supported in wax. Photographs were taken of the fractured surface in each case using a Leitz Panfort microscope.

8. Infra red Analysis of Eggshell Pigment

8.1 Preparation of Material

Cuticular Pigment

The cuticular pigment was examined in the quail and Black Headed Gull by mechanically scraping it from the shell surface into a watch glass.

Shell Pigment

The cuticle layer was removed from the eggs of the quail, Black Headed Gull, White Leghorn and Brown Ranger by dissolving it in E.D.T.A. The eggs were dried and small pieces of shell ground in an agate mortar and pestle.

Quail Pigment and potassium bromide (KBr)

In order to test the effect of sublimation on the pigment some of the latter was scraped from a quail shell and ground up with potassium bromide to give a KBr/pigment disc.

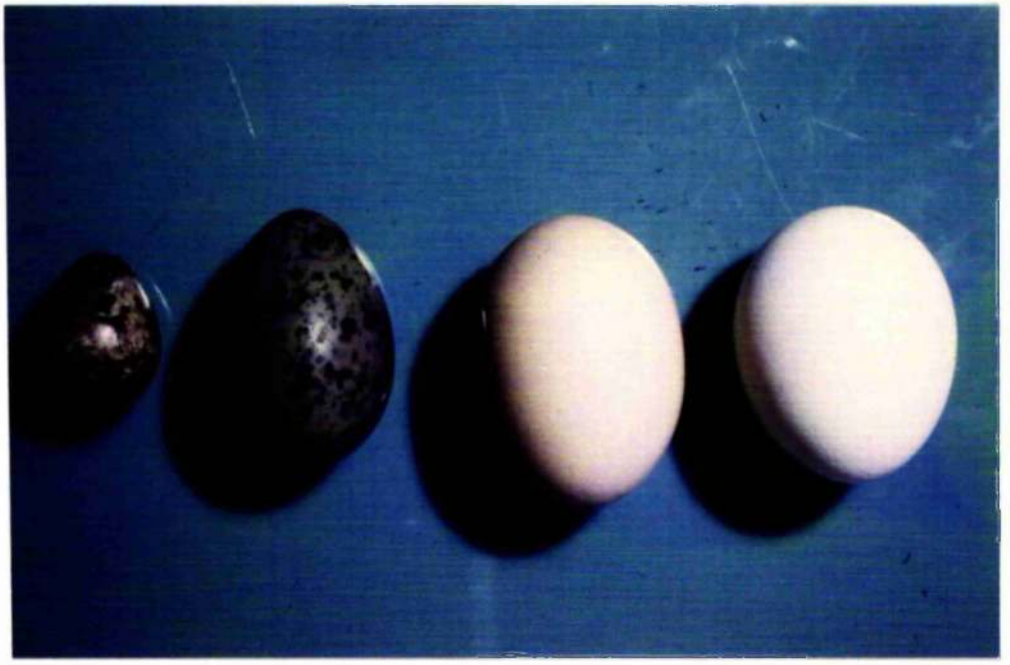


Fig 3. Eggs used in pigment analyses.
Left to right: Quail, Black headed Gull, Brown Ranger, White Leghorn.

8.1 (Cont'd.) Shell + KBr

Quail shell from which most of the pigment had been removed by repeated sublimation was ground up with KBr to see if the calcium carbonate in the shell was causing any interference. The KBr discs prepared above were then treated as in 8.3.

8.2 Sublimination of Pigment

The resultant powder arising from the above techniques was placed on a thermally massive stainless steel block, fitted with a thermocouple and a 100 watt heater. The system was evacuated to a pressure of approximately 10^{-5} torr and the temperature of the block was raised to 300°C in order to sublime the pigment, which was collected on either glass discs (1 inch in diameter) or on NaCl discs (0.5 inch diameter) for subsequent analysis. No evidence was found for decomposition at this temperature.

8.3 Analysis of Sublimed Pigment

The NaCl discs obtained in 8.2 were sandwiched together pigment sides adhering - to give a thicker

8.3 (Cont'd.) thicker/ pigment layer and hence a stronger reading. They were fixed in a brass container and placed in the PE257 Spectrophotometer. A clear NaCl disc was used as a reference.

9. X-ray Fluorescence

Elemental analysis was carried out by X-ray fluorescence with the Philipps PW12/20. The technique involves bombarding the specimen (pigment on glass slide) with a monochromatic X-ray beam which is generated in turn by bombardment of a suitable target with electrons. The X-rays emitted during this process are characterised by an analysing crystal (LIF) and the signals are collected in a detector. Examination was made for lead, cobalt, zinc, iron, calcium and magnesium by adjustment of the analysing crystal so that only X-rays characteristic of these elements were recorded.

10. Ultrastructural Analysis of Pigment Crystals

10.1 Sublimed Pigment

The yellow pigment from the shell and cuticle of the quail and Black Headed Gull and the pigment derived from the shell only of the Brown Ranger and White Leghorn was washed off the glass slides with dilute HCl. Drops of the resultant yellow solution were placed on carbon coated grids and allowed to dry before viewing with the Siemens Elmiskop 1A. Both transmission and diffraction studies were carried out on the crystals.

10.2. Crushed Pigment

In order to see if sublimation affected crystal structure, pieces of Black Headed Gull shell were crushed in an agate mortar and pestle. Some of the powder was dissolved in dilute HCl and a drop transferred to a carbon coated grid. Examination was carried out in the Siemens Elmiskop 1A.

10.3 Protoporphyrin di methyl ester (Sigma)

A few grains of esterified protoporphyrin were placed on a glass slide. When this purple powder

10.3 (Cont'd.) powder/ was crushed between two glass slides its colour changed to yellow-gold. Some of this crushed deposit was dissolved in dilute HCl and a drop placed on a carbon coated grid. When dry the grid was examined as above.

11. Biochemical Analysis

11.1 Concentration of Acid Phosphatase in Plasma and Shell Gland Fluid

In view of the cyclical variations observed in the intracellular localisation of acid phosphatase in the shell gland it was decided to investigate the possible fluctuations of this hydrolytic enzyme in the plasma and shell gland fluid during the laying cycle. A number of methods have been devised to estimate serum acid and alkaline phosphatase activity. Of these the most widely used is the King & Armstrong method (1934), which utilises phenyl phosphate as the substrate. In the present investigation Para-nitrophenyl phosphate was used as substrate (Bessey et al 1946) because it is hydrolysed faster than phenyl phosphate and is therefore more sensitive. Sensitivity was considered to be of some importance in the measurement of phosphatase activity in the shell gland fluid since the amount of fluid collected at any one time is minimal.

11.1.1 Materials

A total of 50 White Leghorns were used in these experiments. Each bird was lightly anaesthetised by means of an oxygen/carbondioxide mixture passed through

11.1.1 through/
(Cont'd.) Halothane B.P. with the bird in a supine position, a small midline incision was made in the abdominal wall and the shell gland exposed. The technique for the collection of shell gland fluid with an egg in the oviduct is described by El Jack and Lake (1967). The cloaca was cleaned and pressure applied to the anterior end of the shell gland. Fluid was collected via the vaginal opening into a graduated centrifuge tube. The amount of fluid collected was extremely small 1 - 1.5ml. With an egg in the infundibulum or magnum only 0.3 - 0.5ml was collected.

Simultaneously 2ml blood was drawn into a heparinised syringe from the main artery supplying the shell gland, the hypogastric. The blood was centrifuged at 3,000g and the plasma fraction removed.

11.1.2 Method

In this method 0.5ml substrate (0.015M P-nitrophenyl phosphate in 0.001 N HCl) buffered with citrate at P^H 4.9 was used. After three minutes 0.1ml shell gland fluid/plasma was added. The reaction was

11.1.2 (Cont'd.) was/ allowed to proceed for 1 hour at 37°C when activity was stopped by the addition of 8ml 0.02N NaOH. The optical density of the solution was measured at 415μ in 1cm cells using the S.P.600 spectrophotometer, the result representing the test reading T. A blank reading B was obtained by adding 1 drop concentrated HCl to the solution remaining in the test tube. All readings were done in triplicate. The acid phosphatase activity was obtained by subtracting T - B and reading this value from a calibration curve prepared for alkaline phosphatase (Solomon 1970). The results were divided by 4 since alkaline phosphatase activity was measured over a 15 minute interval.

[1 B.L.B. unit = that quantity of acid enzyme which liberates 1 millimole of P nitrophenol / litre serum / hour at 37°C.]

11.2 Estimation of Tissue Porphyrins

Pieces of tissue were taken from the mid isthmus, shell gland pouch region and the shell gland/vagina of the quail, White Leghorn and Brown Ranger. The

11.2 (Cont'd.) The/ birds were divided into 2 groups - oviduct empty, calcifying egg in shell gland. The tissue was placed in a tube and approximately 3ml glacial acetic acid was added. The whole was homogenised, then 10 volumes of Diethyl ether added. The mulch was spun down at 3,000g and then decanted into separating funnels. The extraction in acetic/ ether was repeated until no more fluorescence was obtained in the ether layer determined by subjecting the solution to a source of UV light. The tissue pellet was retained for further extraction for uroporphyrin.

Coproporphyrin and Protoporphyrin

The combined ether layer was washed twice with 25ml 3% sodium acetate, it was then shaken with 25ml dilute iodine solution and finally with 25ml of water. Coproporphyrin was then extracted by shaking the solution with successive small quantities (2ml) of 0.1N HCl. The optical density of this solution was read at 400 - 402µ on the SP 500.

11.2 (Cont'd) Protoporphyrin was extracted from the ethereal solution after all coproporphyrin had been removed. This was done by shaking the solution with small quantities 2ml of 5% Hcl until the extract no longer showed red fluorescence in UV light. The optical density of this solution was measured at 406-408mμ.

Uroporphyrins

The tissue pellet from the acetic/ether extraction was washed with 2 x 10ml N ammonia hydroxide, spun and decanted. 3% sodium acetate was added and the mixture altered to p^H1.5 - 2.0 with concentrated nitric acid. The same volume of cyclohexanone was added and the solution mixed thoroughly but not vigorously, allowing good separation of the phases. To the combined cyclohexanone extracts were added 2 volumes of diethyl ether and the whole mixed. Uroporphyrin was extracted with 5% Hcl and the absorption read at 406mμ.

Calculations

Uro

$$2D\ max - (D430 + D380) \times 832 \times \text{acid volume} \times \frac{1}{\text{wt taken}}$$

Result µg/g wet wt.

Copro

$$2D\ max - (D430 + D380) \times 73 \times \text{acid volume} \times \frac{1}{\text{wt taken}}$$

Result µg/g wet wt.

Proto

$$2D\ max - (D430 + D380) \times 1.226 \times \text{acid volume} \times \frac{1}{\text{wt taken}}$$

Result µg/g wet wt.

R E S U L T S

I S T H M U S

LIGHT AND ELECTRON MICROSCOPY

The upper limit of the isthmus is defined by the aglandular zone which separates it from the magnum. At its lower limit the isthmus is clearly distinguished from the tubular shell gland by a colour change from white / brown. Such a change is readily discernible in Fig. 1. Observations reported hereafter on isthmian structure assume that the lower limit of this viscus is at the point at which this colour change occurs.

The isthmus is lined throughout by a pseudostratified columnar epithelium comprising a mixed population of ciliated cells and non-ciliated secretory cells. The cells are not of uniform height, tending to become taller posteriorly, accompanied by increased folding of the epithelium, Figs. 4 & 5.

In routine H&E sections the cytoplasm of the ciliated cells is only lightly stained. The non-ciliated cells appear agranular in H&E sections, Fig. 5.

The tubular glands are similar in appearance to those of the magnum (Wyburn et al 1970, Aitken 1971) but their secretions, where these are visible in the lumens, tend to form long threads rather than the amorphous masses which are characteristic of the magnum. The wedge-shaped gland cells

cells/
in routine H&E sections are filled with eosinophil granules which press the nuclei basally, Fig. 5.

The isthmus is well vascularised containing numerous vessels of capillary class. Aggregates of lymphocytes occur in the proprial connective tissue at all levels of the isthmus Fig. 6 together with scattered lymphocytes and plasma cells. The muscularis consists of an inner circular and outer longitudinal layer interspersed between which is vascularised connective tissue.

Ultrastructure

The surface epithelial cells, as elsewhere in the oviduct, are joined by tripartite junctional complexes, comprising a zonula occludens, zonula adhaerens and macula adhaerens. In addition to the ciliated and non-ciliated epithelial cells, a third type of cell has been identified at ultrastructural level and will henceforth be referred to as the mitochondrial cell, Fig. 7.

The ciliated cell has projecting from its apical surface both cilia, with a 9 + 2 arrangement of fibrils and microvilli. These cells contain few organelles, apart from an apically situated nucleus, a few oval-round shaped mitochondria and granules of

of/
variable electron density which have been observed at all levels in the cell. Their appearance however cannot be correlated with any specific phase of the laying cycle. Figs. 7 & 9.

The non-ciliated cell has only microvilli projecting from its apex which range in length from 1.22μ - 3.2μ. The secretory product of the non-ciliated cell is composed of medium dense membrane bound granules. The Golgi complex in these cells is only marginally developed and comprises a few stacks of flattened cisternae circumscribing a mass of small granules in various stages of formation. These granules appear to increase in size by coalescence and move apically. The non-ciliated cell contains variable numbers of mitochondria distributed throughout the cytoplasm. Bundles of filaments have been observed in the cytoplasm. Paranuclear cytoplasmic spaces have also been observed in these cells. Fig. 9.

The mitochondrial cell is characterised by the presence of large numbers of oval shaped mitochondria filled with parallel cristae. The nucleus is centrally placed and the Golgi complex is inconspicuous. Scattered throughout the cytoplasm are

are/
numerous free ribosomes. These cells are non ciliated and have been found throughout the length of the isthmus interspersed between the other two cell types. Figs. 7 & 8.

Tubular glands

The glands in cross section comprise 4 - 5 pyramidal cells joined at their apices by desmosomes. Figs. 10 & 11.
Two morphologically distinct types of cell have been observed in the glands, at all levels of the isthmus, throughout the laying cycle. It is appreciated that these may be different phases in the formation of one mature form, nevertheless they will be described independently. No attempt has been made as yet to estimate the proportions of the two types.
Type 1 Cell is characterised by the presence of numerous granules of variable size and electron density; many of the granules comprising 2 substances of differing electron density. Fig. 12 shows a range of granule types. The basally situated nucleus lies in association with the Golgi complex which surrounds smaller granules in various stages of development. Figs. 13 & 14. The rough endoplasmic reticulum (R.E.R.) varies in its location and degree of distension, sometimes appearing as parallel profiles

profiles/
in the lower two thirds of the cell in close association with the oval shaped mitochondria. Fig. 14. At other times it occurs throughout the cytoplasm in the form of dilated cisternae filled with a fibrillar type of material. Fig. 13. Microvilli project from the lumenal surface of these cells. In some lumena the material is organised into an electron dense core surrounded by material of low electron density. Fig. 10. The density of the secreted material is similar to the density of the intracellular granules, which fuse with the lumenal plasmalemma and release their contents as amorphous masses. Fig. 15. This initial disorganisation of electron dense and less electron dense material is depicted in Fig. 16.

Type 2 cell also contains granules of variable density, although those of high electron density now predominate. The Golgi complex which is supranuclear is fairly inconspicuous. The most conspicuous feature of these cells is the R.E.R. which takes the form of short cords scattered throughout the cytoplasm. Fig. 17. Within the reticular cisternae are numerous discrete electron dense granules. Fig. 18. Frequently the tips of

of/
these cisternae are expanded to form smooth surfaced vesicles. Dilatations, however, have also been observed at points along the length of the cisternae. The material filling the gland lumena of these cells is of high electron density, similar to that of the granules filling the gland cells. Fig. 17.

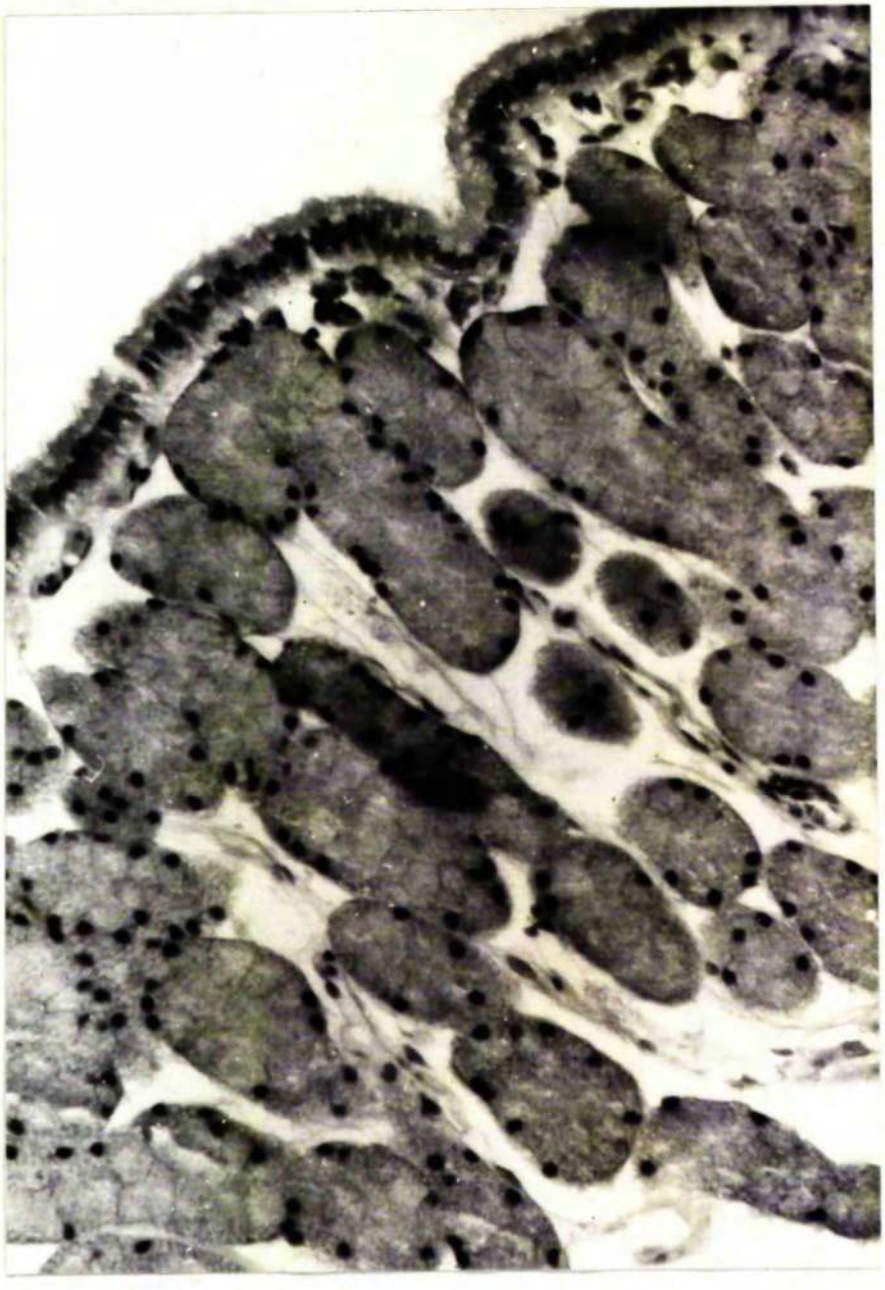


Fig 4. Upper isthmus showing the entire depth of the mucosa. The cytoplasm of both the surface and glandular cells is poorly stained. Note the basal location of the gland cell nuclei. x240 H&E

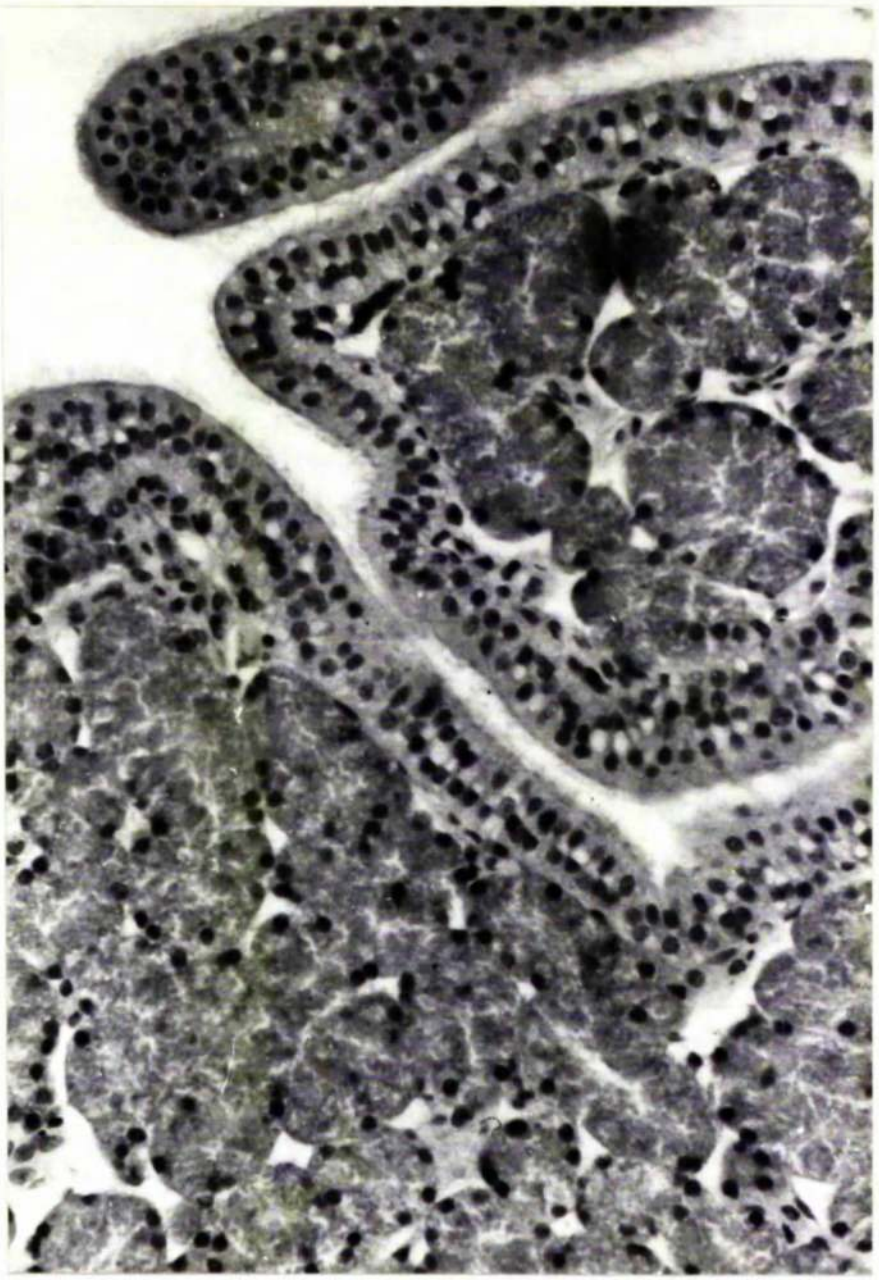


Fig 5. Lower isthmus preceding passage of the egg. The epithelium is more folded and the surface cells are taller (c.f. Fig 4.). Note the granular nature of the gland cells. x240 H&E

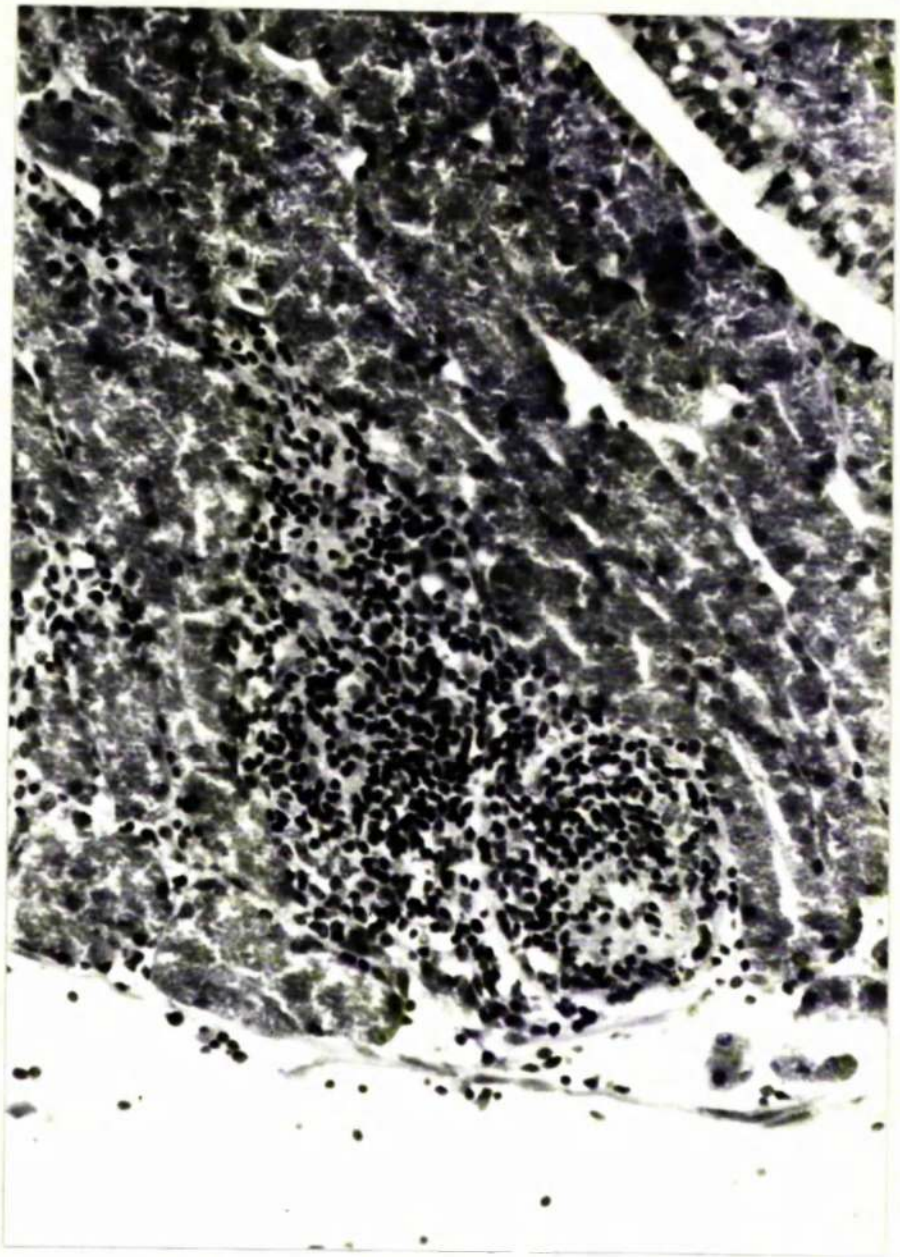


Fig 6. A patch of lymphoid tissue in the lower isthmus. x240 H&E

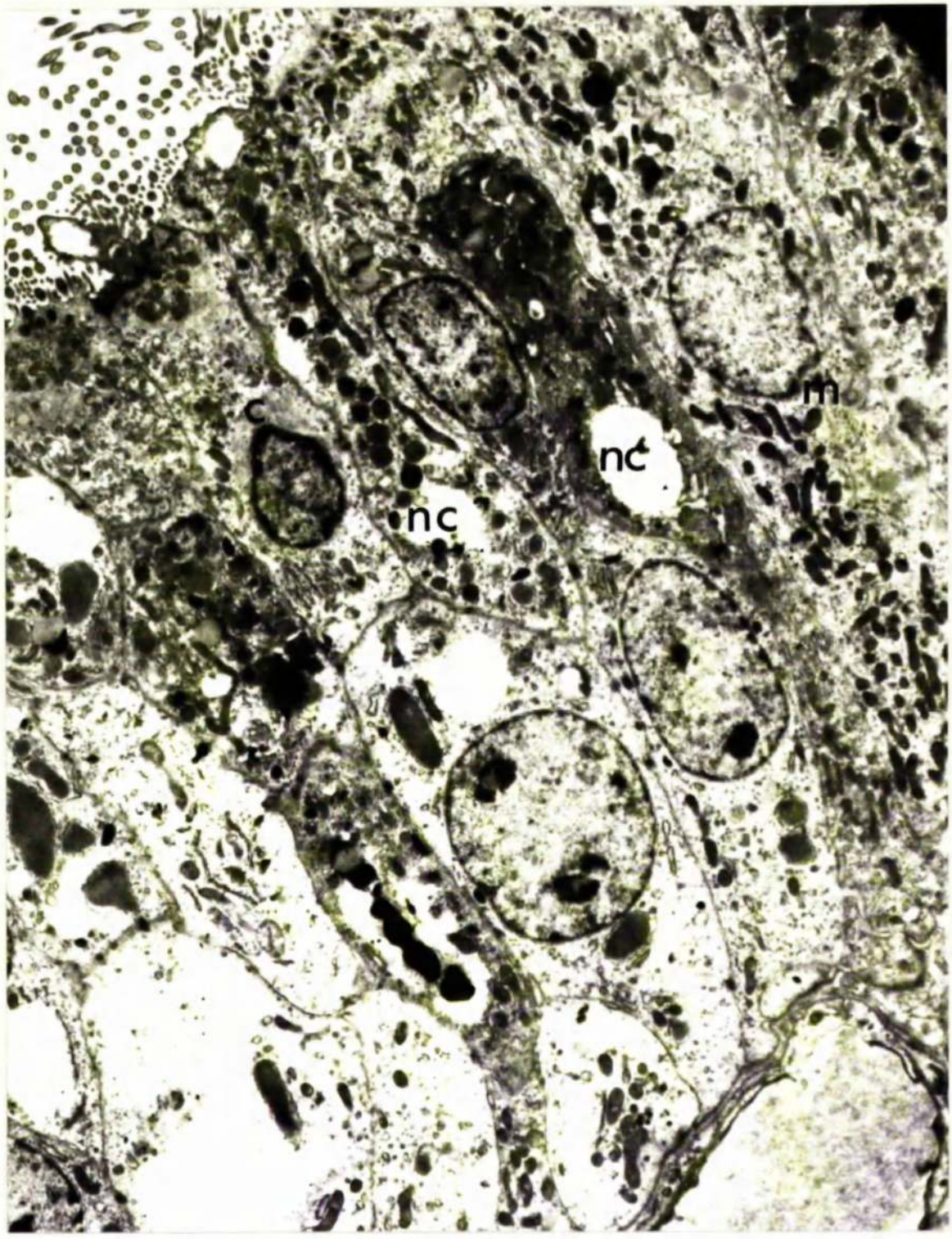


Fig 7. Surface epithelial cells lining the isthmus showing ciliated (c) and non ciliated (nc) cells and a mitochondrial cell (m). Note supranuclear cytoplasmic spaces. x7,000

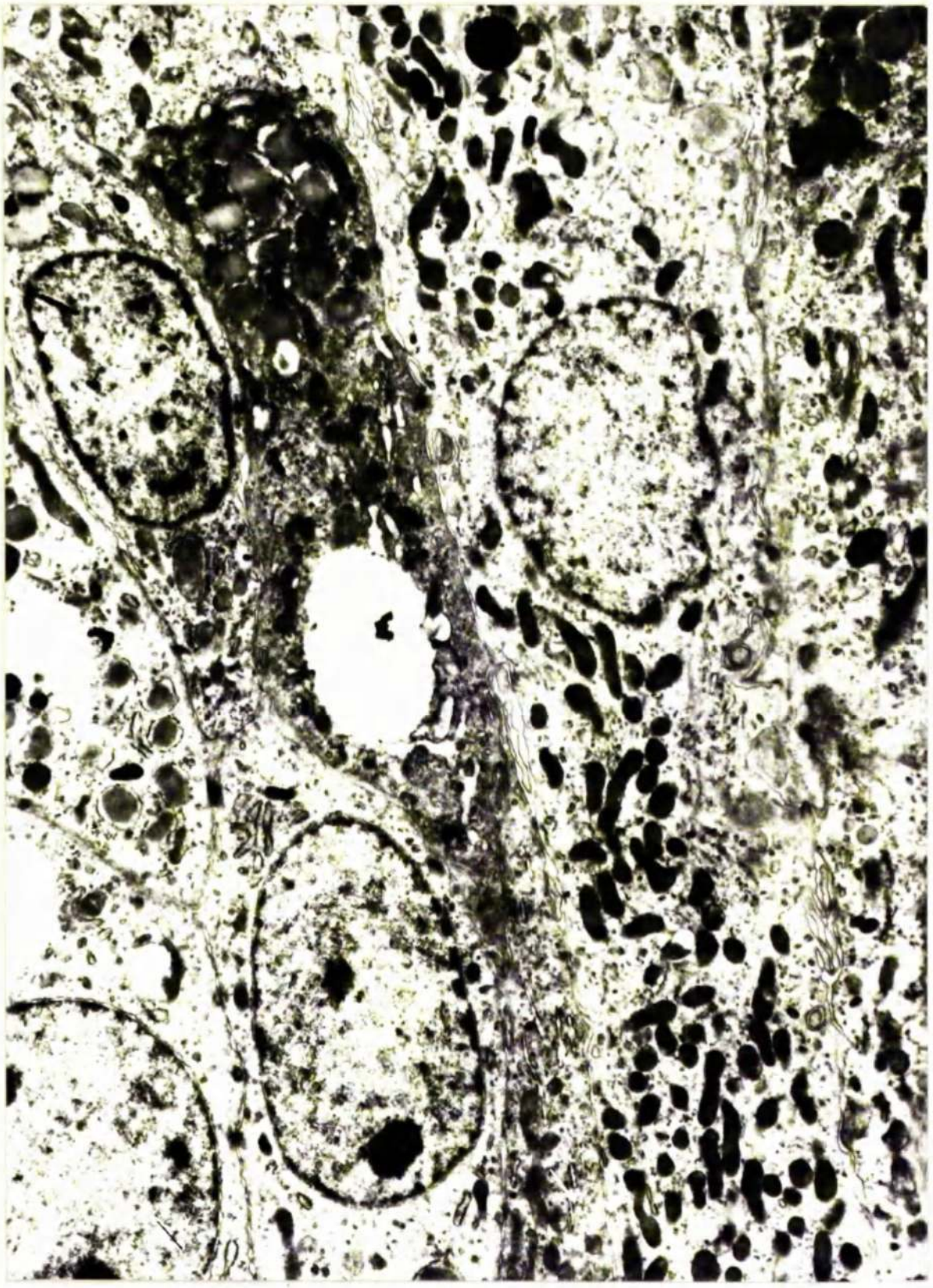


Fig 8. Higher power of mitochondrial cell between two non ciliated cells. x15,000

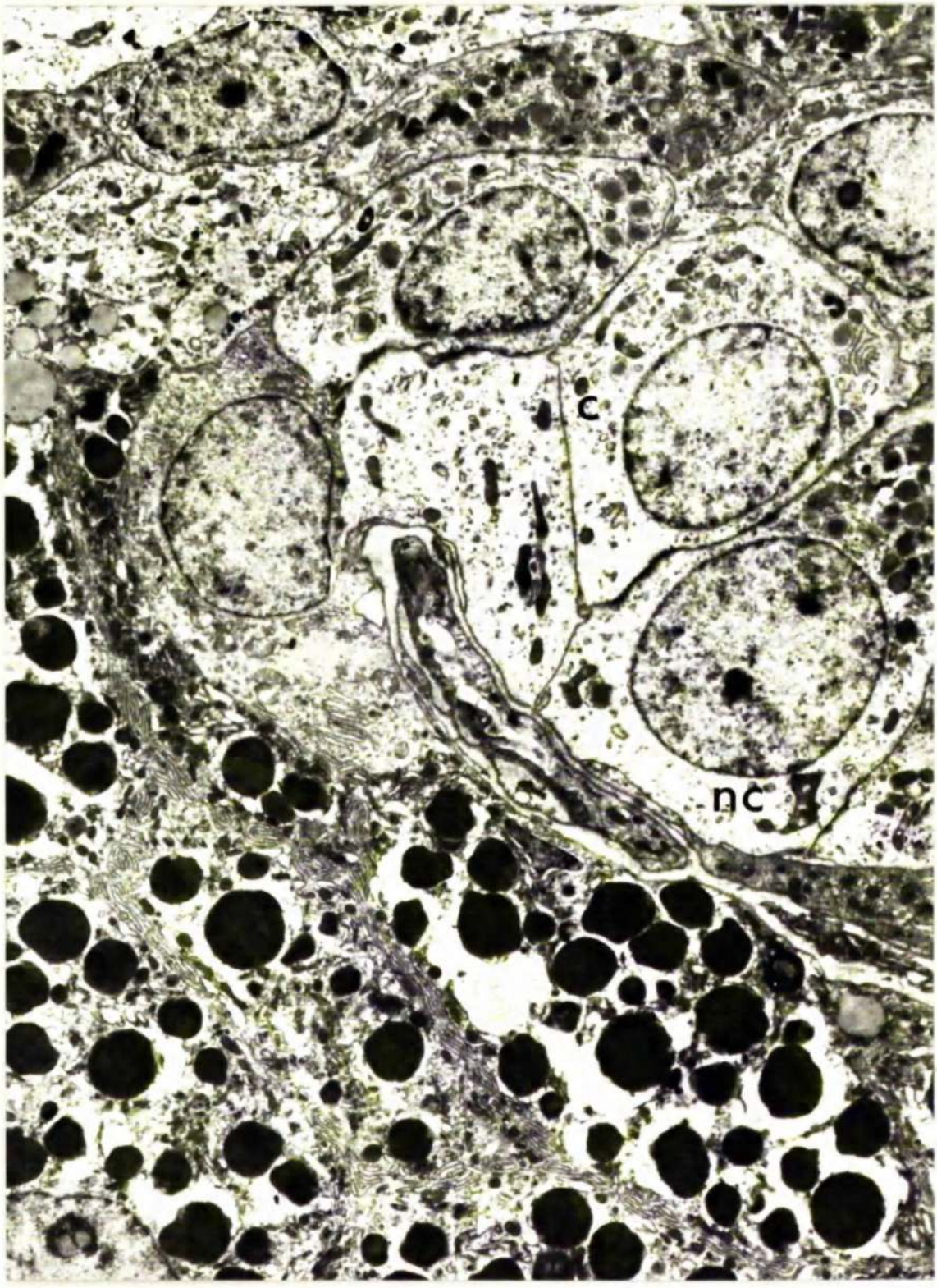


Fig 9. Tangential section of surface epithelial cells i.e. ciliated (c) and non ciliated (nc) lining lower isthmus. These cells overlie the tubular glands which are filled with electron dense granules. x5,600

x 27

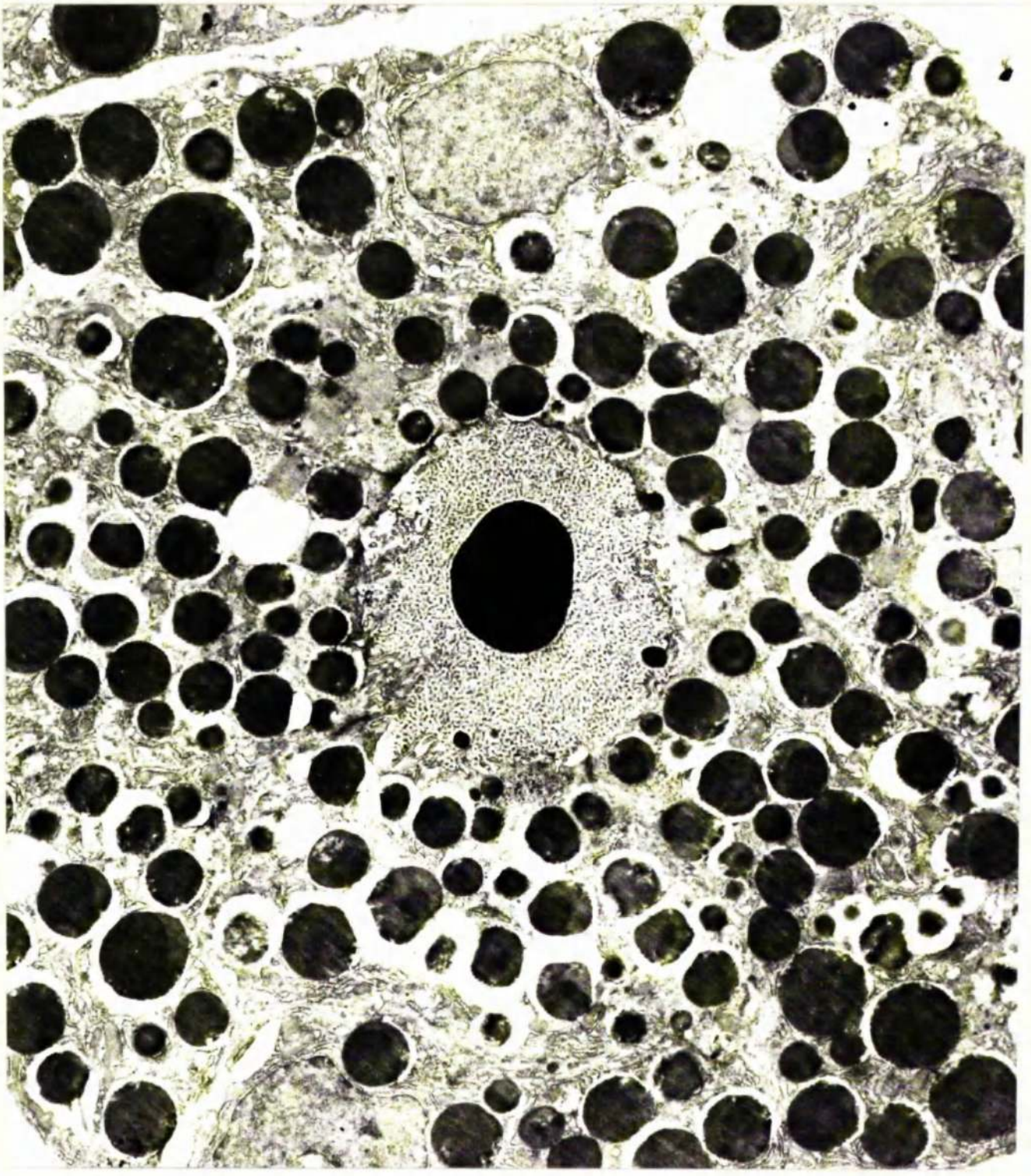


Fig 10. Isthmian gland cells. The spaces round the granules are due to shrinkage. The endoplasmic reticulum occurs throughout the cytoplasm. Note material filling gland lumen which is organised into an electron dense core surrounded by fibrillar material of lower electron density. x5,600

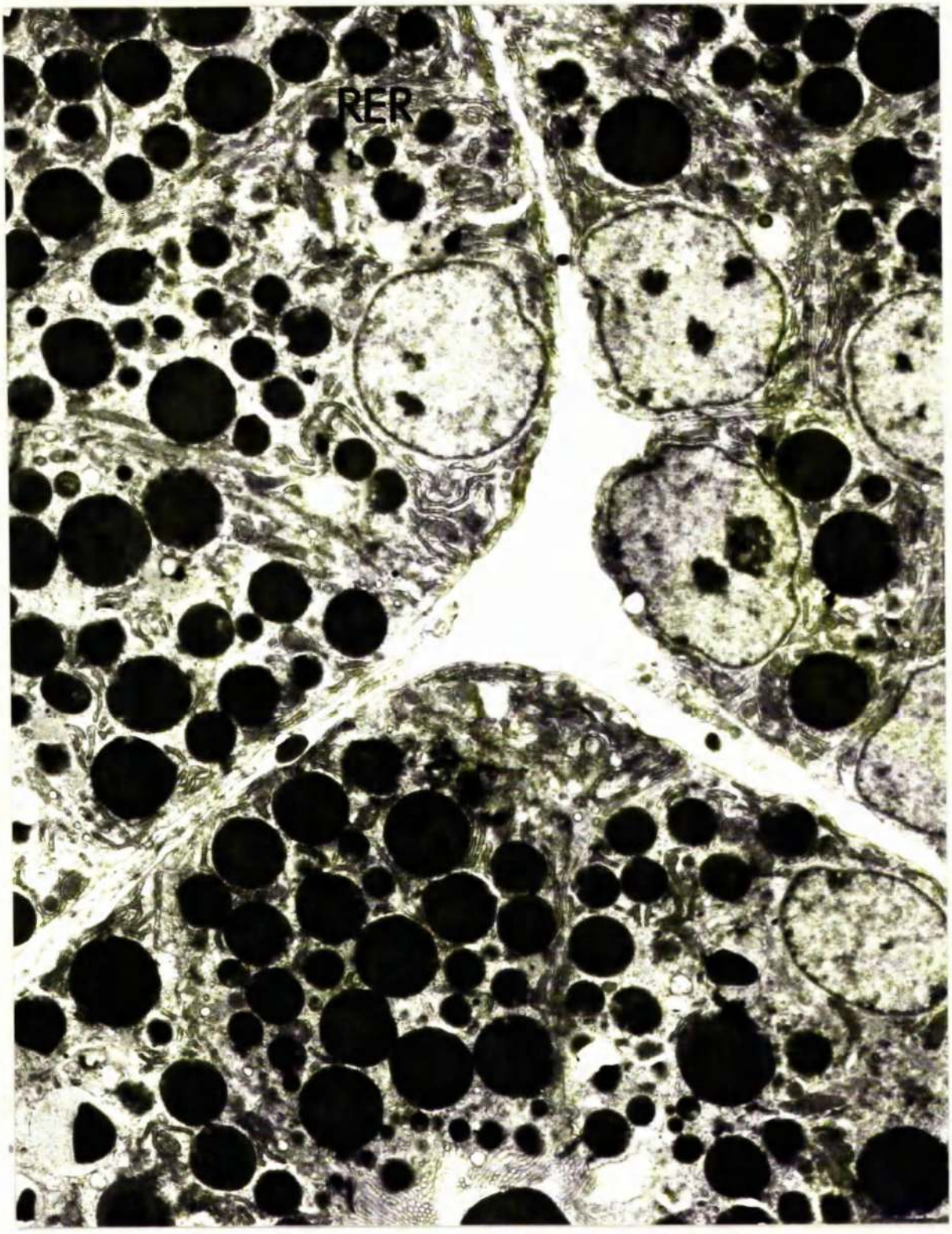
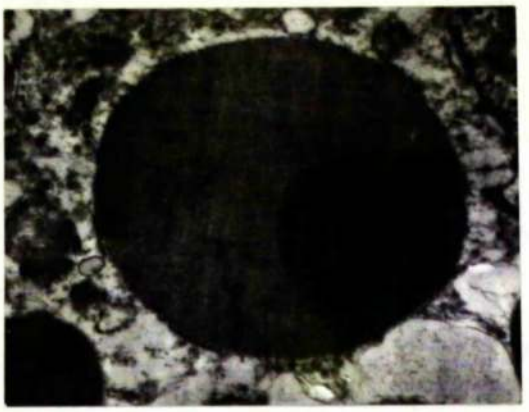
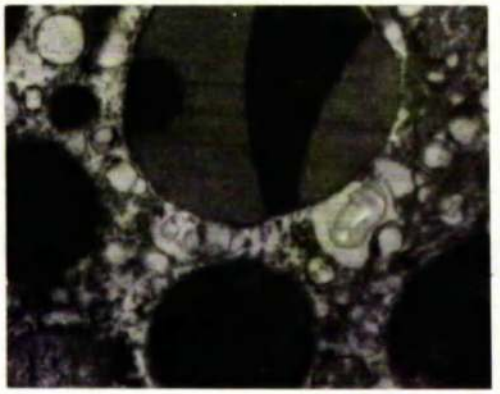


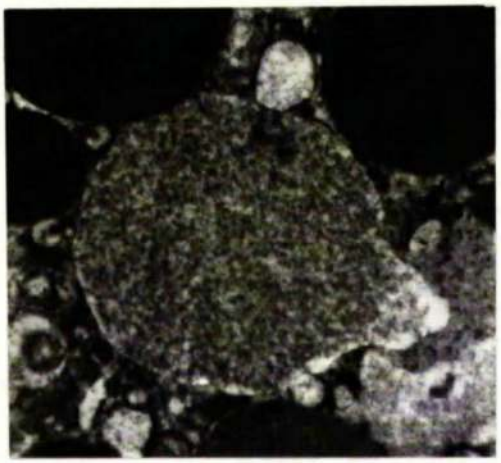
Fig 11. The wedge-shaped gland cells are well depicted. Parallel profiles of R.E.R. filled with material of low electron density occupy the basal half of the cell in association with the nucleus. x10,000



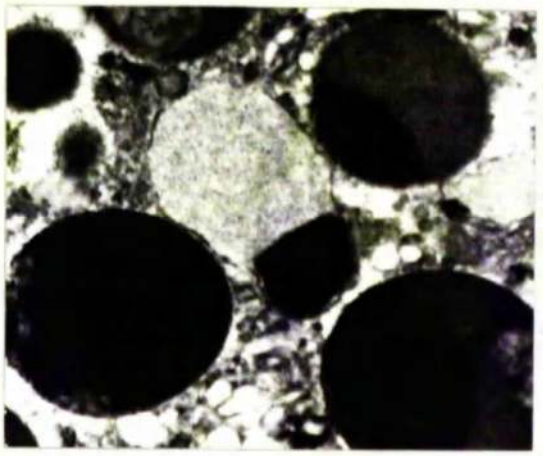
x10,000



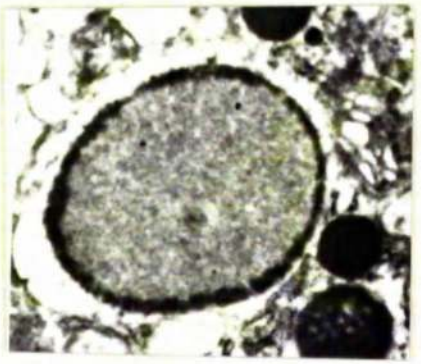
x8,000



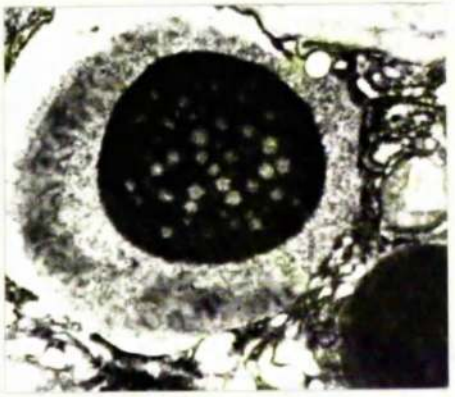
x9,800



x9,000



x7,800



x9,800

Fig 12. A range of granule types of variable electron density observed at all levels of the isthmus.

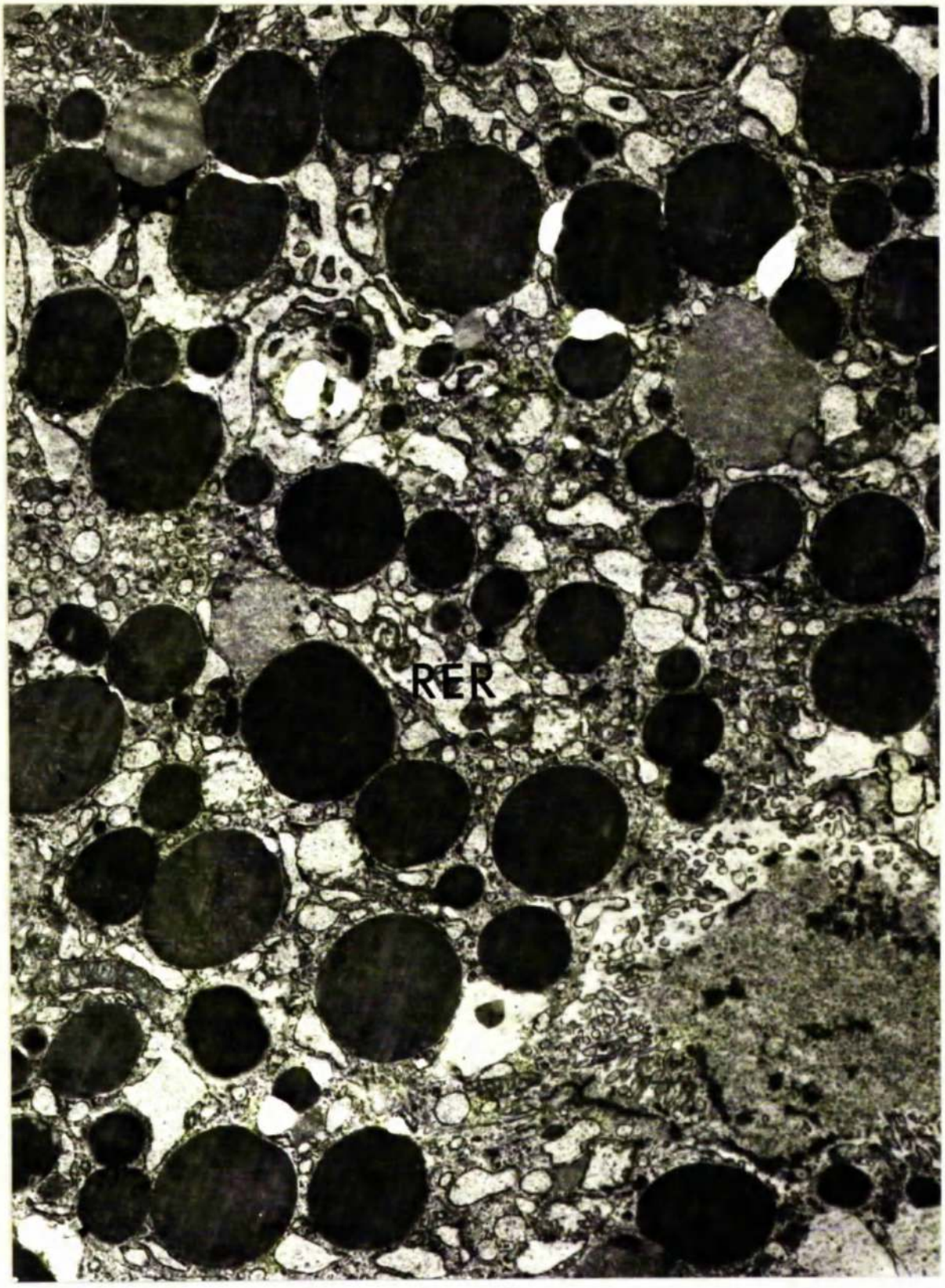


Fig 13. Type 1 cell showing granules of variable electron density, surrounded by dilated R.E.R. filled with material of low electron density. The secretory material filling the gland lumen is similar in density to the intracellular granules. Note its disorganised appearance. x12,000

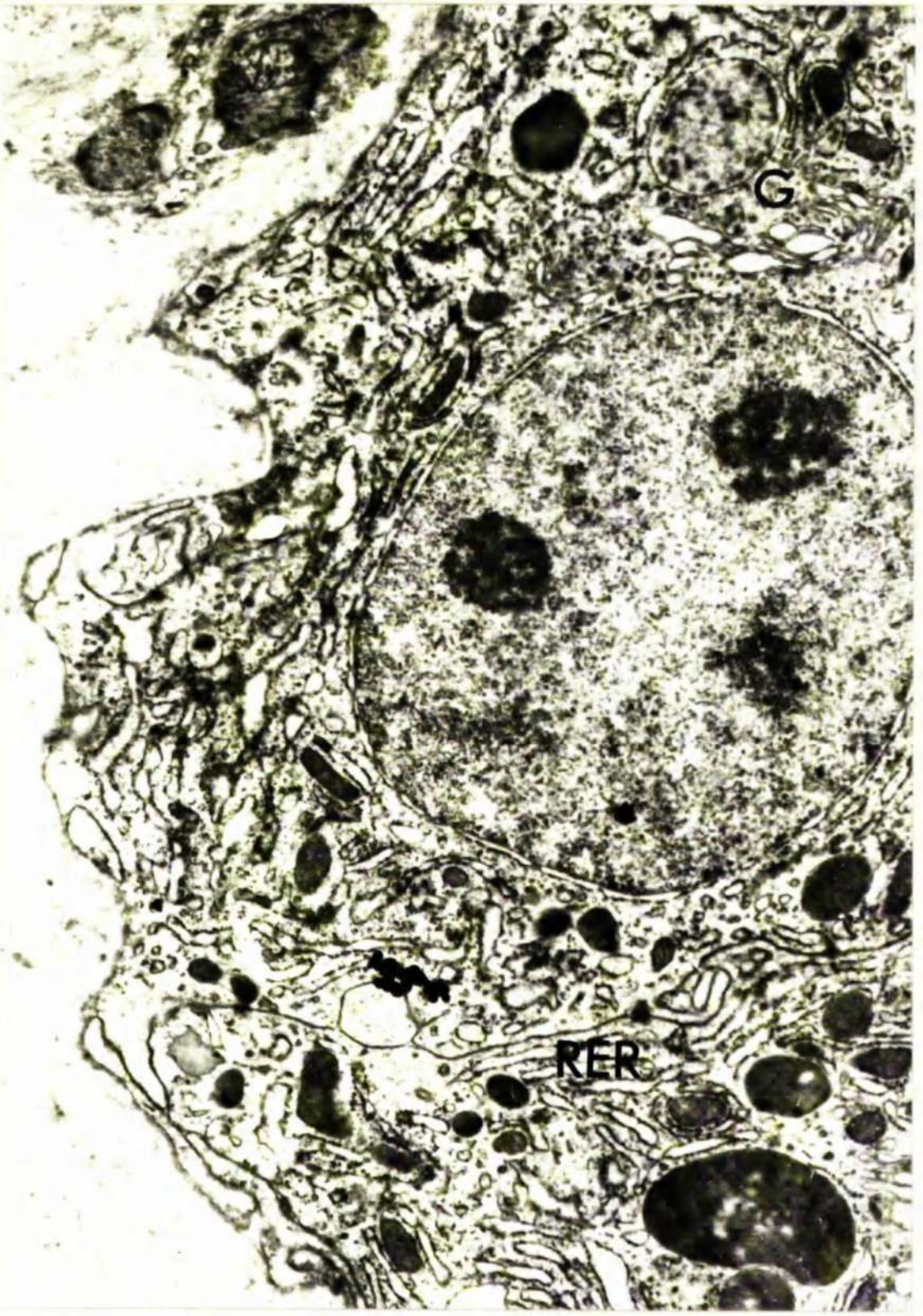


Fig 14. Basal portion of type 1 cell showing parallel profiles of R.E.R. in association with the nucleus. Note well developed Golgi complex (G) and associated developing granules. x10,000

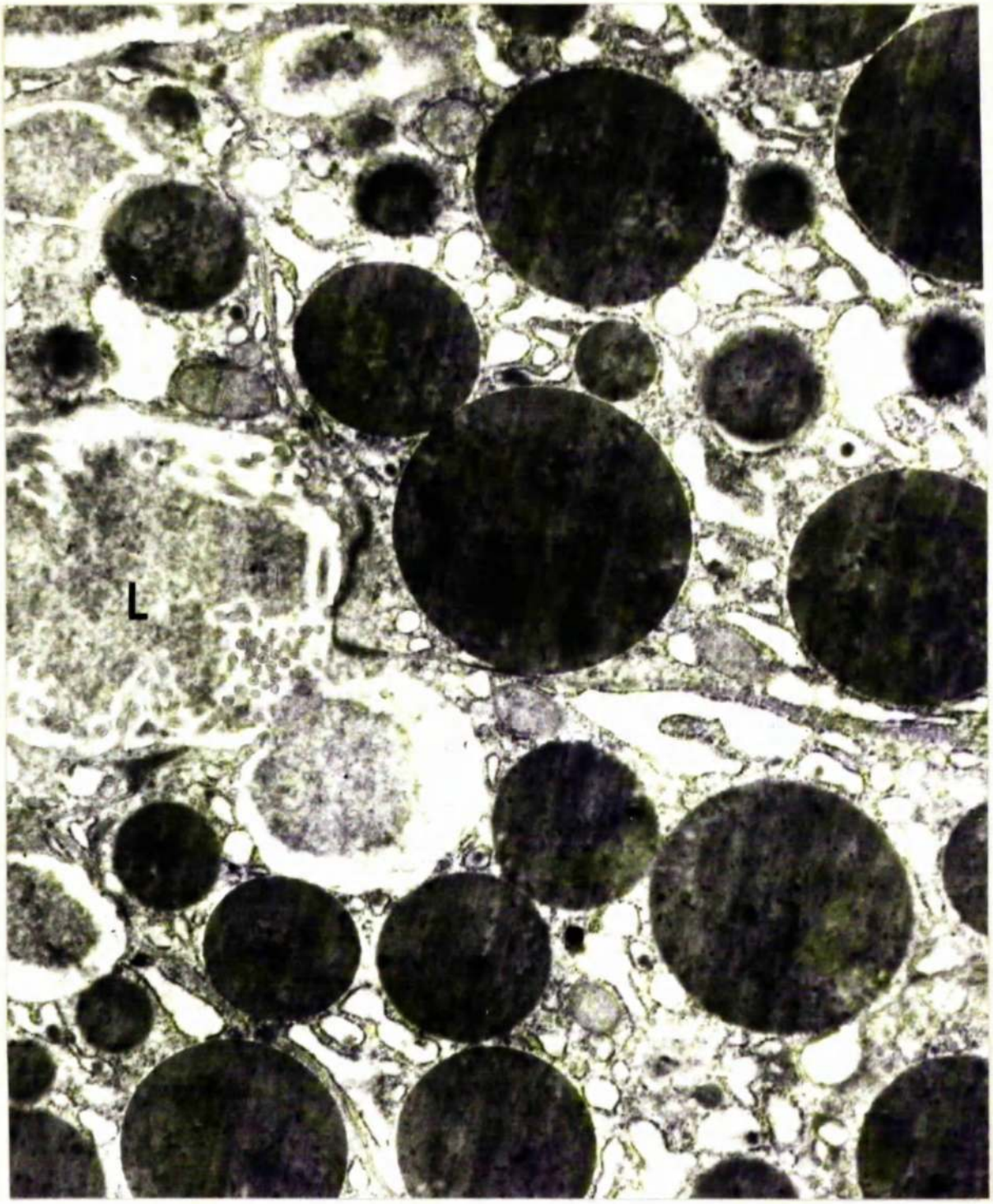


Fig 15. Type 1 cell showing the process of secretion in the tubular gland cells. Note invagination of lumenal surface (L). x 12,600

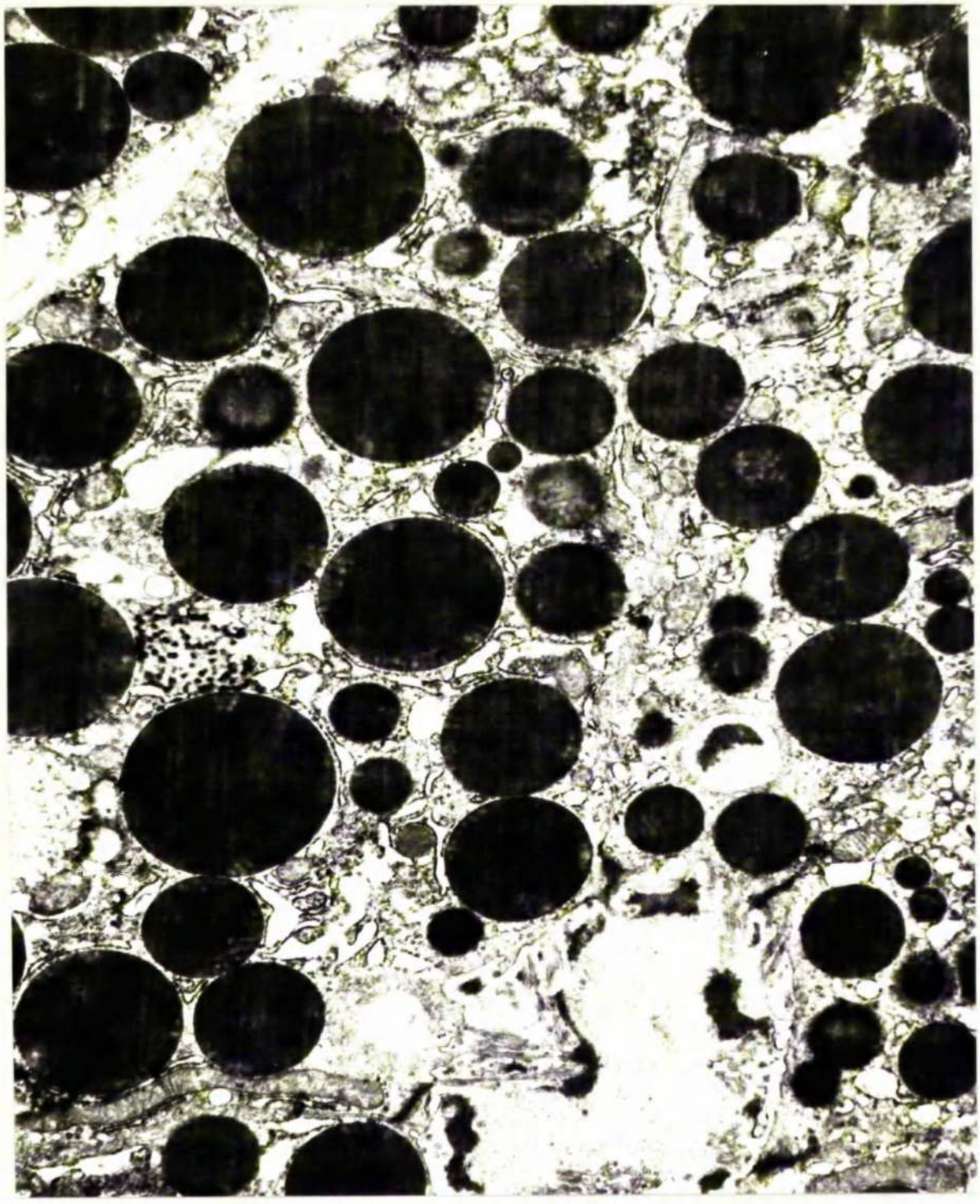


Fig 16. Type 1 cell showing initial disorganisation of secretory material in gland lumen. x10,000

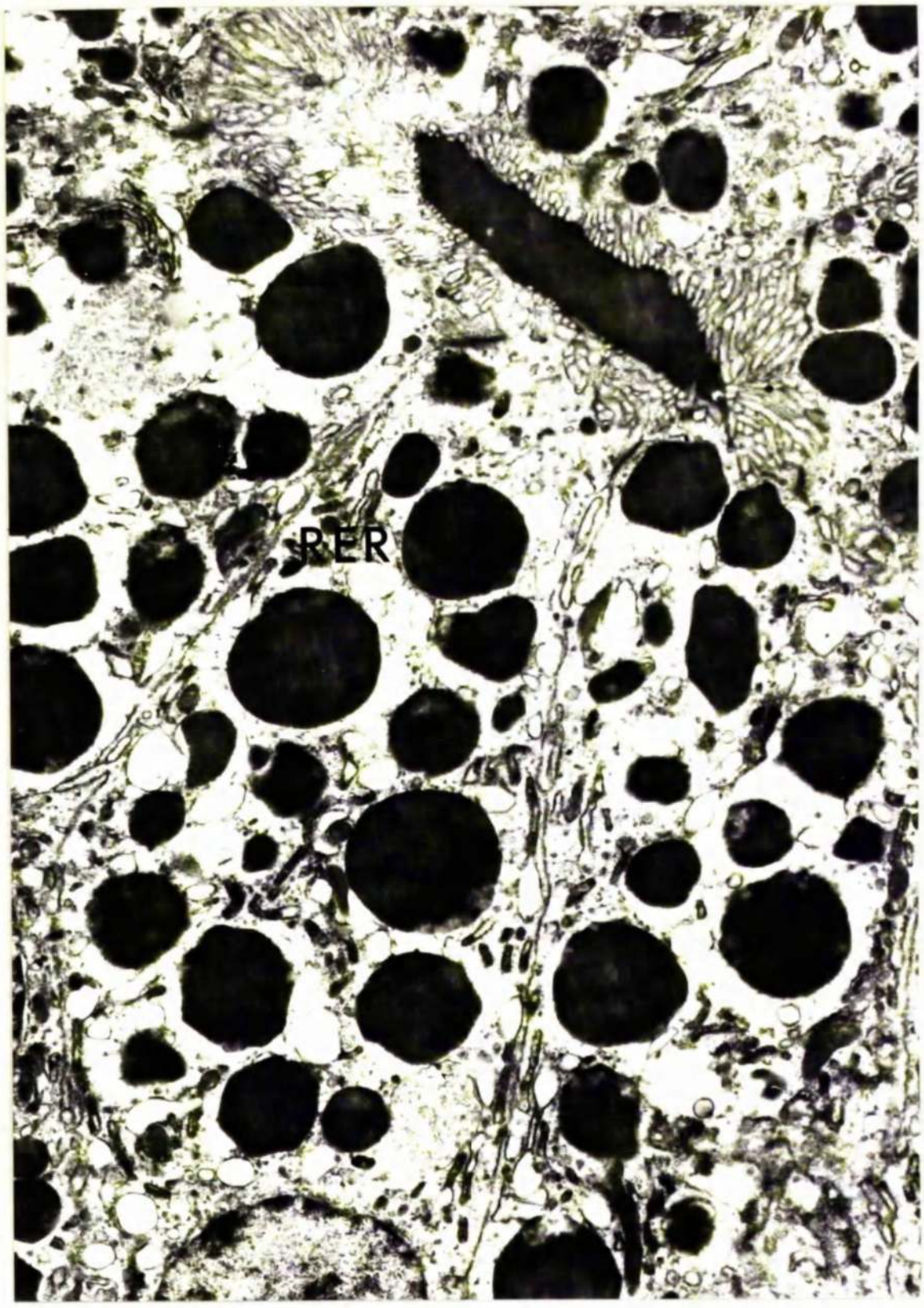


Fig 17. Type 2 cells. The material filling the gland lumen is similar in electron density to the intracellular granules. The most conspicuous feature of these cells is the R.E.R. filled with material of high electron density. The R.E.R. expands into smooth surfaced vesicles giving the cytoplasm a vacuolated appearance. x12,000

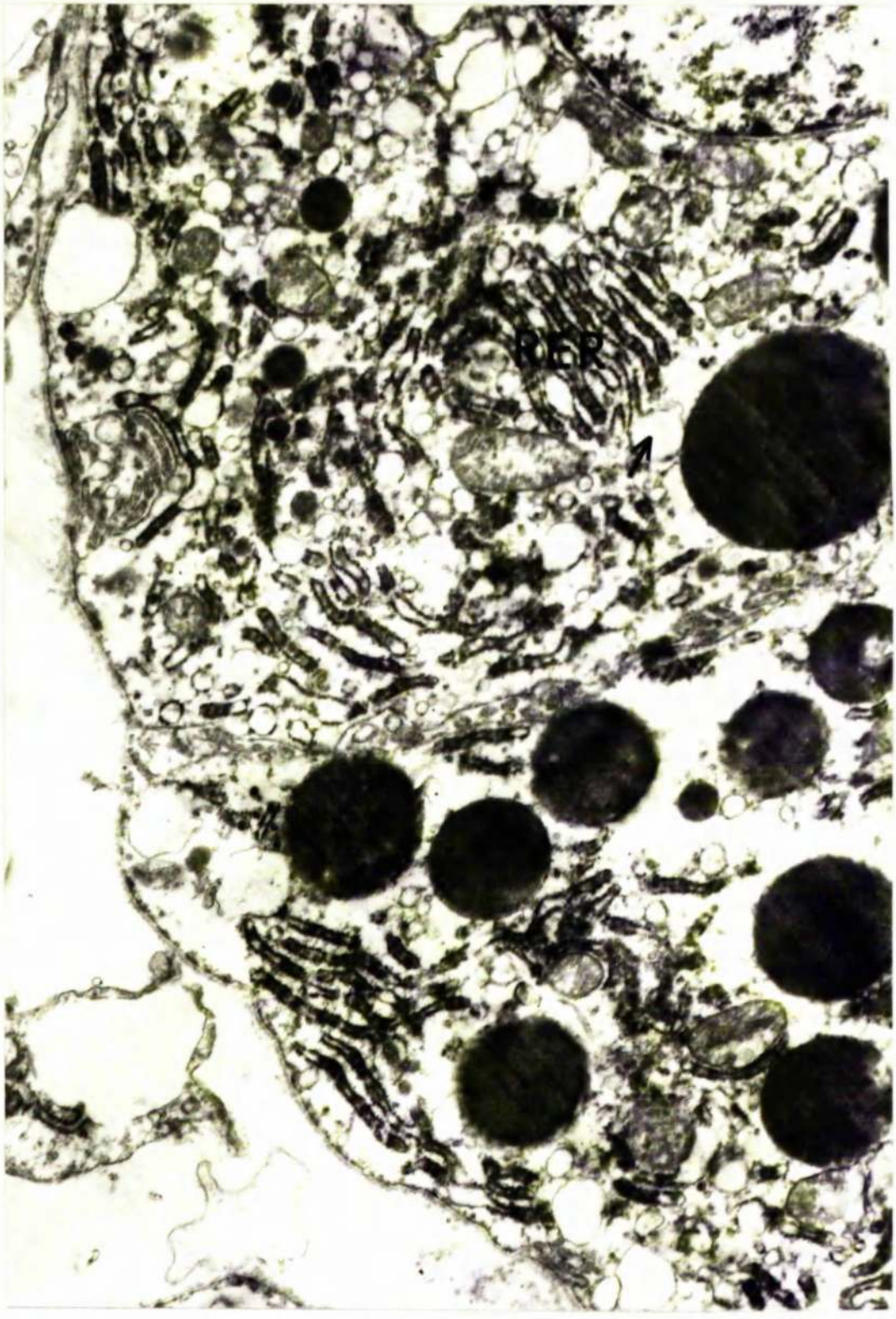


Fig 18. Higher power of type 2 cell showing intracisternal granules in R.E.R. Note expansions of R.E.R. into smooth surfaced vesicles —>. x15,000

S H E L L G L A N D

Light and Electronmicroscopy

SHELL GLAND

Light Microscopy

The term shell gland is here taken to include a relatively narrow tubular section of oviduct measuring some 4cms in length and a dilated pouch-like portion immediately following this which measures up to 8cms in length when occupied by an egg. Both parts are similarly coloured brown when not actively engaged in shell formation and therefore are readily distinguished from the isthmus. Both tubular portion and pouch become bright red in colour during the process of shell formation due to vascular engorgement. Figs. 19, 20 & 21. Nevertheless, as will be shown hereafter, structural features distinguish tubular and pouch parts which might be thought to justify recognition in nomenclature. Studies of the mechanism of calcium secretion in shell formation show however that both parts are involved in this process and because of this identity of function it has seemed reasonable to classify both parts as shell gland.

As in other oviducal parts the entire shell gland is lined by a pseudostratified columnar epithelium, comprising ciliated and non ciliated cells or "apical" and "basal" cells

cells/
as they are frequently termed because of the regular arrangement and location of their respective nuclei, Fig. 22.

Studies with the optical microscope confirm published reports regarding the presence of basophil granules in the luminal cytoplasm of the ciliated cells, particularly in the shell gland pouch region; there is no evidence however of a cyclic variation in the number of these associated with the egg cycle. The non ciliated cells in H&E sections in contradistinction appear agranular at all stages of the laying cycle. Fig. 22.

In sections of the tubular part of the shell gland taken from tissue removed when the egg is lodged here the surface epithelium is indented at regular intervals. fig. 23. Where shell membranes are retained in contact with this epithelium these indentations are seen to correspond with the mammillae projecting from the surface of the outer membrane which appear in such preparations as amorphous masses of eosinophilic material. Fig. 24, 25.

The gland cells lining the tubular shell gland are distinctive and have a vacuolated appearance in conventional

conventional/
paraffin sections somewhat reminiscent of the glands reported to occur in the pouch, but contain in addition moderate numbers of eosinophil granules. The latter however are markedly less numerous than those characteristic of the isthmian glands at all stages of the egg cycle. Fig. 26.

Where the tubular portion expands into the pouch region, these glands are replaced by the typical vacuolated cells of the shell gland with their basally situated nuclei. Fig. 22. The connective tissue and outer muscle layers are slightly thinner than elsewhere in the oviduct, and there are more capillaries in all layers.

Ultrastructure

The structural features of the ciliated and non ciliated cells respectively lining the tubular and pouch regions of the shell gland are similar. The ciliated apical cells are consistently paler in appearance than the adjacent basal cells which are compressed laterally. They contain a number of medium electron dense granules. These granules are most frequently apically situated but they have also been observed in the basal part of the cell. The Golgi complex is well developed and consists

consists/
of stacks of flattened cisternae adjacent to the nucleus. These cells contain few mitochondria. Ribosomes occur scattered throughout the cytoplasm. Fig. 27.

The non ciliated cells contain a complement of secretory granules approximately ½ the size of those in the ciliated cells. These granules are of high electron density and occur at all levels in the cell, maximum numbers occurring during the isthmian phase of egg formation. The Golgi complex is well developed and is medially placed in the cytoplasm. Occasionally these cells have been observed to have a supranuclear vacuole filled with flocculent material. The appearance of these vacuoles has not been correlated with any particular phase of the laying cycle. In the absence of the vacuole the nucleus is capped by a parallel array of cisternae of rough endoplasmic reticulum. Fig. 28. Mitochondrial cells have also been observed in the tubular portion of the shell gland but never in the pouch region.

The gland cells lining the tubular portion of the shell gland differ markedly from those lining the pouch and those in the isthmus. In cross section each gland comprises 5 - 6 wedge-shaped cells, each with a basally situated nucleus. Fig. 29.

Fig.29./
The cytoplasm contains variable numbers of oval-round mitochondria and a prominent Golgi complex. The secretory granules at this level are of low electron density only and are bounded by smooth membranes. Figs. 30, 31 & 32. A further feature of the glands of the tubular shell gland is the constant presence of glycogen which is particularly abundant cranially but diminishes in amount as the pouch is approached. Figs. 33, 34, 35. The basal and lateral cell walls are uncomplicated and show no surface specialisation. The microvilli are shorter than those lining the isthmian glands.

The gland cells in the distal tubular shell gland and pouch region are characterised by the presence of complex infoldings of their lateral cell walls, numerous, bizarre-shaped mitochondria, and the absence of secretory granules. The nucleus is basally located, R.E.R. is sparse and the Golgi complex inconspicuous. Fig. 36.

During the laying cycle ultrastructural changes have been observed in the gland cells of the distal tubular shell gland and shell gland pouch regions. As calcification proceeds the microvilli projecting from the luminal surface become short and stubby, at the same time their tips become swollen. Fig. 37.

Fig. 37/
It has been suggested that this is a form of apocrine secretion. An outstanding feature of the pouch gland cells is a significant variation in the number of mitochondria per cell at different stages of the egg cycle; thus before the egg arrives in the shell gland the cells contain an average of 14.5 $\pm$ 4.7 mitochondria/cell. During the initial 4 hour period of calcification this number increases to 33.4 $\pm$ 6.37 per cell and is maintained at this level until the egg is laid. These values are the means $\pm$ S.D. of 200 observations. Figs. 38, 39 & 40. At this time also the lateral cell membranes increase in complexity, the result of increased infolding. The basal plasmalemma also becomes more interdigitated at this time. Fig. 41, 42. After laying the cells revert in the course of 1 - 2 hours to the state they were in before the egg had reached the shell gland.

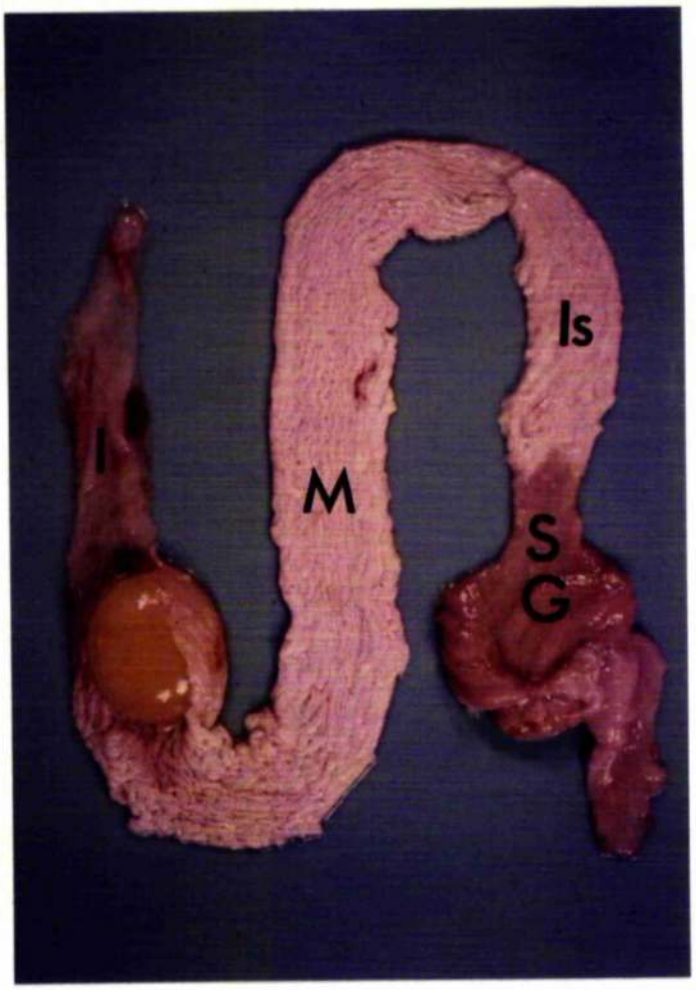


Fig 19. Entire oviduct showing egg in infundibulum (I). Magnum (M), Isthmus (Is), Shell gland (SG).

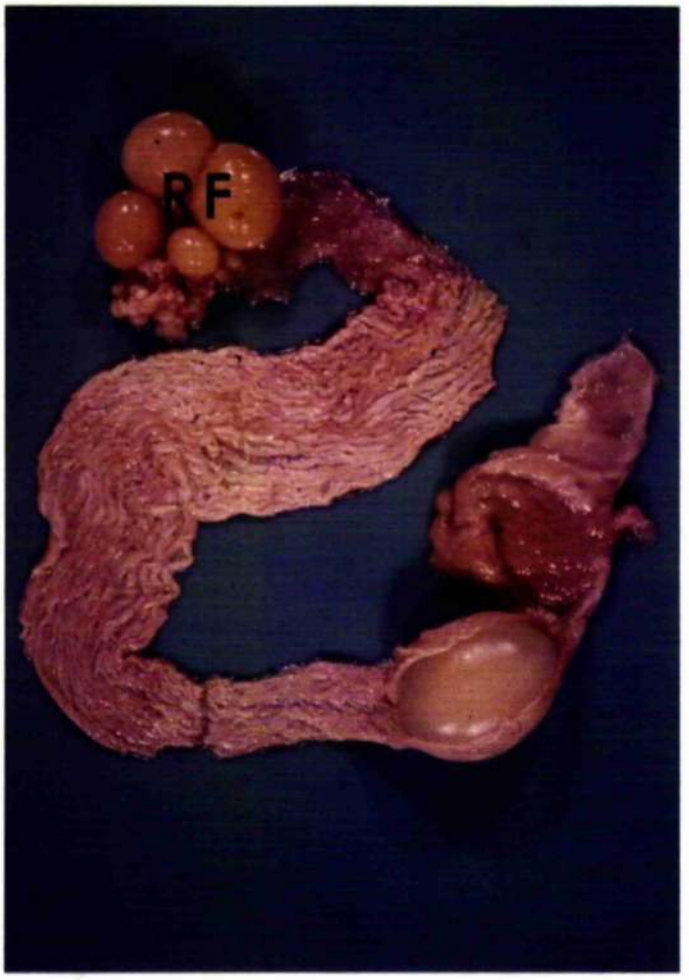


Fig 20. Entire oviduct with egg in lower isthmus/ tubular portion of shell gland. Ripening follicles (RF).

Fig 21. Entire oviduct with egg in shell gland pouch. Note bright red colour of the shell gland compared with the creamy white of the isthmus.

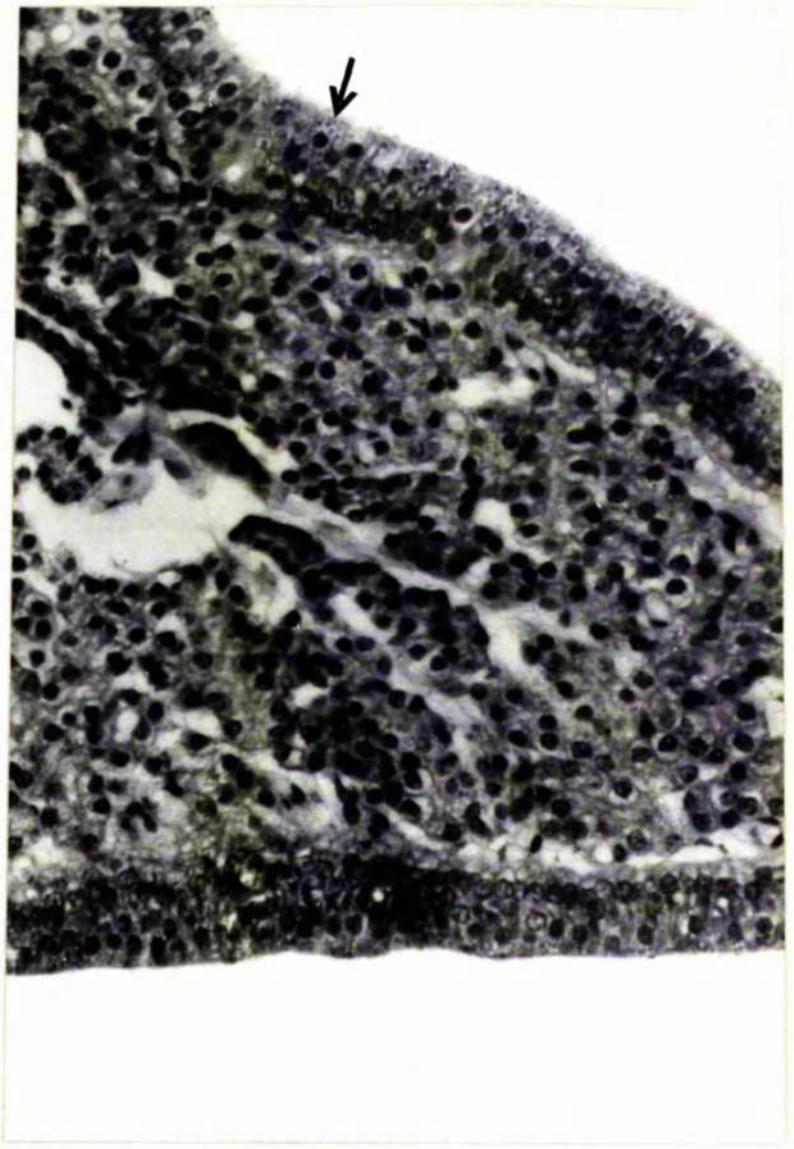


Fig 22. Section of shell gland pouch showing 'apical' ciliated cells and 'basal' non ciliated cells, overlying a mass of tubular glands. Basophil granules are present in the ciliated cells —>. x240 H&E

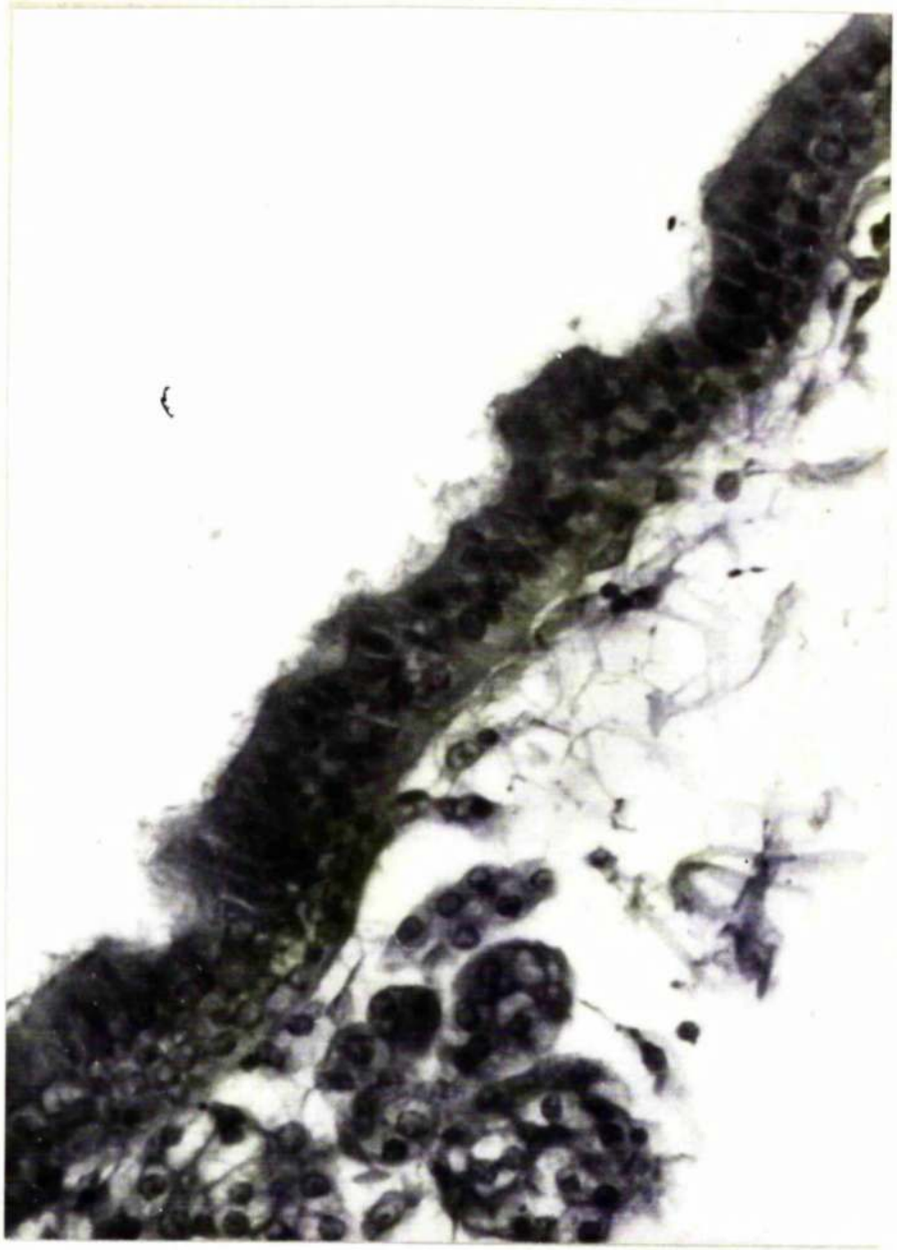


Fig 23. Section of tubular shell gland showing indentations in surface epithelium caused by mamillae. x600 H&E

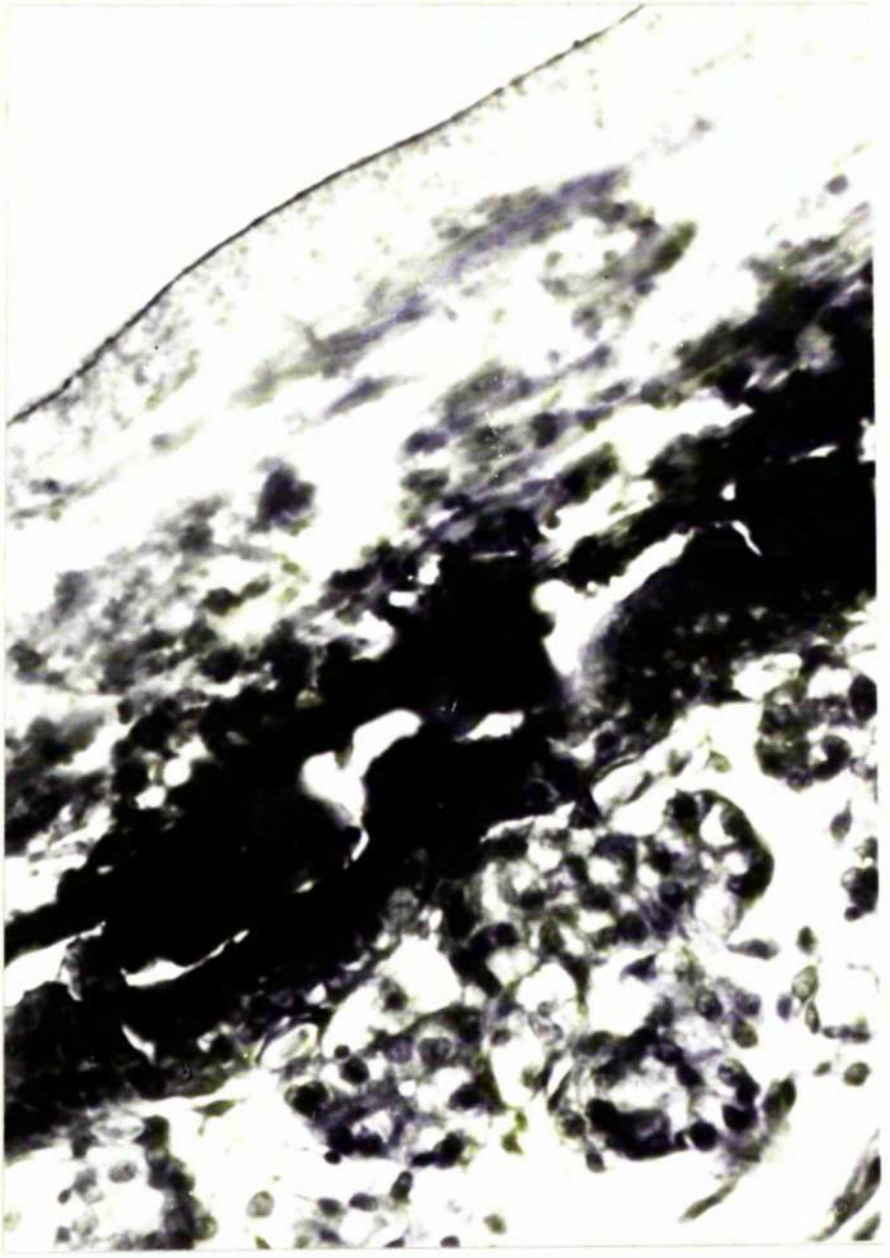


Fig 24. Section of tubular shell gland with membrane adhering. Note mamillae projecting into surface epithelium. x600 H&E

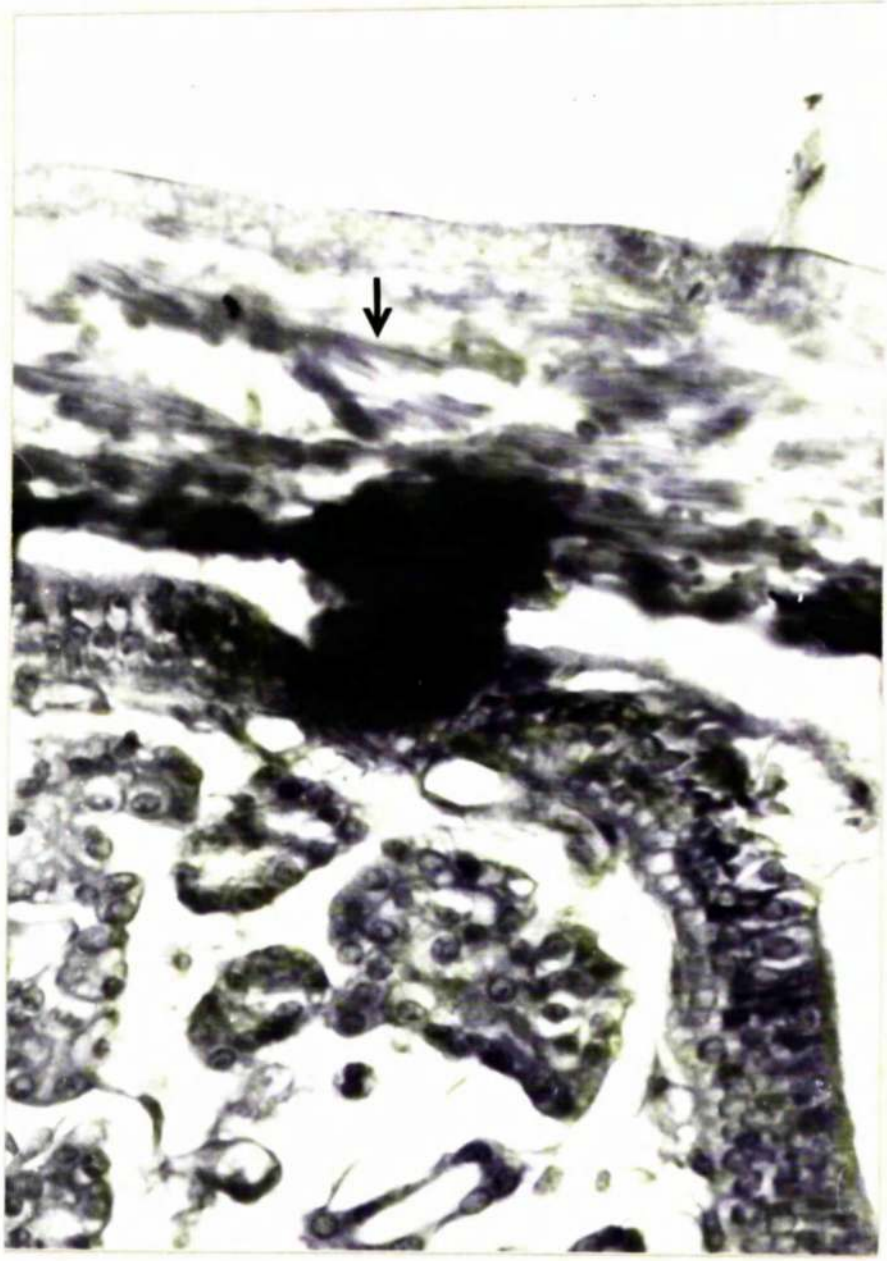


Fig 25. Higher power of mamillae formation.
The two toned appearance of the membrane fibres can be seen —>.
x850 H&E

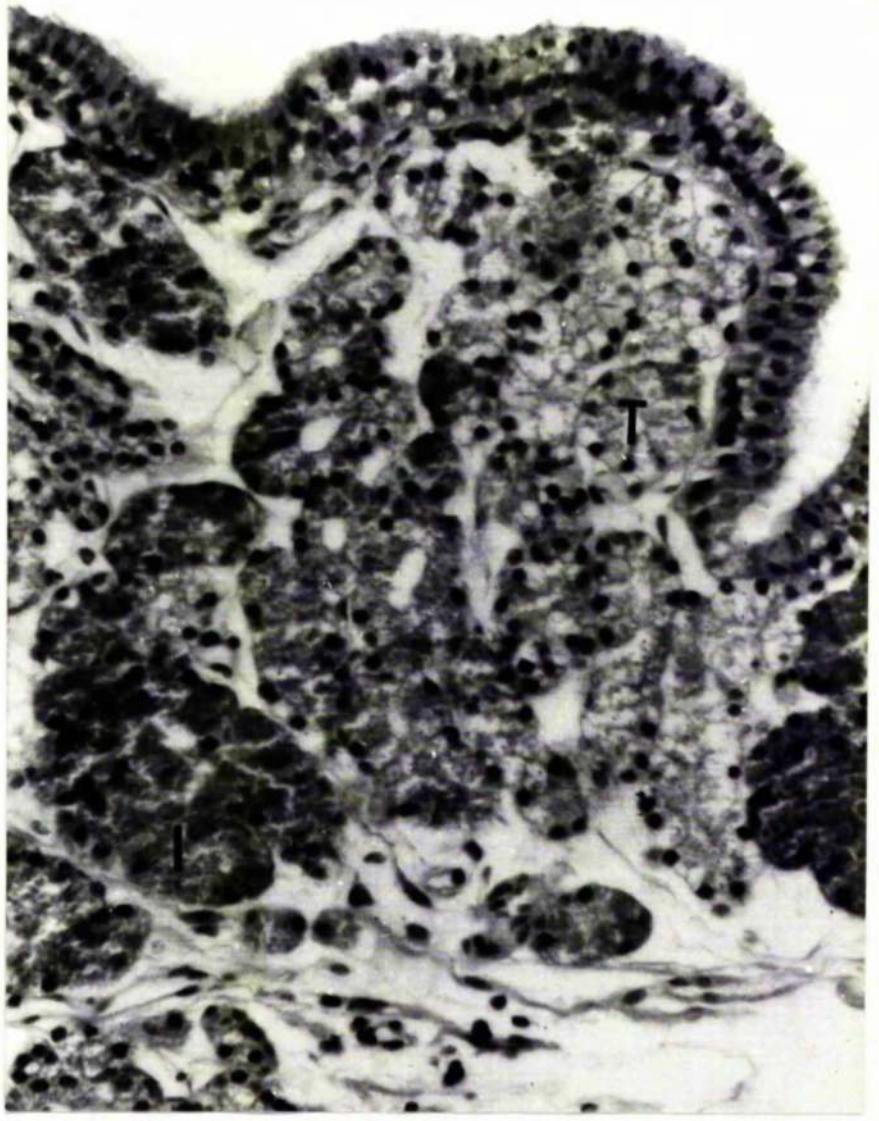


Fig 26. Junctional region between isthmus and tubular shell gland. The typical isthmian gland cells (I) are packed with eosinophil granules. The paler gland cells of the tubular region (T) also contain moderate numbers of eosinophil granules. x240 H&E

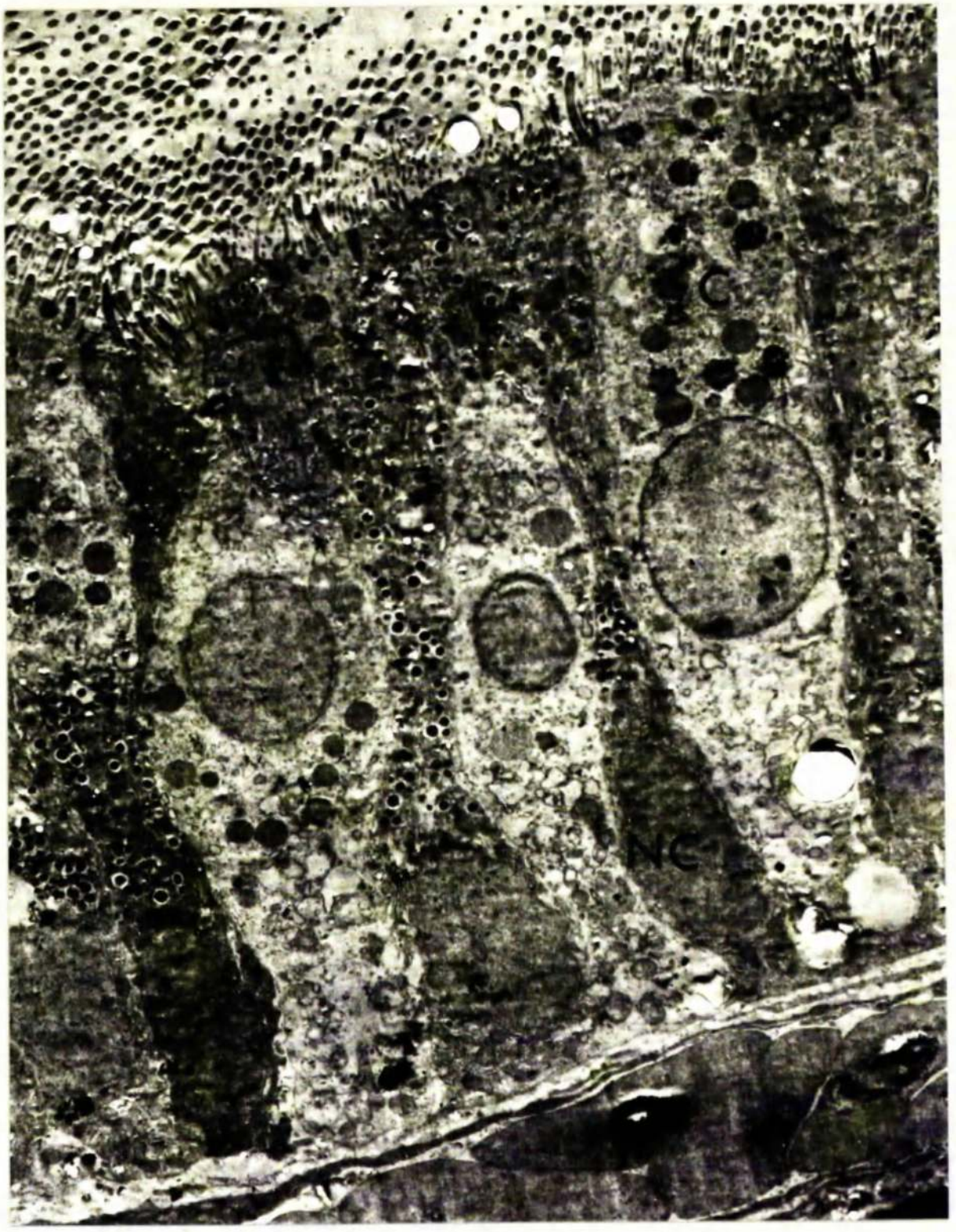


Fig 27. Surface epithelium of shell gland pouch showing ciliated cells (C) which are pale in appearance, and non ciliated cells (NC) which are consistently darker. x9,000

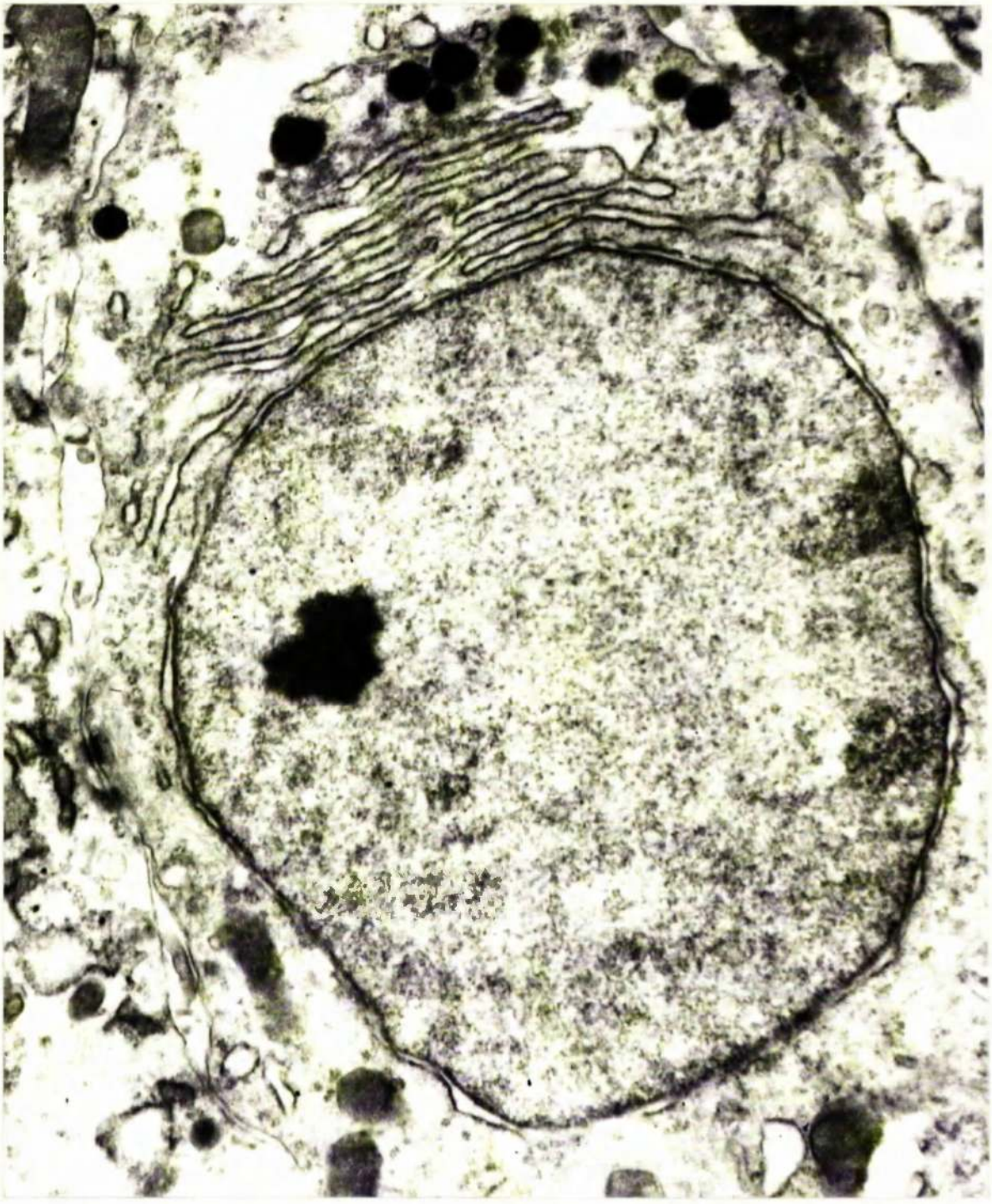


Fig 28. Parallel folds of R.E.R. capping the nucleus in the non ciliated cells. x16,000

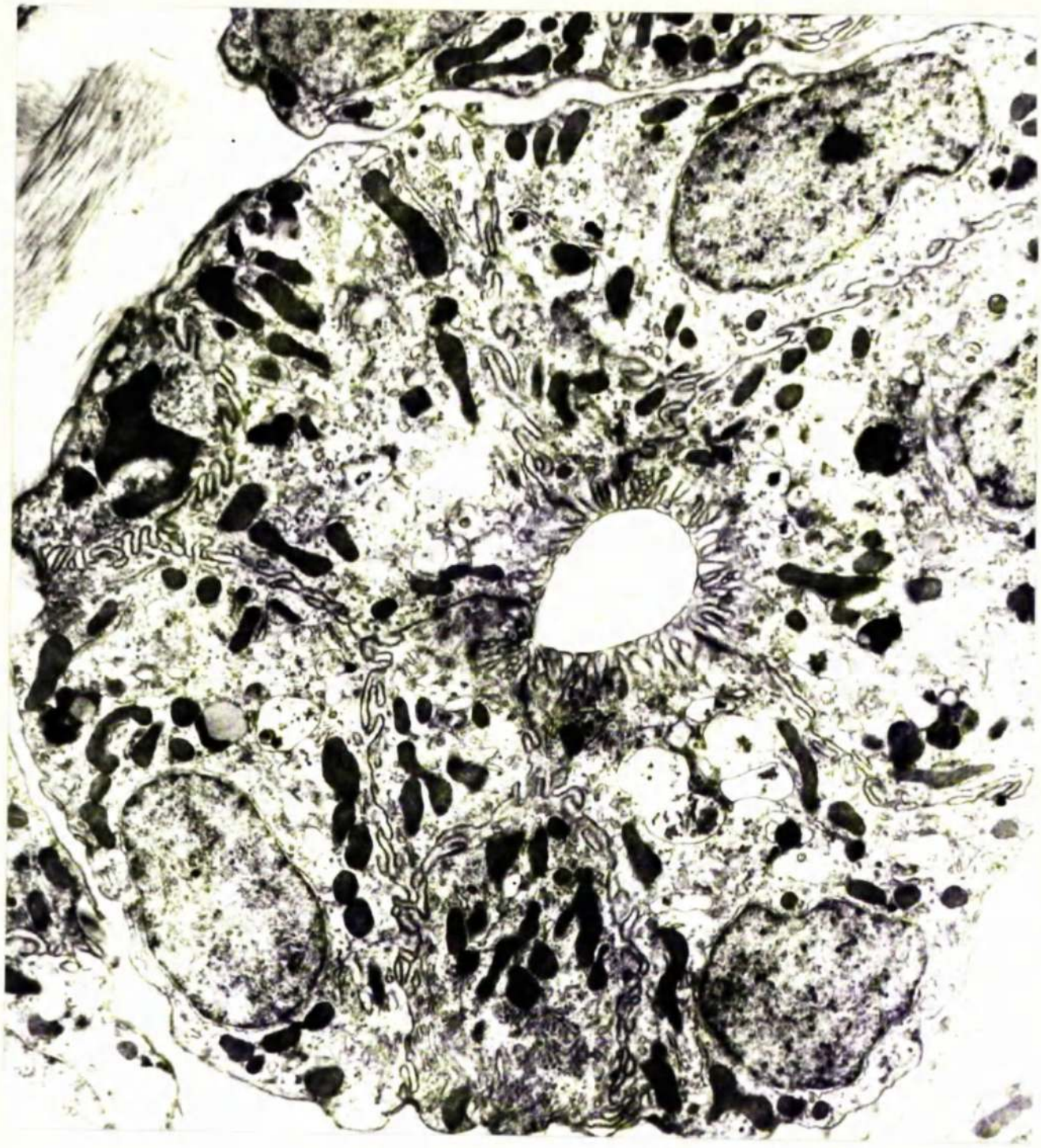


Fig 29. Mid tubular shell gland showing wedge shaped cells with basally situated nuclei. x12,000

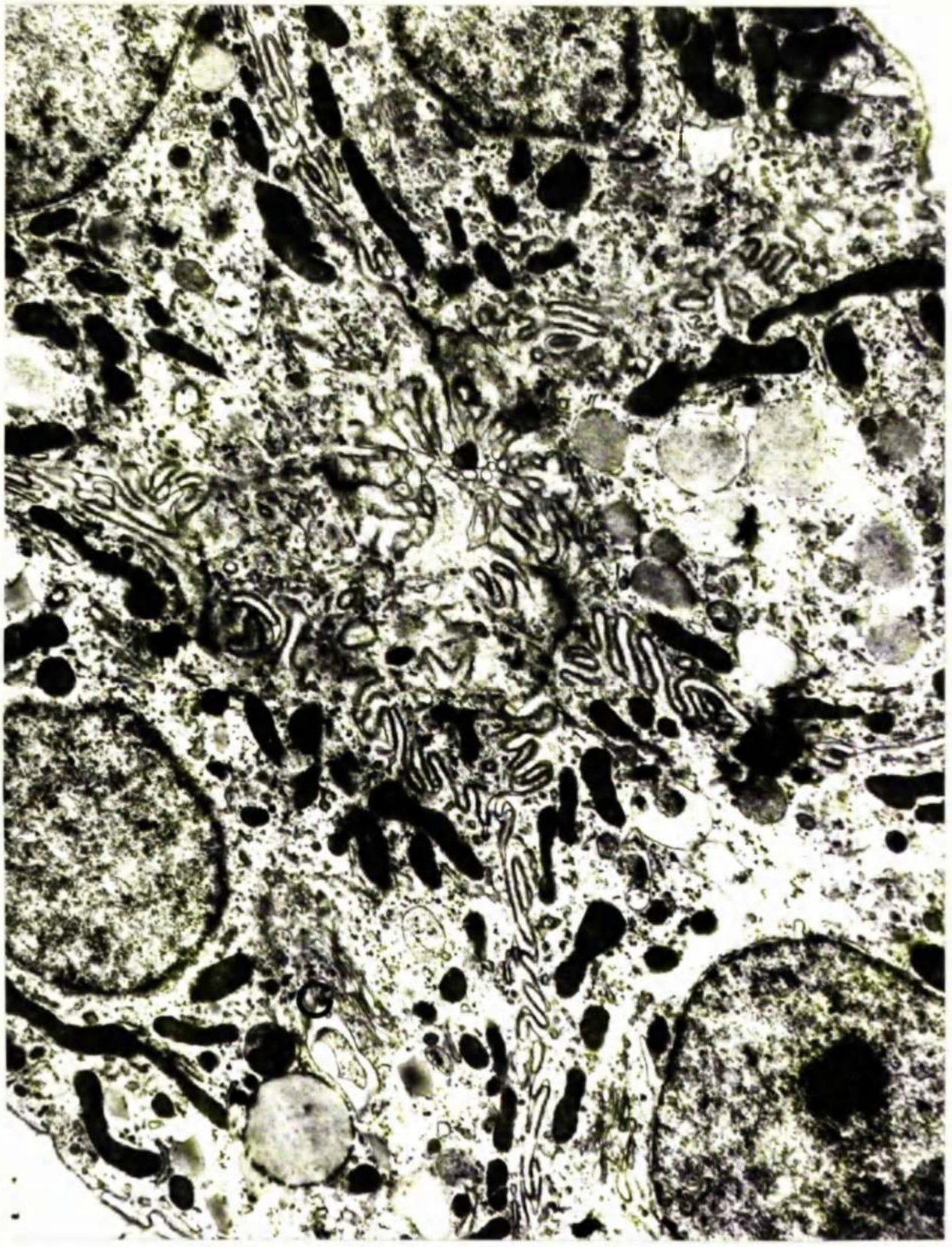


Fig 30. Mid tubular shell gland. The lateral cell membranes are fairly well infolded - a feature more characteristic of the pouch region. The cytoplasm, however, contains granules of low electron density bounded by smooth membranes. The Golgi complex (G) is well developed. x12,000

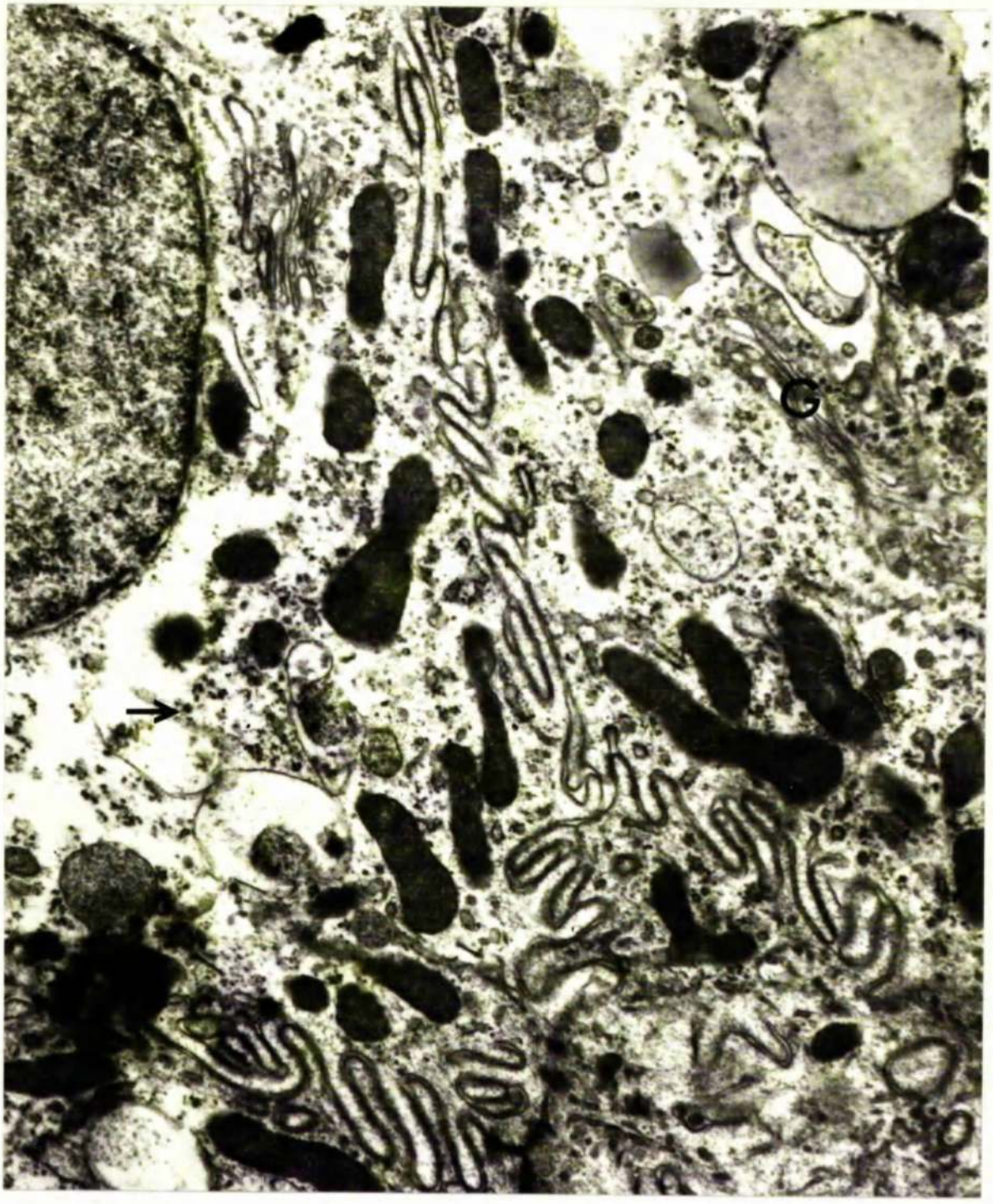


Fig 31. Higher magnification of part of Fig 30. showing folded cell walls, Golgi complex (G) and glycogen granules (->), scattered throughout the cytoplasm. x24,000

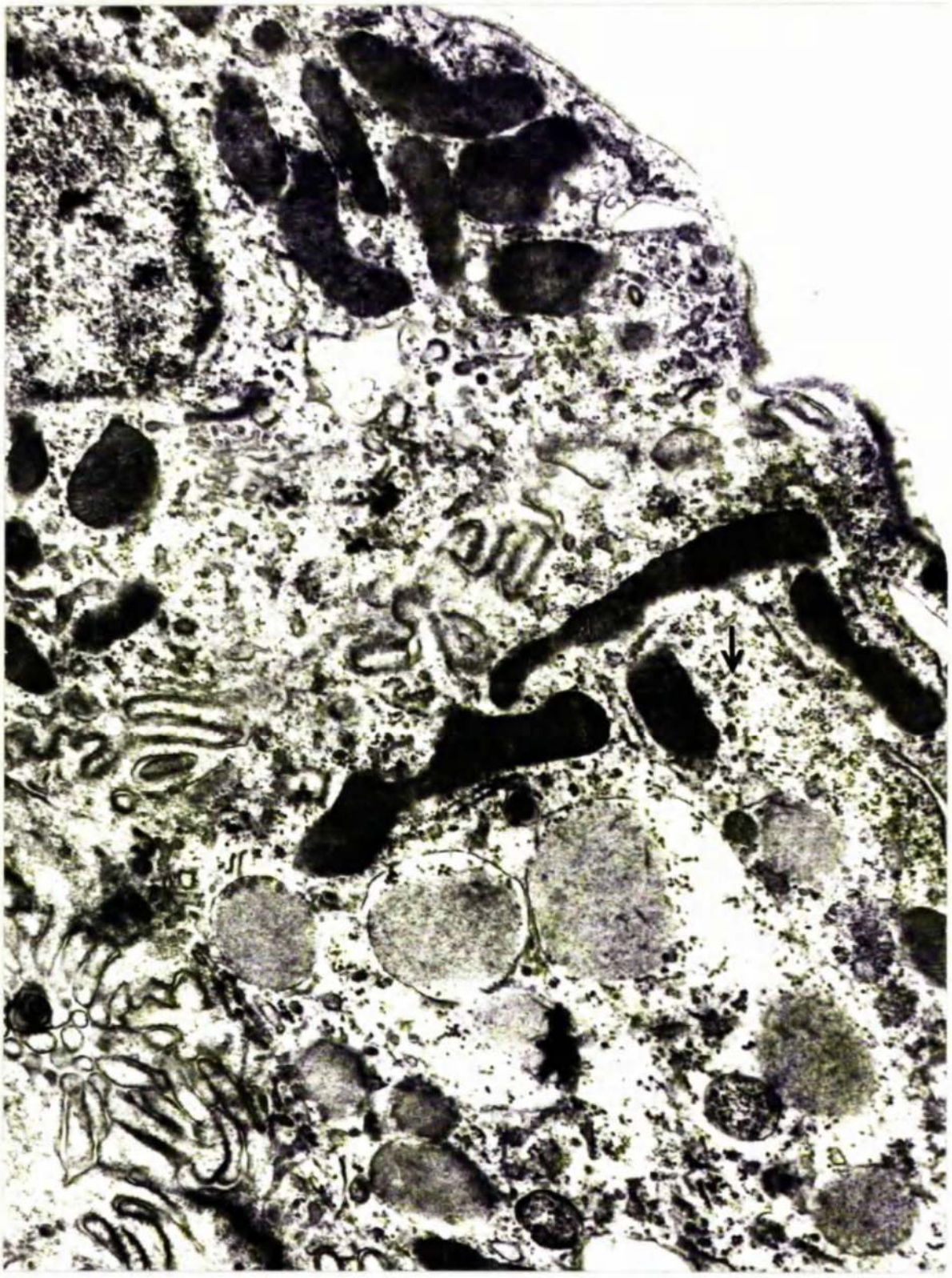


Fig 32. Section of mid tubular shell gland showing granules of low electron density and glycogen particles —>. x14,000

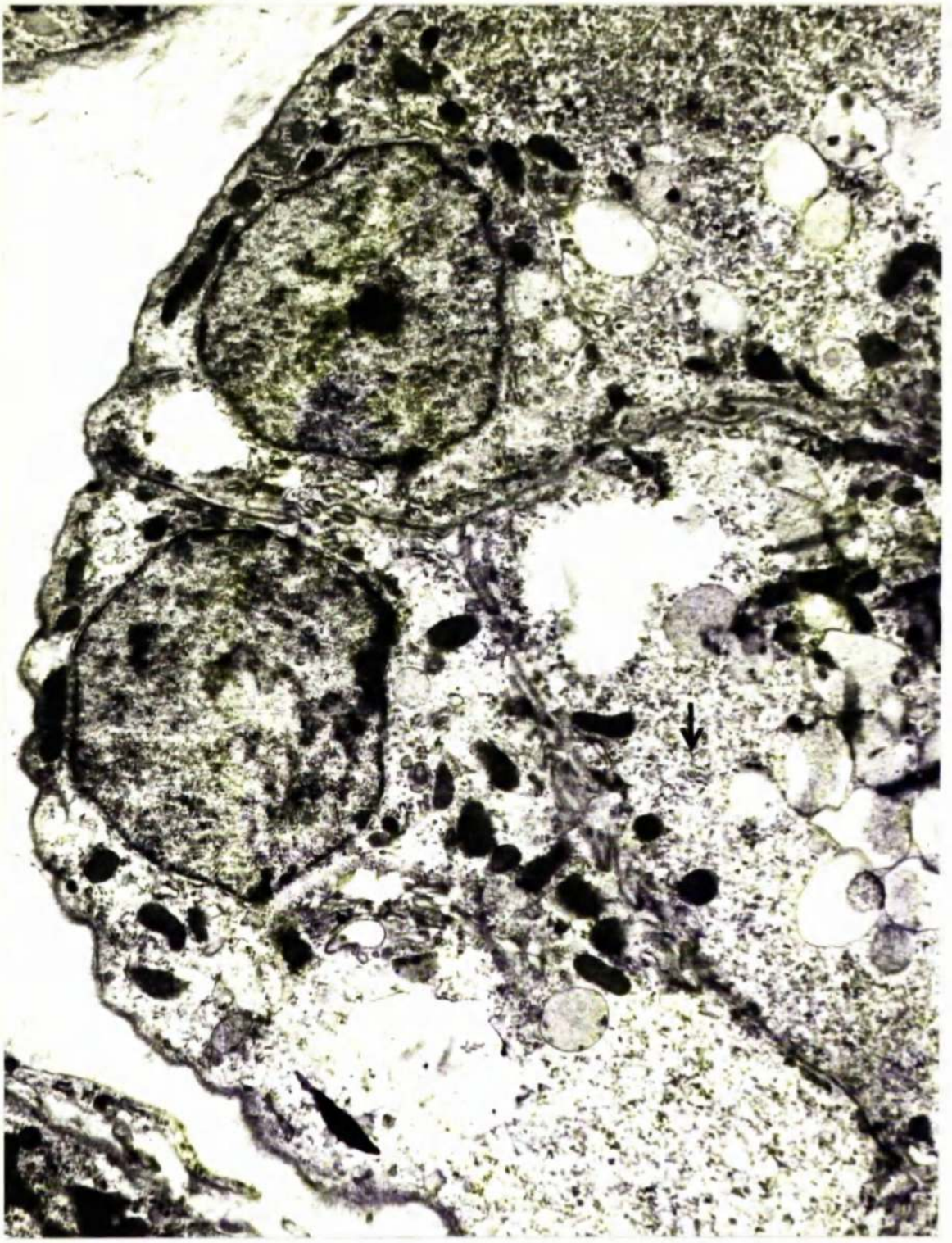


Fig 33. Upper tubular shell gland. Lateral cell membranes are only moderately folded, and the cytoplasm is filled with glycogen particles (->) and granules of low electron density. x8,000

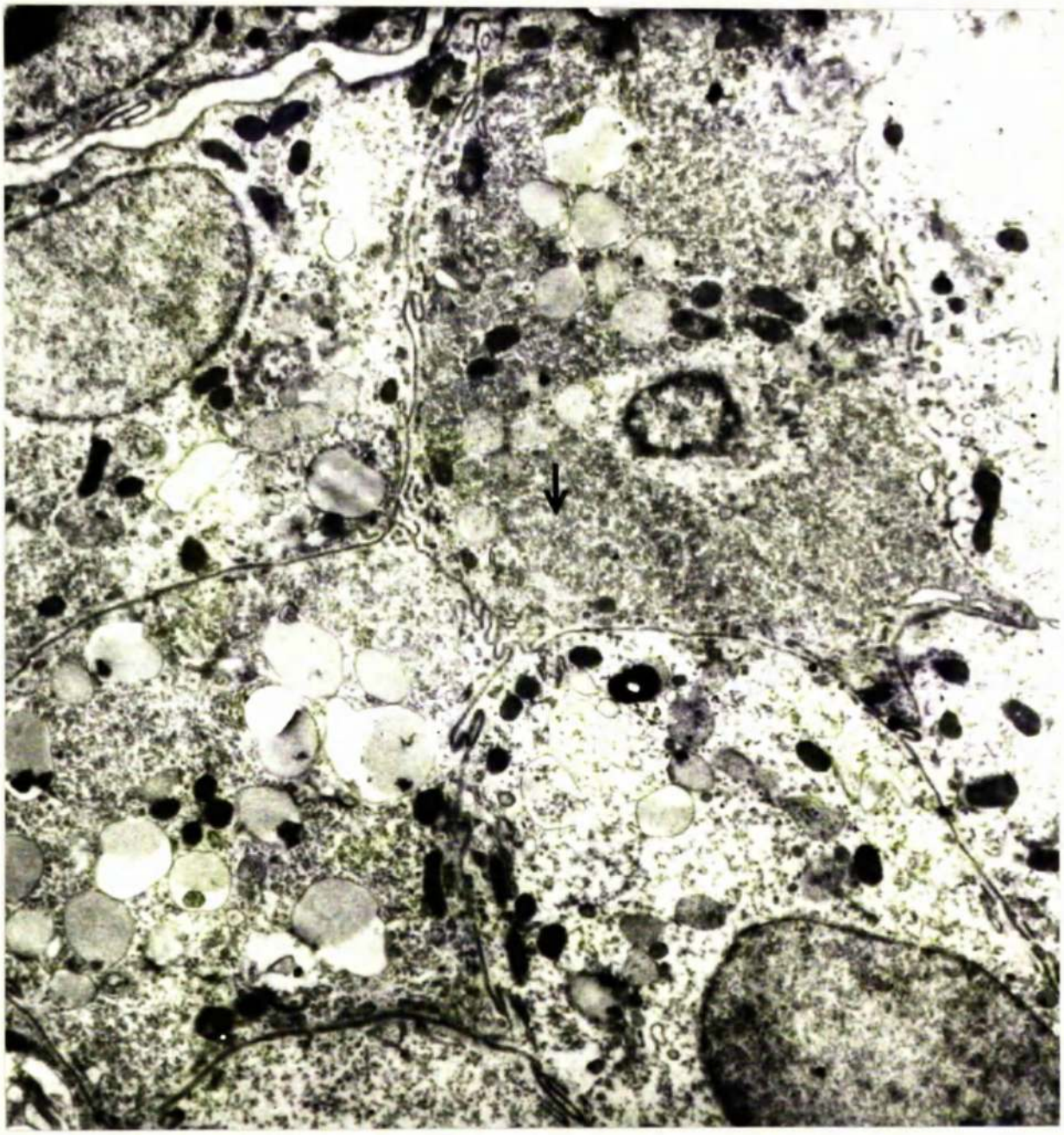


Fig 34. Upper tubular shell gland showing gland cells filled with glycogen (->) and granules of low electron density. x8,500

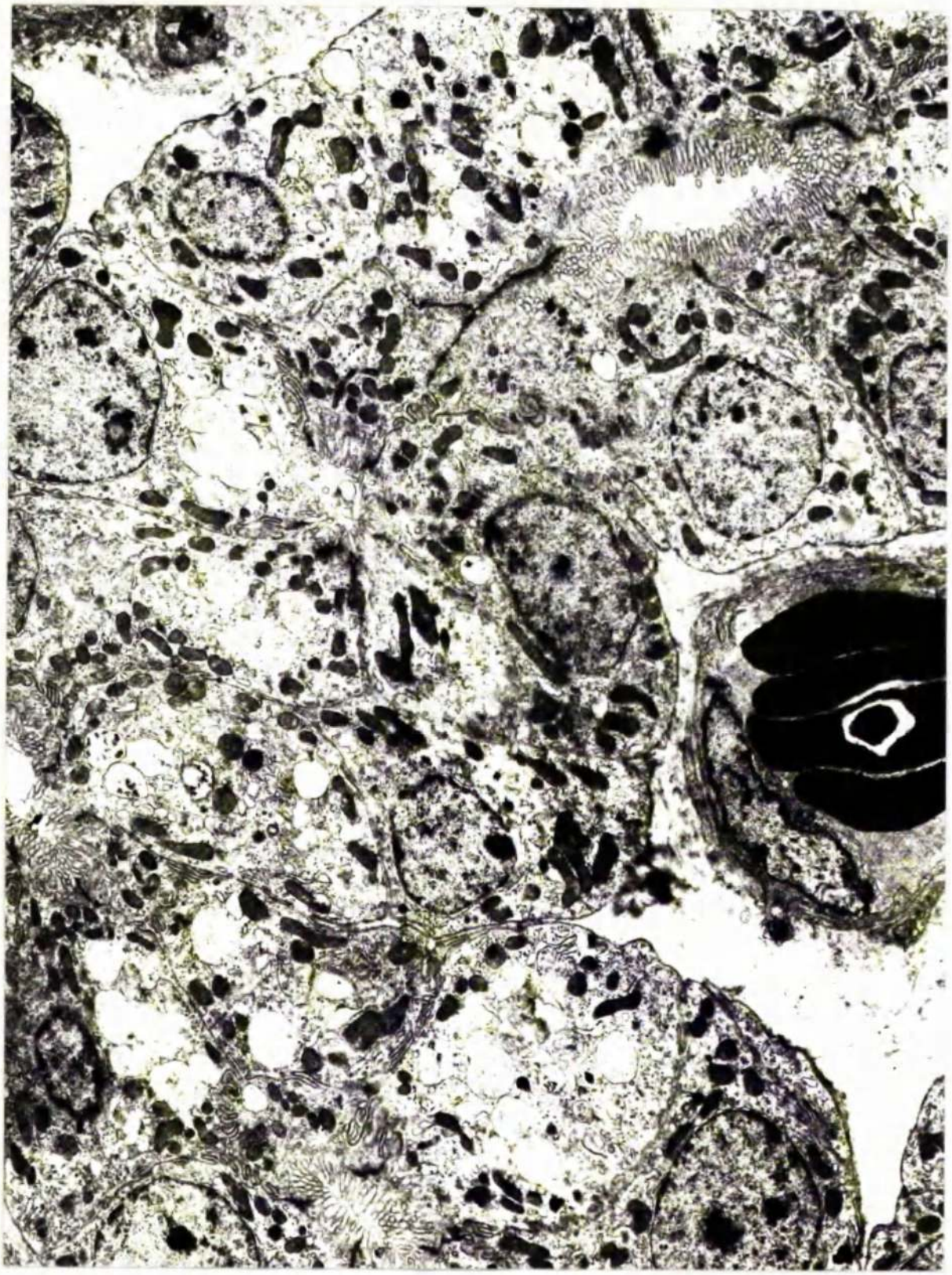


Fig 35. Mid tubular shell gland. Gland cells contain less glycogen than those depicted in Fig 34. x6,000

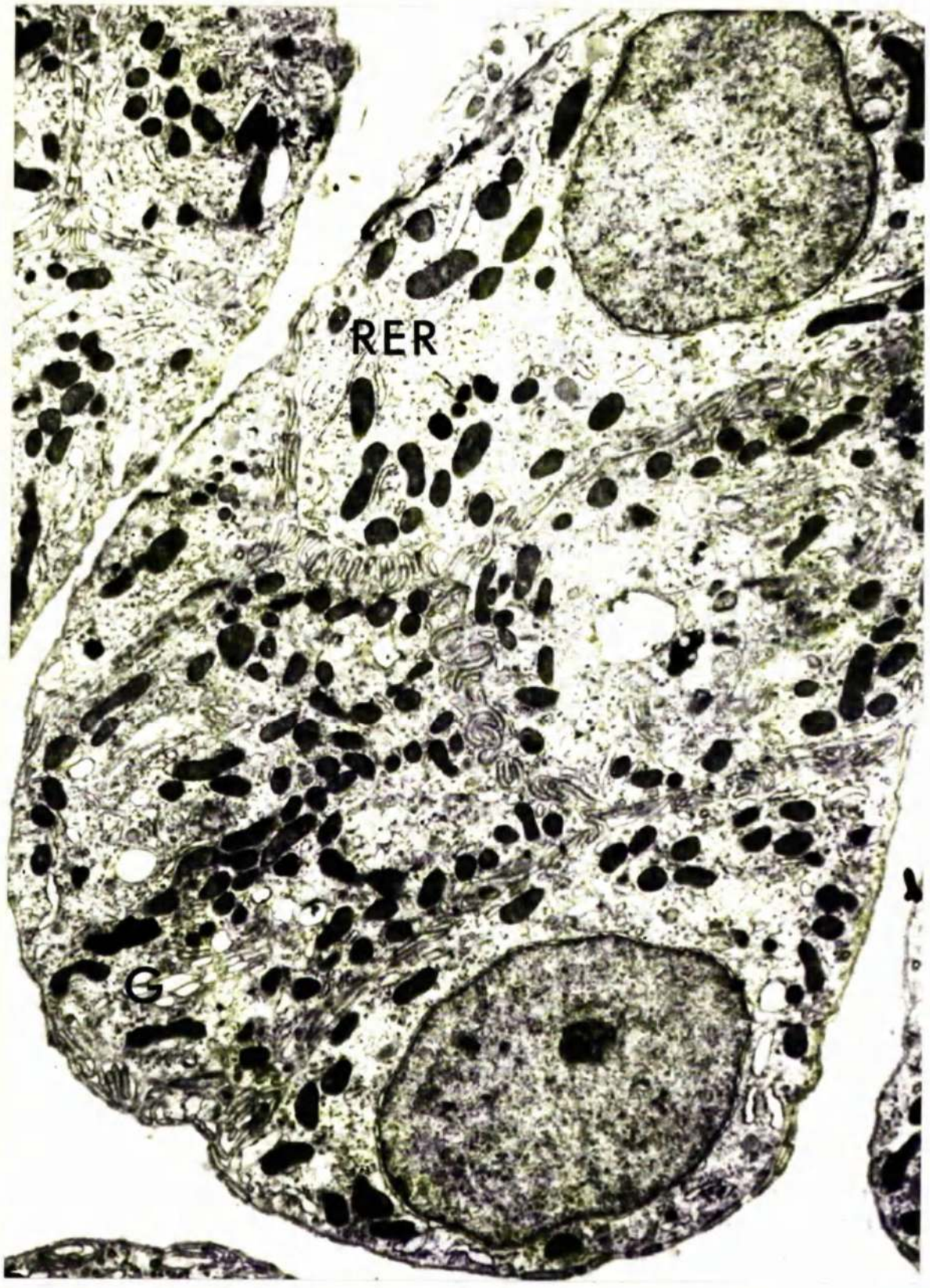


Fig 36. Distal tubular shell gland showing complex infoldings of lateral cell membranes. Secretory granules are absent, although a few glycogen particles still persist. Ribosomes can be seen scattered throughout the cytoplasm, as can a few R.E.R. cisternae. Golgi complex (G). x12,000

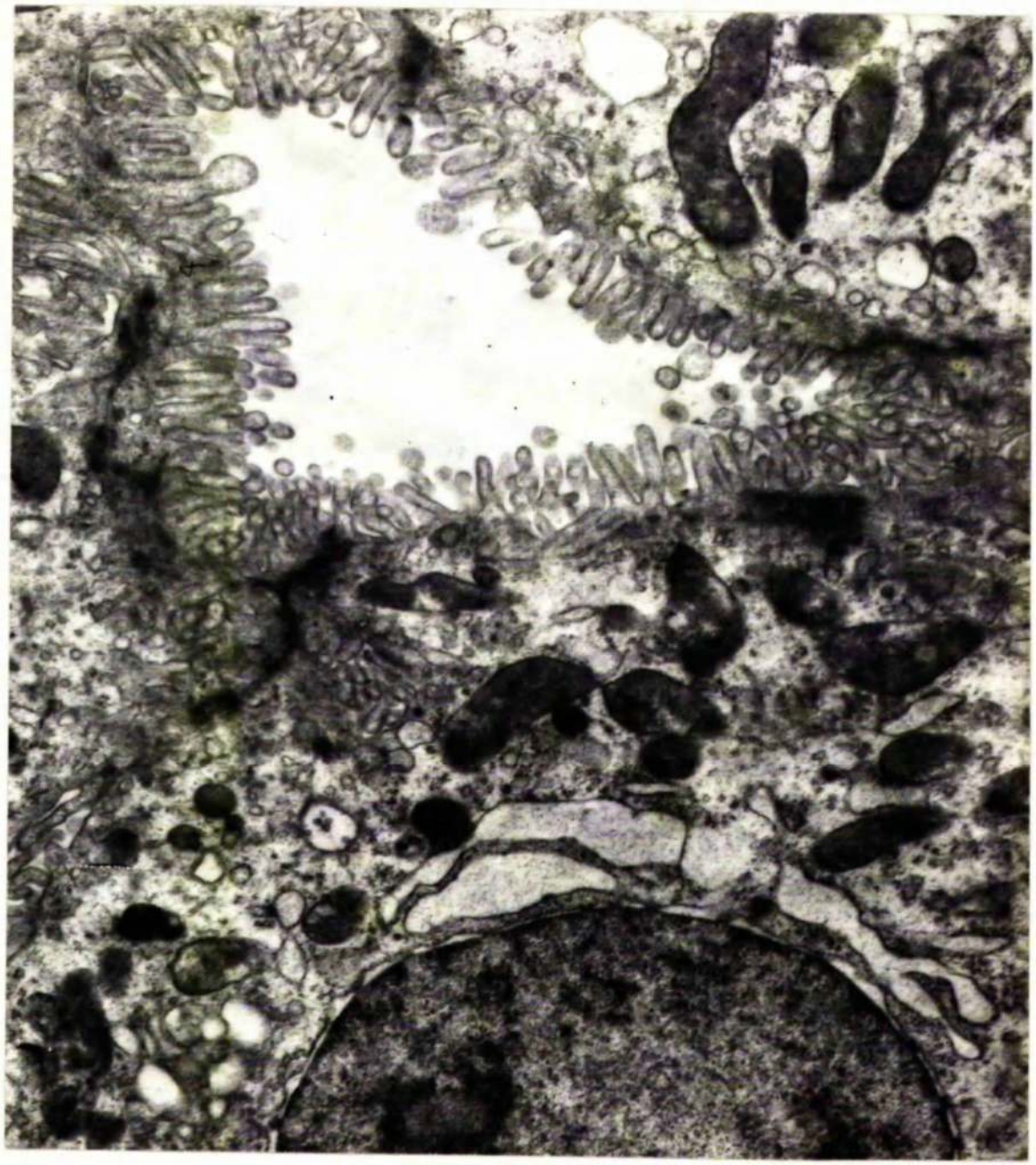


Fig 37. Distal tubular shell gland showing blebbing of microvilli. x18,000

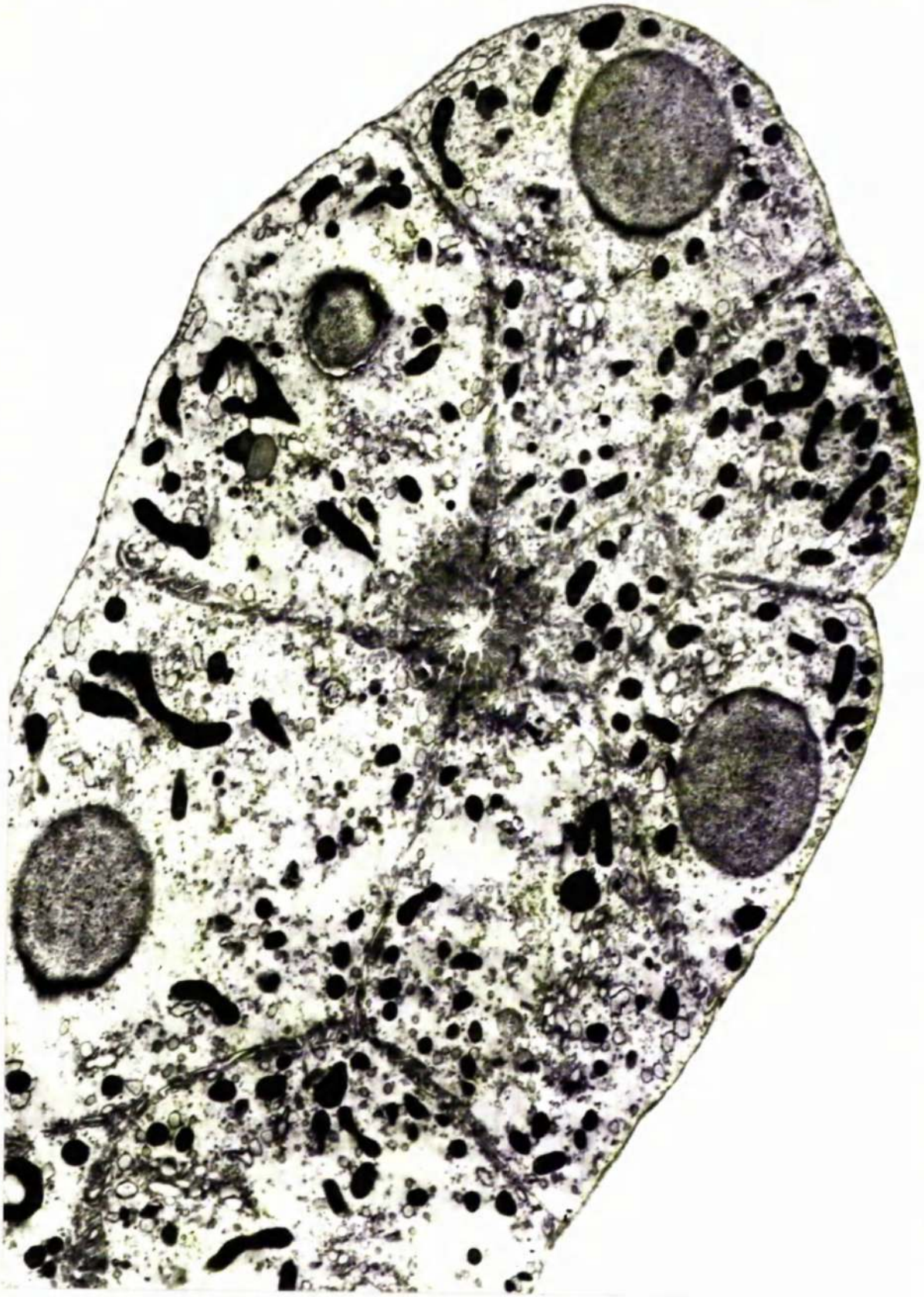


Fig 38. Shell gland pouch region preceding calcification. The gland cells contain few mitochondria at this time. x6,000

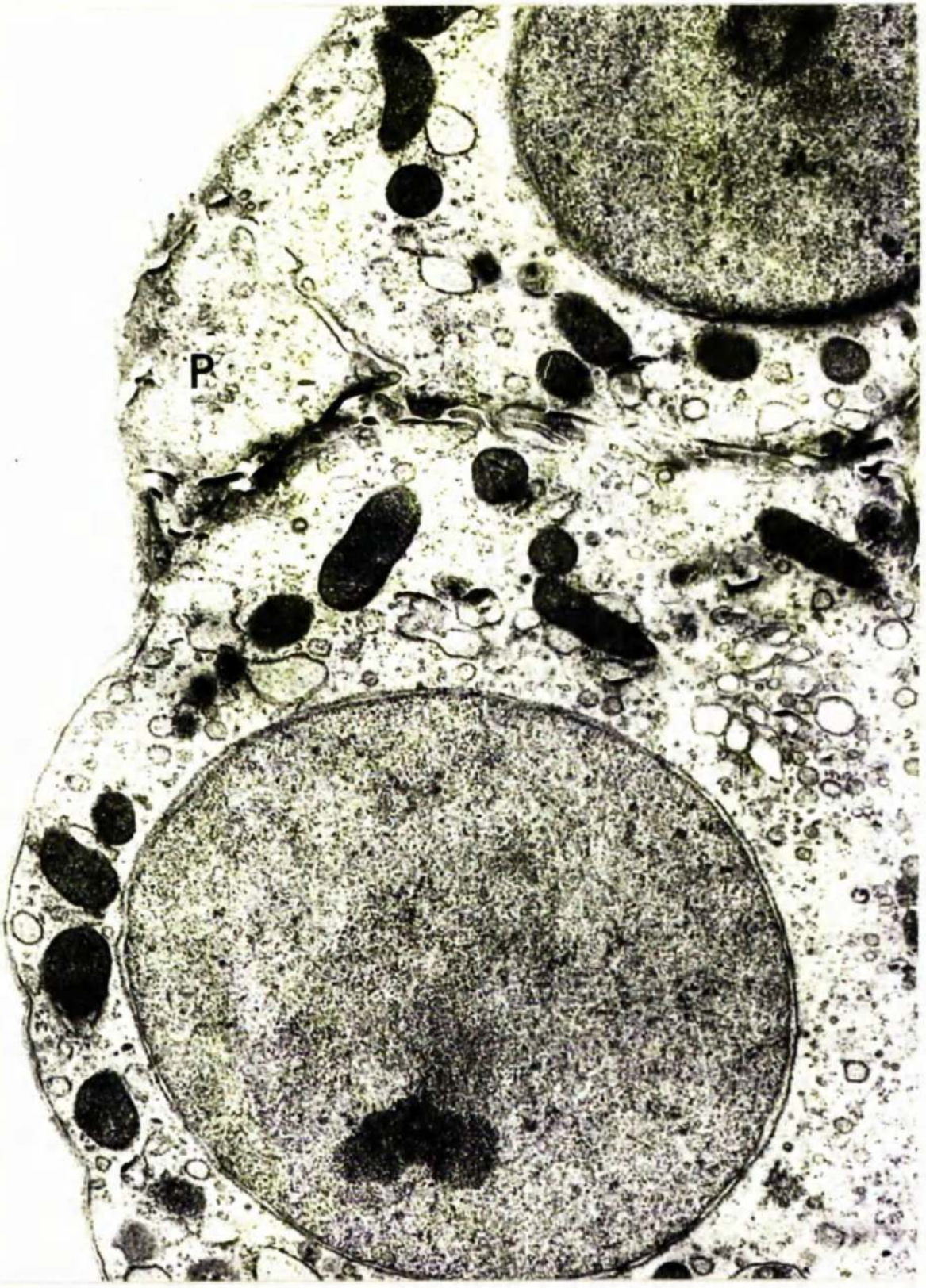


Fig 39. Higher power of part of two tubular gland cells preceding calcification showing specialisation of the basal plasmalemma (P). During calcification this region becomes more infolded. x14,000

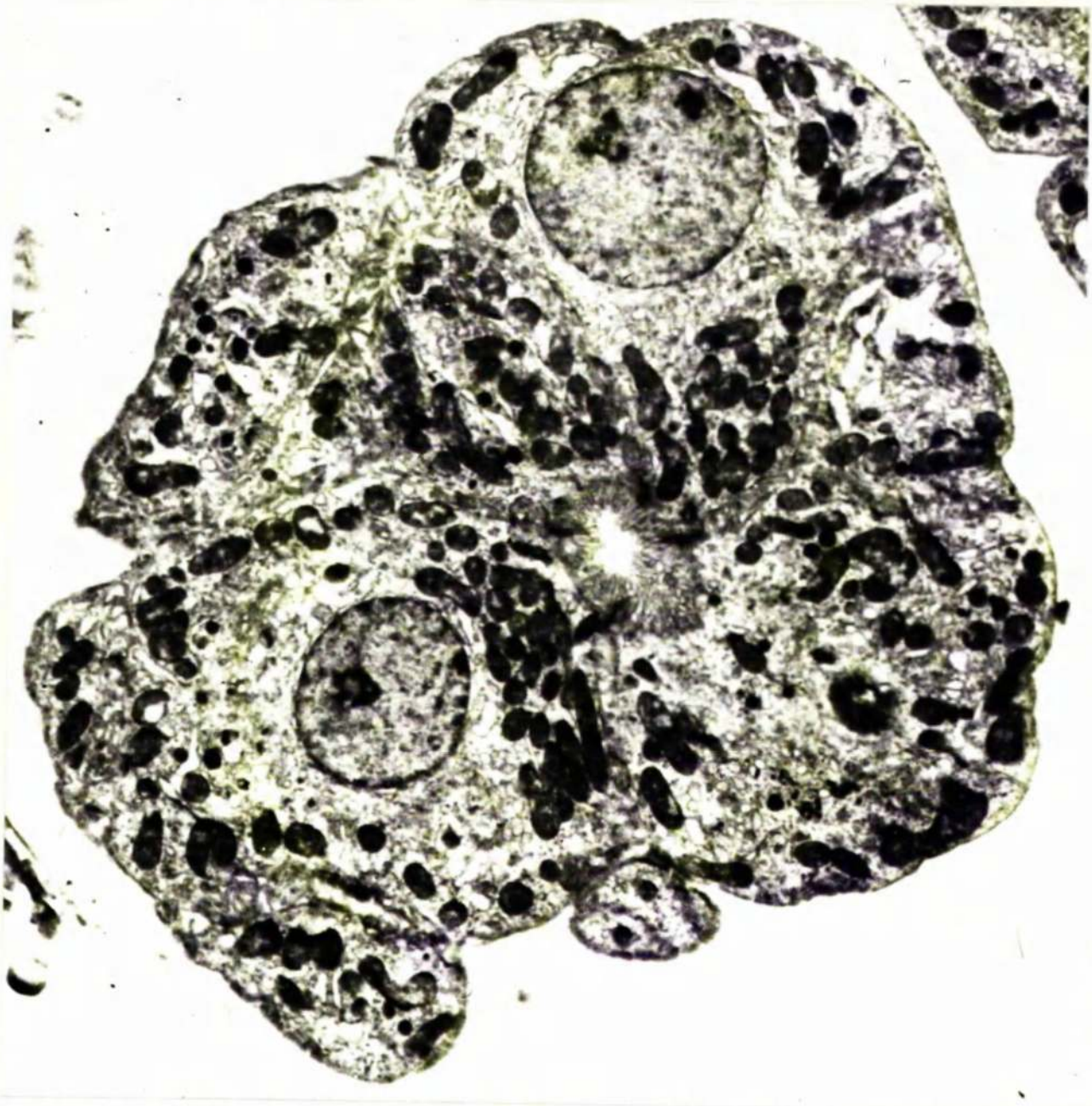


Fig 40. Tubular gland cells in pouch region during calcification. The cells are filled with mitochondria. x6,000

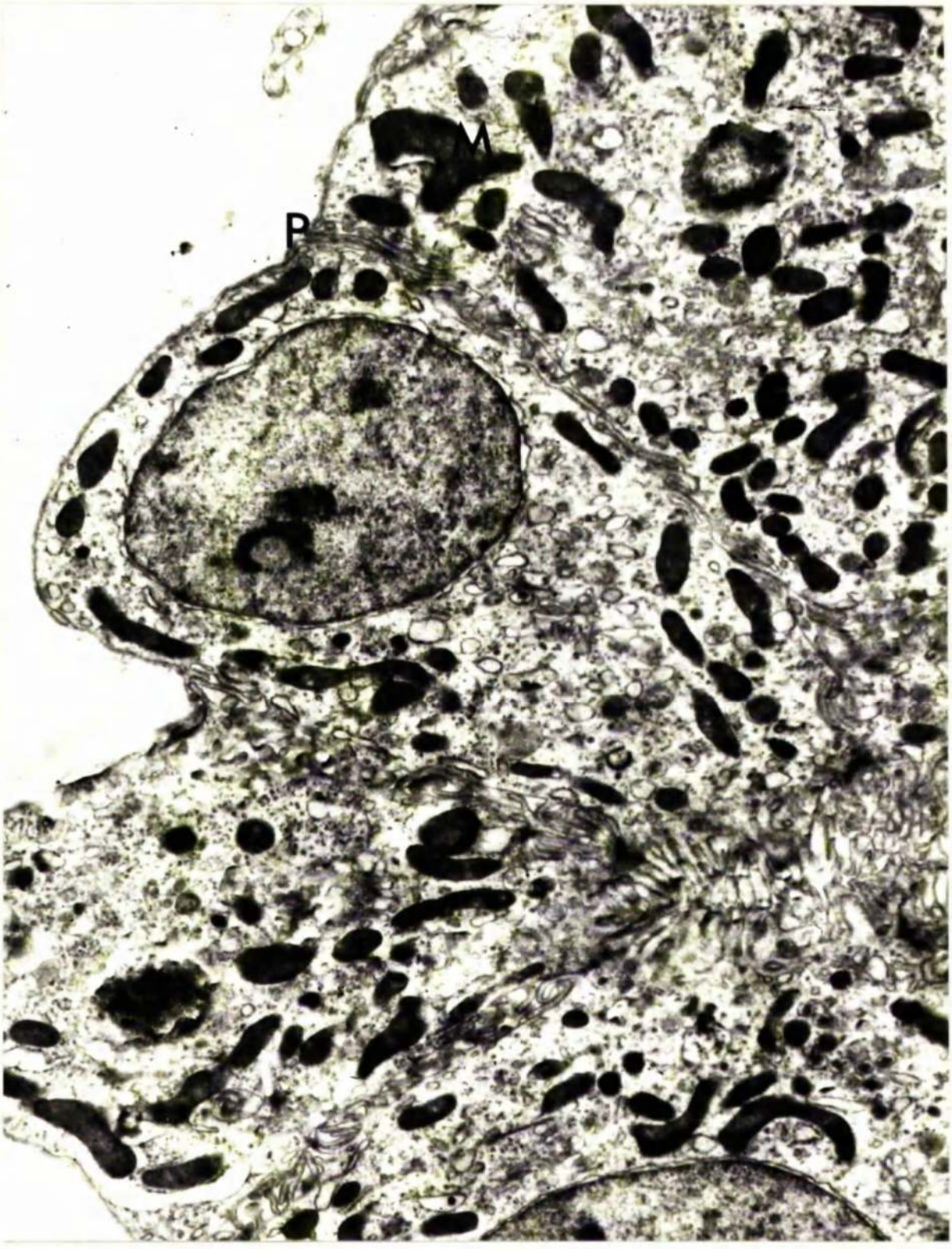


Fig 41. Shell gland pouch during calcification showing complex infolding of lateral cell walls and adjacent plasmalemmae (P). Note bizarre shape of mitochondria (M). x13,000

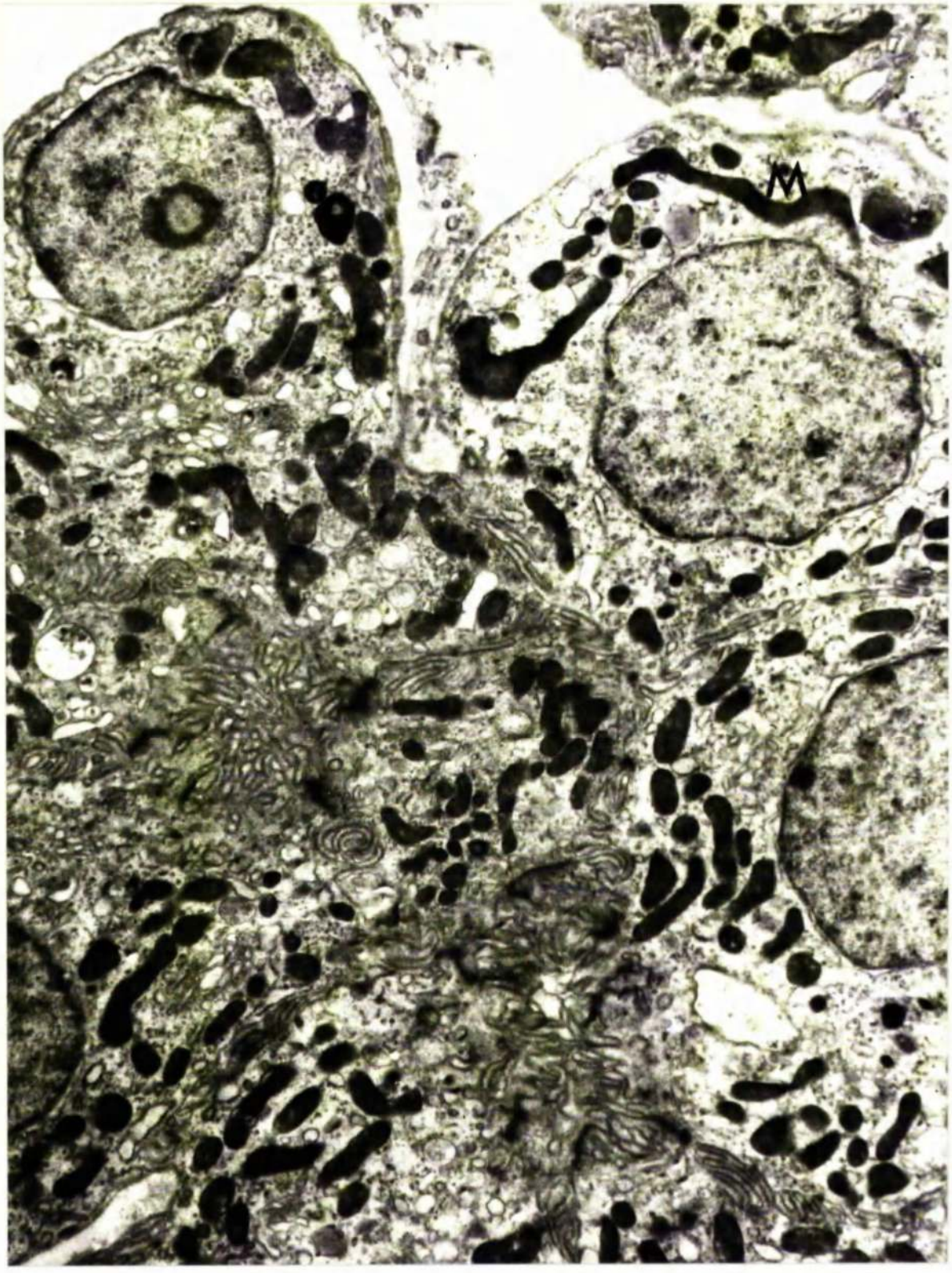


Fig 42. Shell gland pouch during calcification as in Fig 41. Note folding of lateral cell walls and elongate mitochondria (M). x12,500

H I S T O C H E M I S T R Y

The passage of the egg down the oviduct will be described here in terms of 5 phases defined thus:

No egg in transit - oviduct active	Phase 1
Egg in infundibulum or magnum	" 2
Egg in isthmus	" 3
Egg in tubular shell gland	" 4
Egg in pouch shell gland	" 5

P.A.S.

The ciliated surface epithelial cells are P.A.S. non-reactive during all 5 phases of the laying cycle. The secretory granules of the non-ciliated cells on the contrary are P.A.S. reactive. Fig. 43. During phases 1 & 2 the reaction is most intensive in the mid and lower isthmus possibly reflecting the greater height of the cells at this level, rather than a qualitative difference in secretion. The P.A.S.+ve granules are located for the most part in the mid/lumenal cytosome and frequently cause these cells to bulge prominently into the isthmian lumen. Fig. 43.

During phase 3 the intensity of staining is reduced above the level of the egg although none of the cells are by any means completely exhausted of secretory granules. Fig. 44. The non ciliated cells adjacent to the developing membranes are filled with P.A.S./

P.A.S./
reactive granules. Fig. 45.

During phases 4 & 5 all the non ciliated cells lining this isthmus are P.A.S.+ve. The gland cells are P.A.S.+ve at all levels of the isthmus and show no variation in staining intensity with the phase of egg development. Figs. 43, 45.

Shell Gland Tubular Portion

During phases 1 & 2 all the non ciliated cells exhibit some degree of P.A.S. reactivity, the reaction being most intense in the initial 1-2cms of this region. The gland cells although vacuolated contain P.A.S.+ve granules. Fig. 46.

P.A.S. reactivity is still evident in all of these cells during phase 3. During phases 4 & 5 however the intensity of staining in the non ciliated cells is very weak. The gland cells at this time also exhibit reduced reactivity.

Shell Gland Pouch

During phases 1 & 2 the non ciliated surface cells contain a few P.A.S.+ve granules distributed evenly throughout the height of the cells. During phase 3, and to a lesser extent phase 4, the P.A.S.+ve granules assume an apical position in these cells giving the impression of increased activity. During phase 5 reactivity is at a minimum/

minimum/
and remains minimal throughout the period of shell formation.

The gland cells are P.A.S. unreactive at all stages of the cycle.

P.A.S. / Diastase

Treatment with diastase removes much of the P.A.S. reactivity in the gland cells lining the tubular portion of the shell gland, although P.A.S. positive granules still persist in this region. Diastase has no effect on the isthmian gland cells and the surface non ciliated cells lining the isthmus and shell gland. This reaction which is specific for the presence of glycogen corroborates the findings at ultrastructural level. Fig. 47.

P.A.S. / Alcian Blue

In the combined P.A.S. / alcian blue stain, P.A.S. reactivity predominates and is as described above. Alcianophilia is confined to the nuclei of the surface epithelial cells and the gland cells.

Alcian Blue $p^H 2.6$

Only the non ciliated surface epithelial cells lining the tubular portion of the shell gland are alcian blue positive. The reaction is most intense during the isthmian phase of development, i.e. phase 3. Fig. 48.

Alcian Blue p^H 1

Increased levels of acidity abolished the reaction in the non ciliated surface epithelial cells of the tubular shell gland.

Sudan Black

Sudanophilia is confined to the mitochondria of the surface epithelial cells and gland cells lining the isthmus and shell gland.

Biebrich Scarlet

The isthmian gland cell granules are positive with Biebrich scarlet at all stages of the laying cycle, indicating the presence of basic protein. Fig. 49. The granules in the gland cells of the tubular shell gland in contradistinction are negative at all phases of the laying cycle with respect to this stain.

Light Microscope Localisation of Acid Phosphatase

Acid phosphatase activity was demonstrable only in the shell gland pouch region. During the initial four hour phase of calcification only the surface epithelium gave a positive reaction. At all other phases of egg formation the enzyme was restricted to the gland cells. Fig. 50, 51.

ELECTRON MICROSCOPY

Ultrastructural Localisation of Acid Phosphatase

The distribution of acid phosphatase in the shell gland alters significantly according to the location of the egg in transit 4 hours after the entry of the egg into the shell gland non membrane bound acid phosphatase (i.e. not lysosomal) is localised principally in the non ciliated "basal cells of the surface epithelium where it is in contact with the lateral cell membranes and in the intercellular space between the basal and ciliated "apical cells". It is also found over the microvilli of the basal cells which appear as extremely dense structures.

The microvilli of the adjacent ciliated cells, on the contrary, are devoid of reaction product, Fig. 52. and although very occasional small dense bodies occur over the cilia and nuclei of these cells they are in no way comparative in number or size to those in the basal cells. During the first four hours of shell formation the only reaction noted in the gland cells is a diffuse moderate nuclear deposition possibly the result of adsorption and insignificant compared with the massive lysosomal concentration noted in these cells at other phases of the egg cycle. At all other times during egg development the surface epithelium exhibits no phosphatase activity.

Lysosomal acid phosphatase (i.e. membrane bound) is maximal in the gland cells with an egg in the isthmus. Figs. 53 & 54 and minimal during the initial four hour period of calcium transfer. During the later stages of shell deposition lysosomes again increase in number preceded by an increase in the number of multivesicular bodies. Fig. 55. Acid phosphatase containing bodies were observed in the lumena of several gland cells and may be indicative of a secretory process. Fig. 53.

ULTRASTRUCTURAL LOCALISATION OF CALCIUM

Autoradiography

No isotopic label was identified in either the surface epithelium or glandular lamina propria of the isthmus or shell gland at any stage during the laying cycle. Tissue which at light microscope level appeared to be well preserved, Fig. 56, at higher magnification was distorted because of ice crystals.

LOCALISATION OF PYROANTIMONATE

Isthmus

No deposit of pyroantimonate was visualised either in the surface epithelial cells of the tubular gland cells at any stage of the laying cycle.

Shell Gland Tubular and Pouch Portions

Electron dense deposits of pyroantimonate were observed in all the surface epithelial cells, including the mitochondrial cells, Fig. 57 of the entire shell gland. These crystalline aggregates of variable size were found in association with the lateral and basal cell walls, free in the cytoplasm, in the mitochondria and over the microvilli and cilia. Figs. 58, 59, 60, 61 & 62. No deposit was seen in the gland cells. Crystals were observed at all stages of the laying cycle, but appeared to be in greatest concentration in the pouch region during the initial four hour period of calcification. No attempt has been made as yet to quantify these results. In view of the small size of many of the crystals, parts of cells only are presented at high magnification.

LOCALISATION OF TISSUE PORPHYRINS

Isthmus

Only the basal portion of the surface epithelial cells of the quail isthmus autofluoresced under ultraviolet light. The intensity of fluorescence was initially very weak and faded after 2-3 seconds.

Shell Gland Tubular and Pouch Regions

The surface epithelial cells lining the distal portion of the tubular shell gland and the pouch region of the quail, White Leghorn and Brown Ranger all autofluoresced in ultraviolet light, Fig. 63. The reaction was strongest in the quail and weakest in the White Leghorn. In every case the reaction faded with time. The reaction did not appear to be confined to one particular cell type, although it did show a predilection for the basal portion of the surface cells. Autofluorescence was never observed in the tubular gland cells.

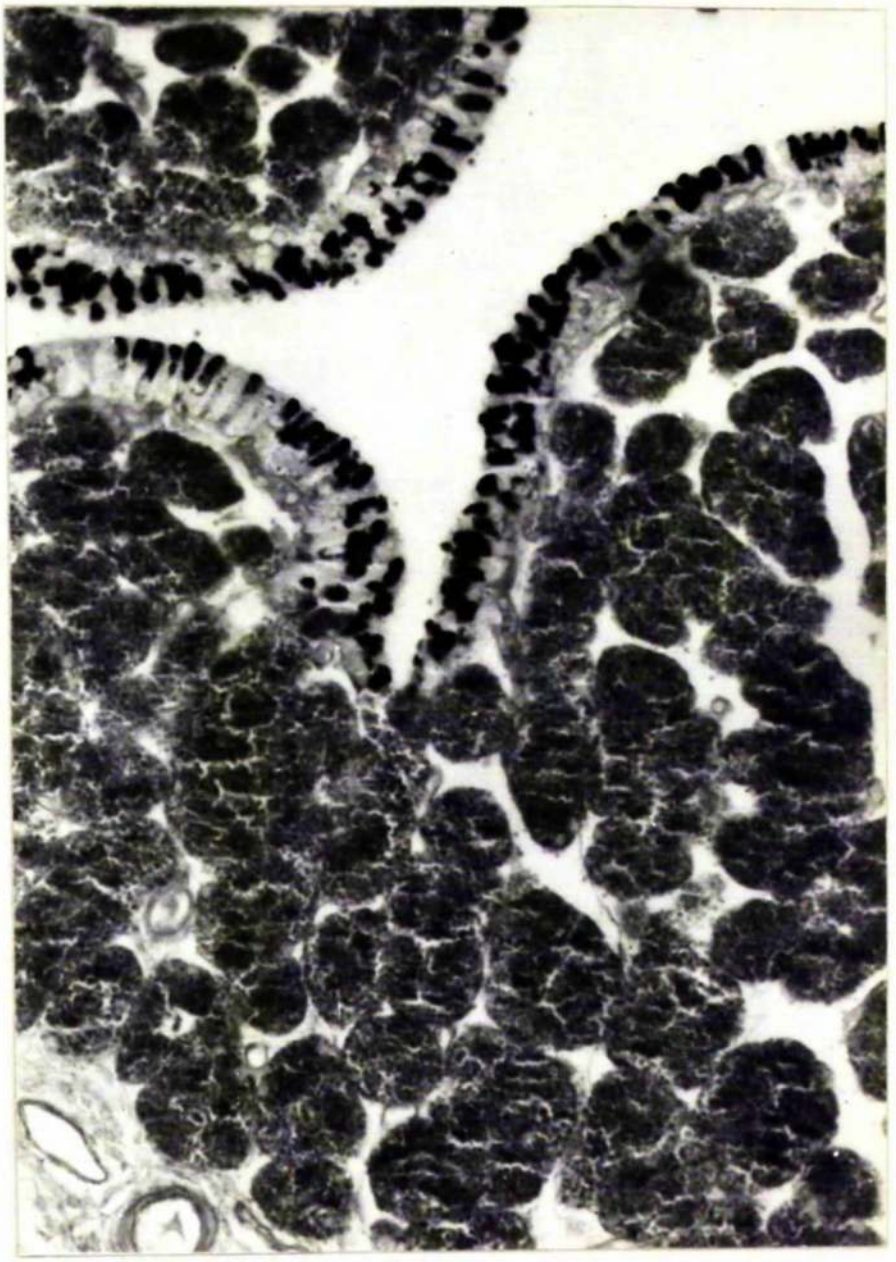


Fig 43. Mid isthmus showing P.A.S.+ve material in surface non ciliated epithelial cells, some of which bulge into the lumen of the oviduct. The adjacent ciliated cells are P.A.S.-ve. The granules filling the gland cells are P.A.S.+ve. Egg in infundibulum. x240

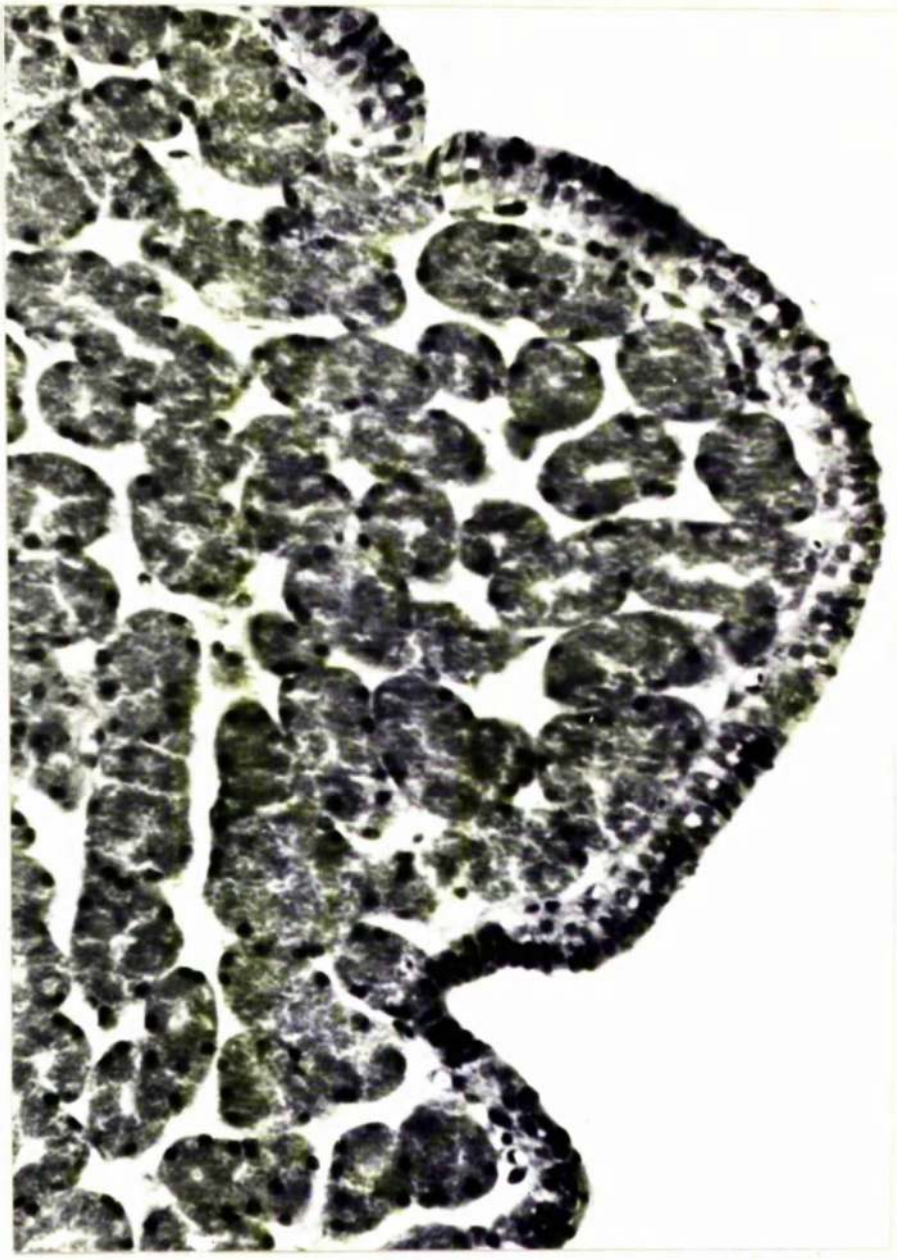


Fig 44. Mid isthmus showing reduced P.A.S.+ve reactivity in surface epithelial non ciliated cells. Gland cells are still filled with P.A.S.+ve granules. Egg in lower isthmus. x240

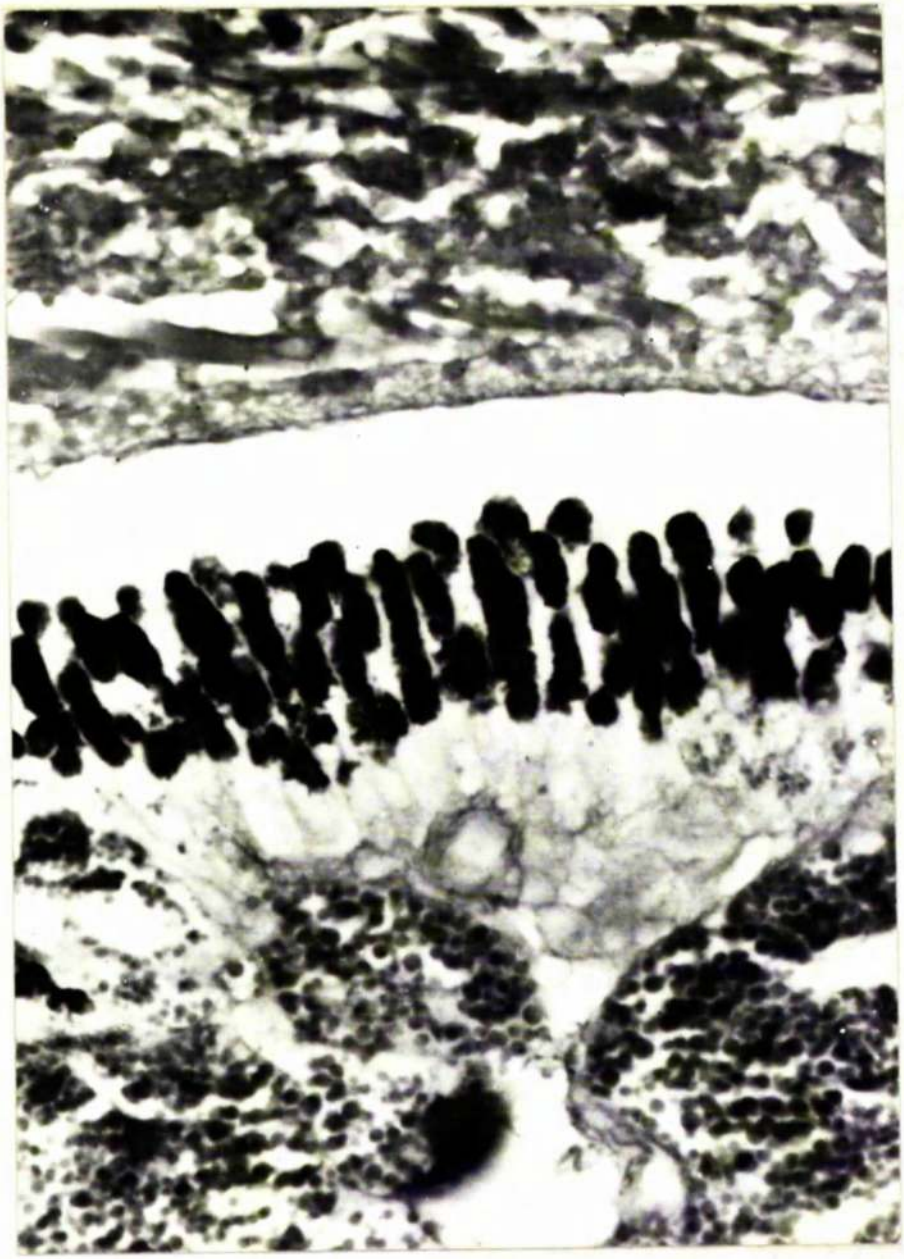


Fig 45. Lower isthmus with an egg <u>in situ</u>, showing the bulging appearance of the P.A.S.+ve non ciliated cells. x600

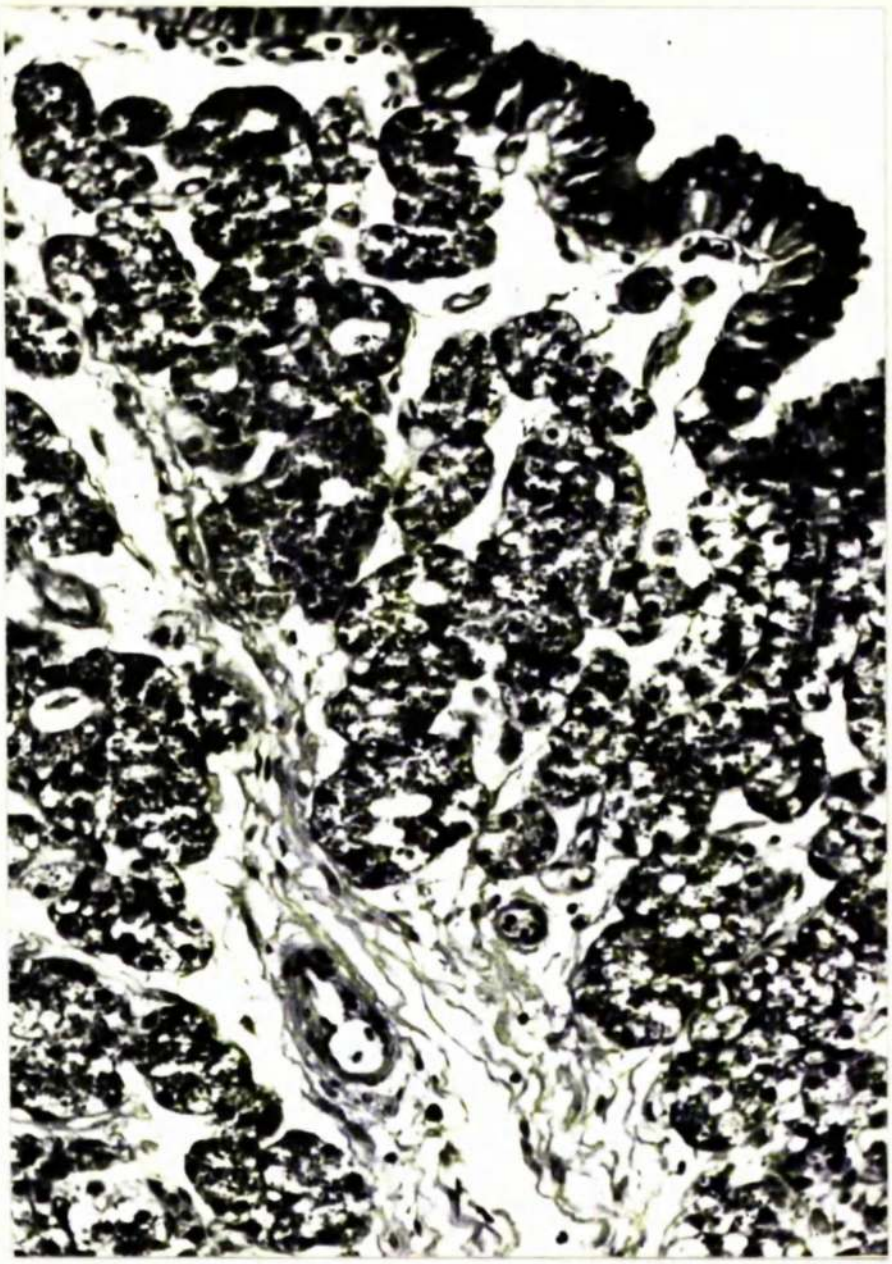


Fig 46. Upper tubular shell gland showing P.A.S. reactivity in the surface non ciliated epithelial cells and the tubular gland cells. No egg in transit. x240

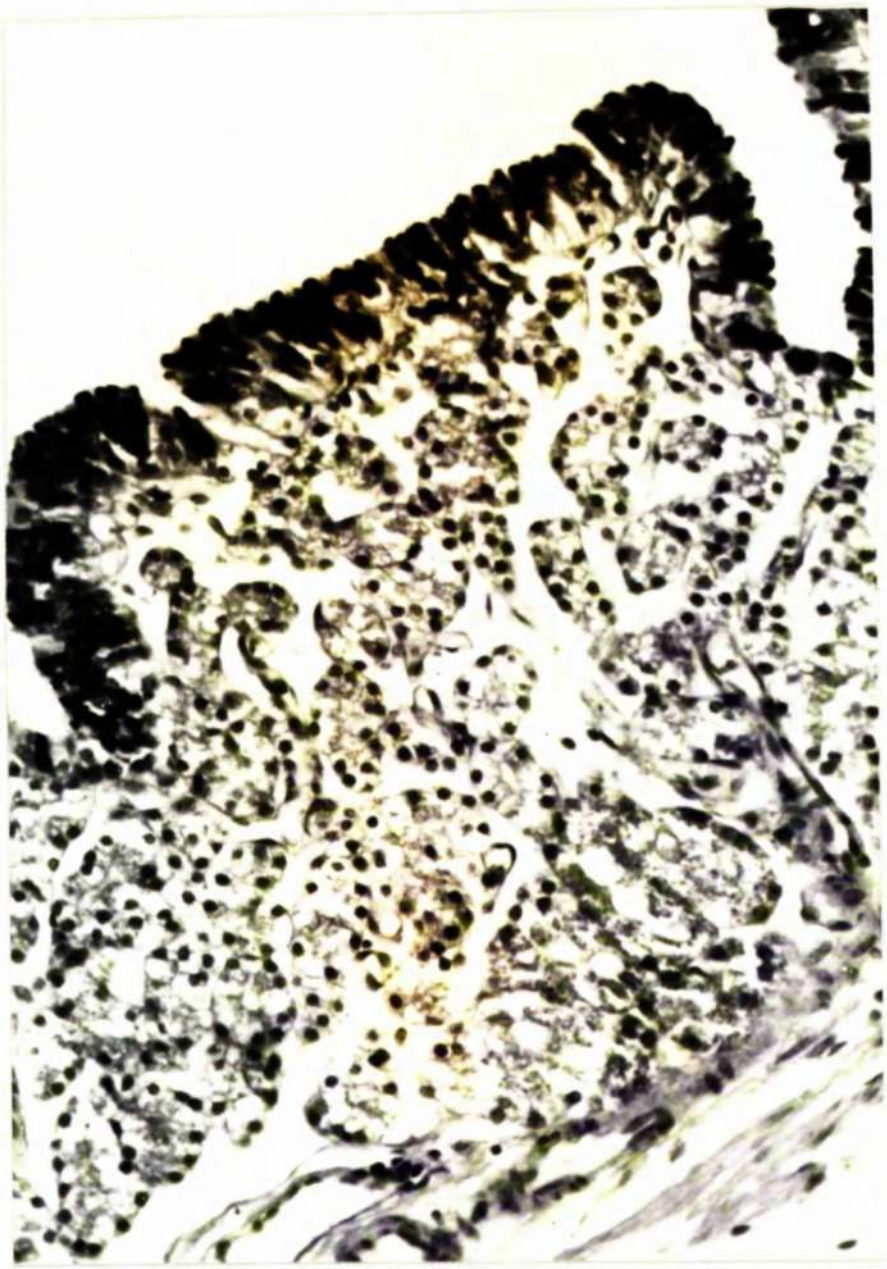


Fig 47. Upper tubular shell gland after treatment with diastase. Note reduced activity in gland cells c.f. Fig 46., although P.A.S.+ve material is still present both in these cells and in the surface non ciliated cells. x240

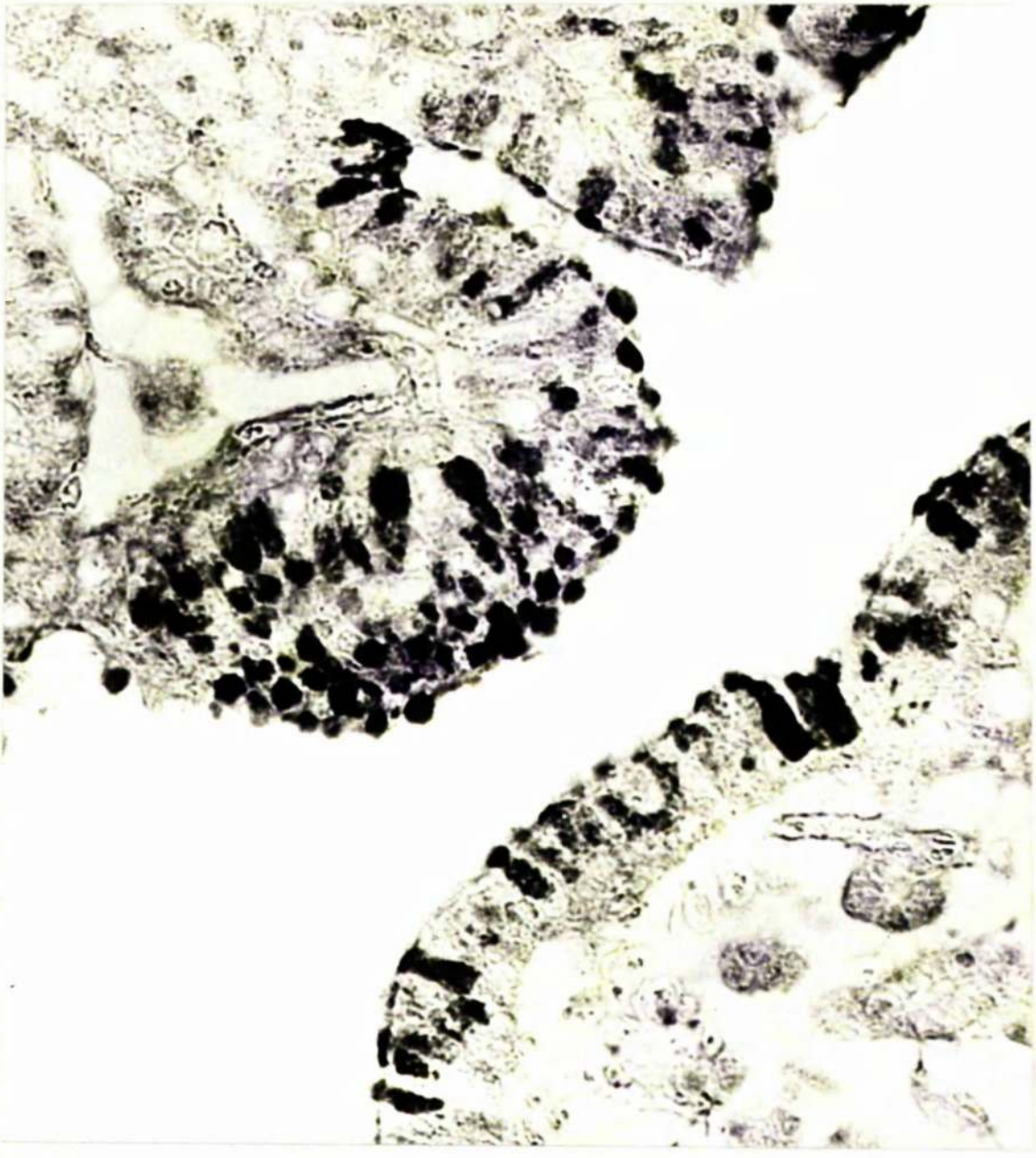


Fig 48. Mid tubular shell gland showing a positive reaction for alcian blue pH2.6 in many of the non ciliated surface cells. Egg in isthmus. x500

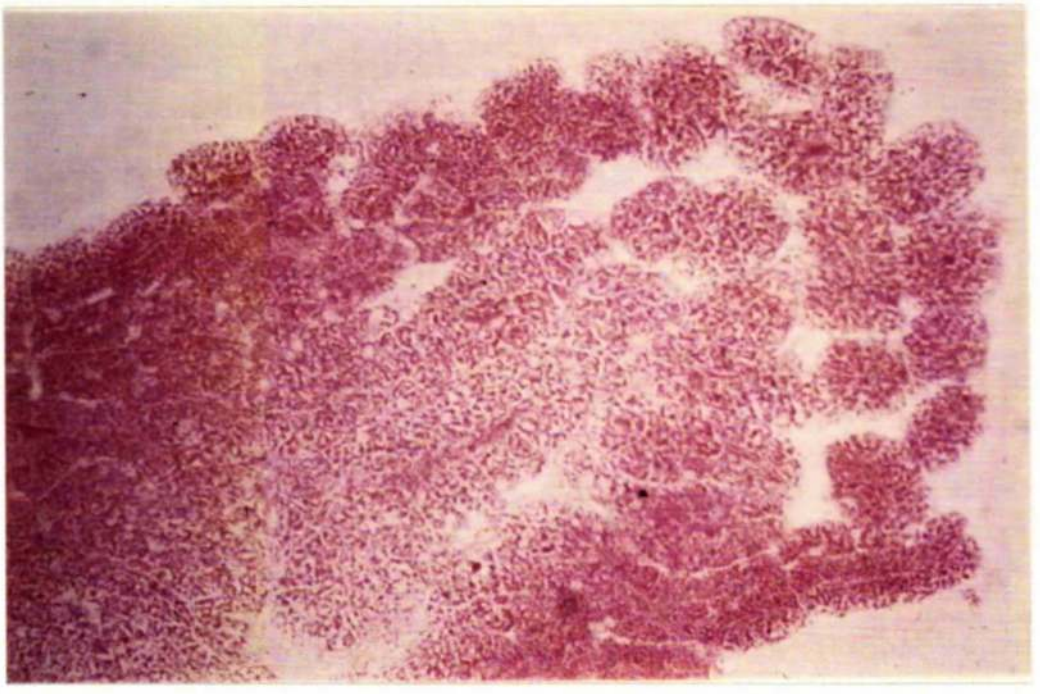


Fig 49. Mid isthmus. Granules in gland cells are positive for Biebrich scarlet. Egg in magnum. x120

Fig 50. Acid phosphatase reactivity in surface epithelial cells of shell gland pouch. Soft shelled egg in shell gland. x300

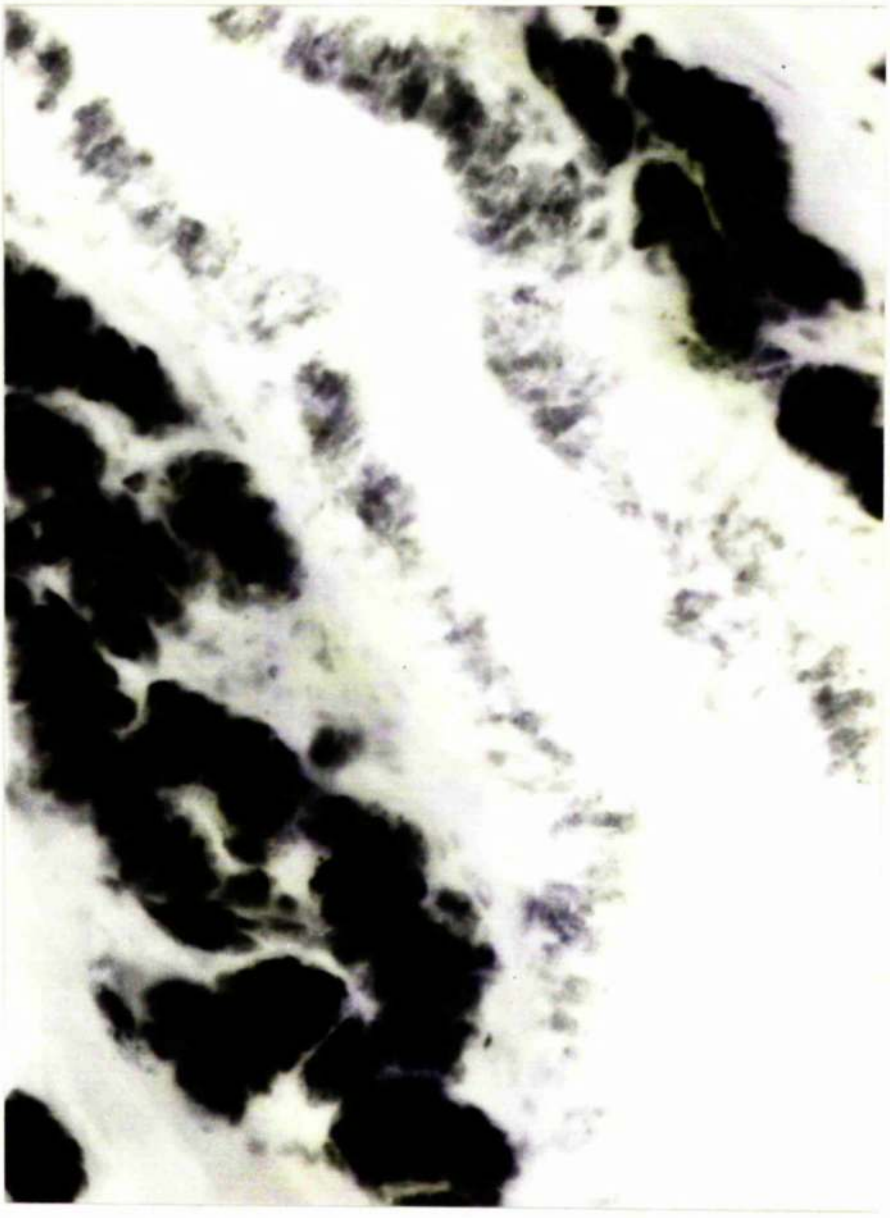


Fig 51. Acid phosphatase reactivity in gland cells of shell gland pouch. Egg in magnum. x300

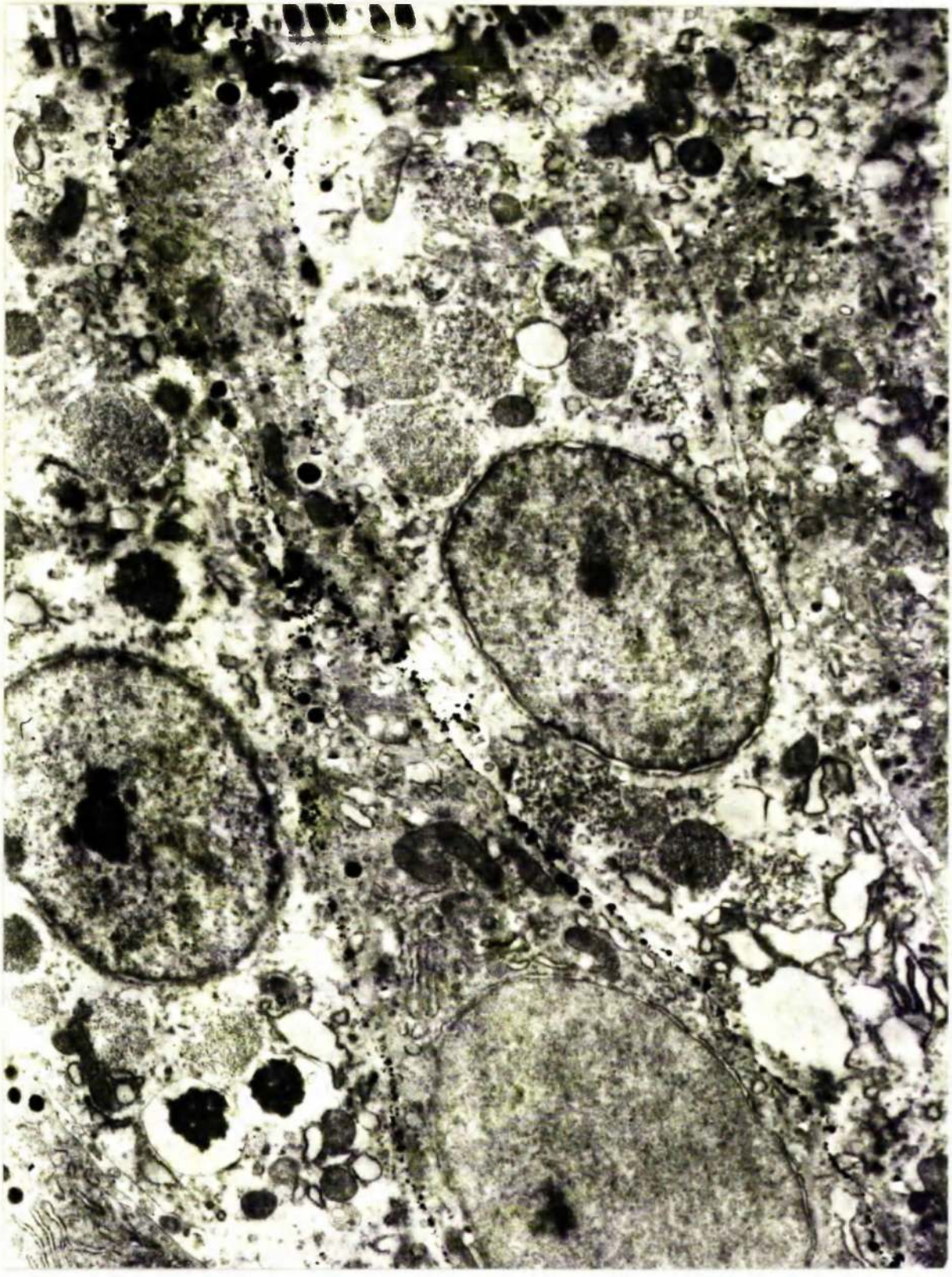


Fig 52. Shell gland pouch surface epithelium showing acid phosphatase reactivity principally in the basal cells, although electron dense deposits can also be seen in the adjacent apical cells. Soft membraned egg in shell gland. x10,800

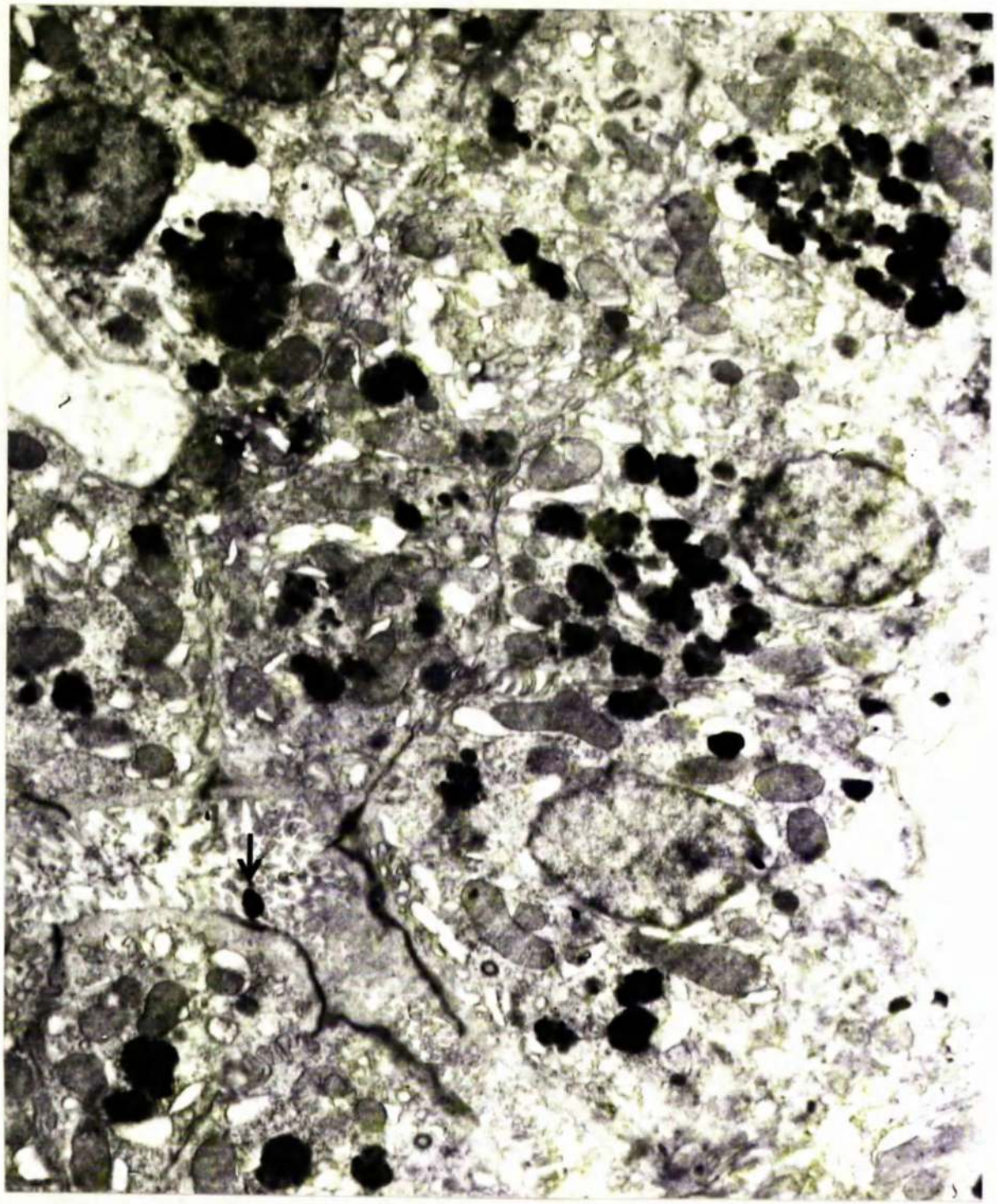


Fig 53. Lysosomal acid phosphatase in gland cells of shell gland pouch. Phosphatase reactivity is present in the gland lumen —>. Egg in isthmus. x10,000

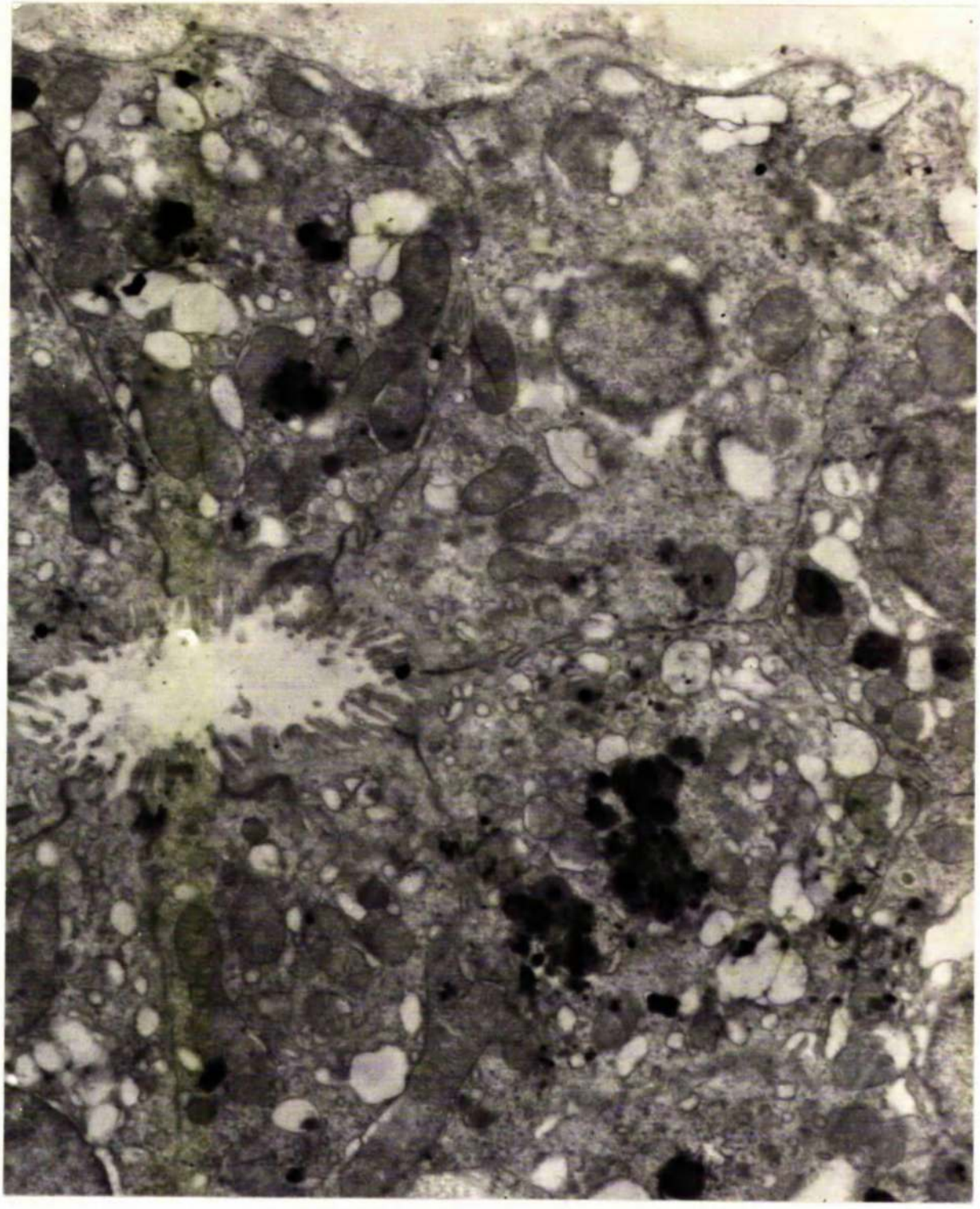


Fig 54. Junction of tubular shell gland and shell gland pouch. Lysosomal acid phosphatase is present in gland cells. Egg in isthmus. x10,000

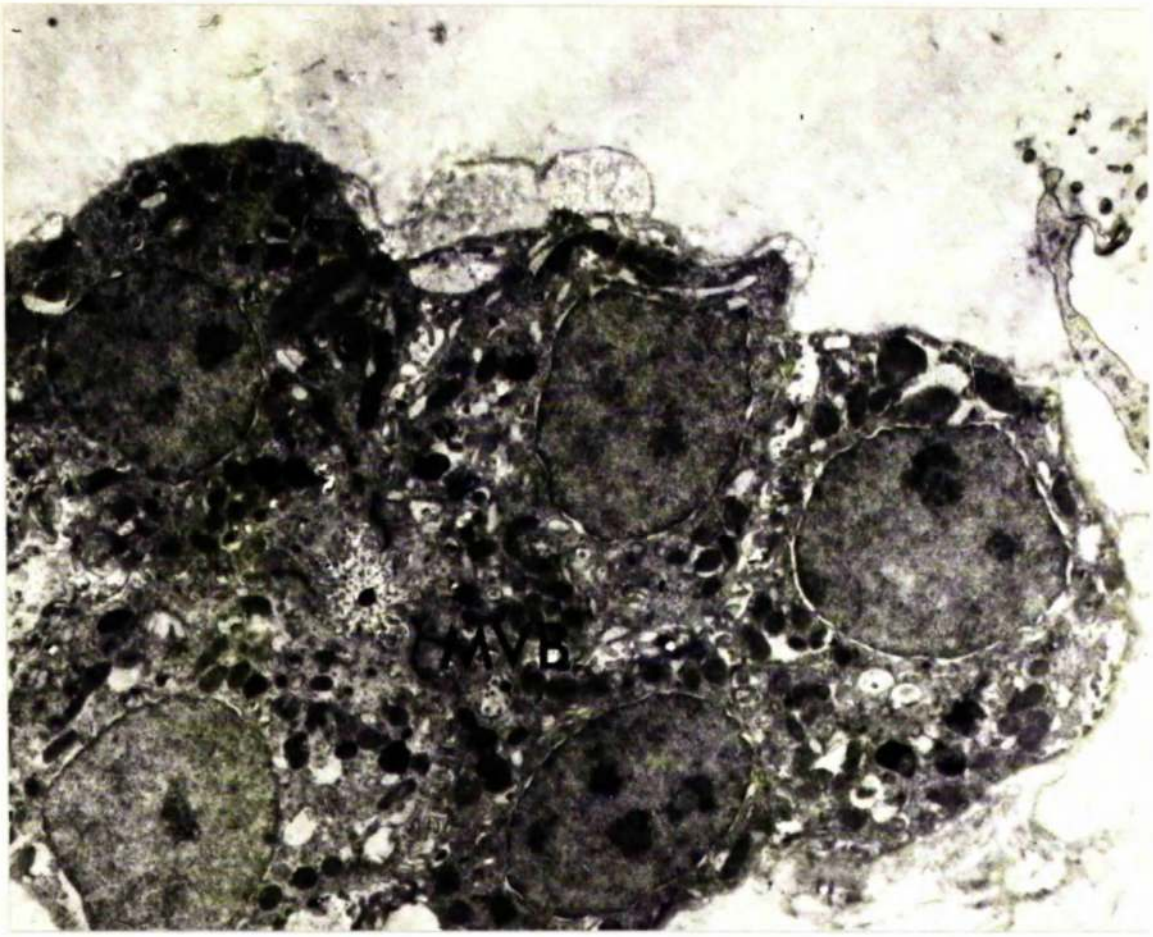


Fig 55. Shell gland pouch towards the end of calcification. Note the presence of multivesiculated bodies (MVB). x6,000

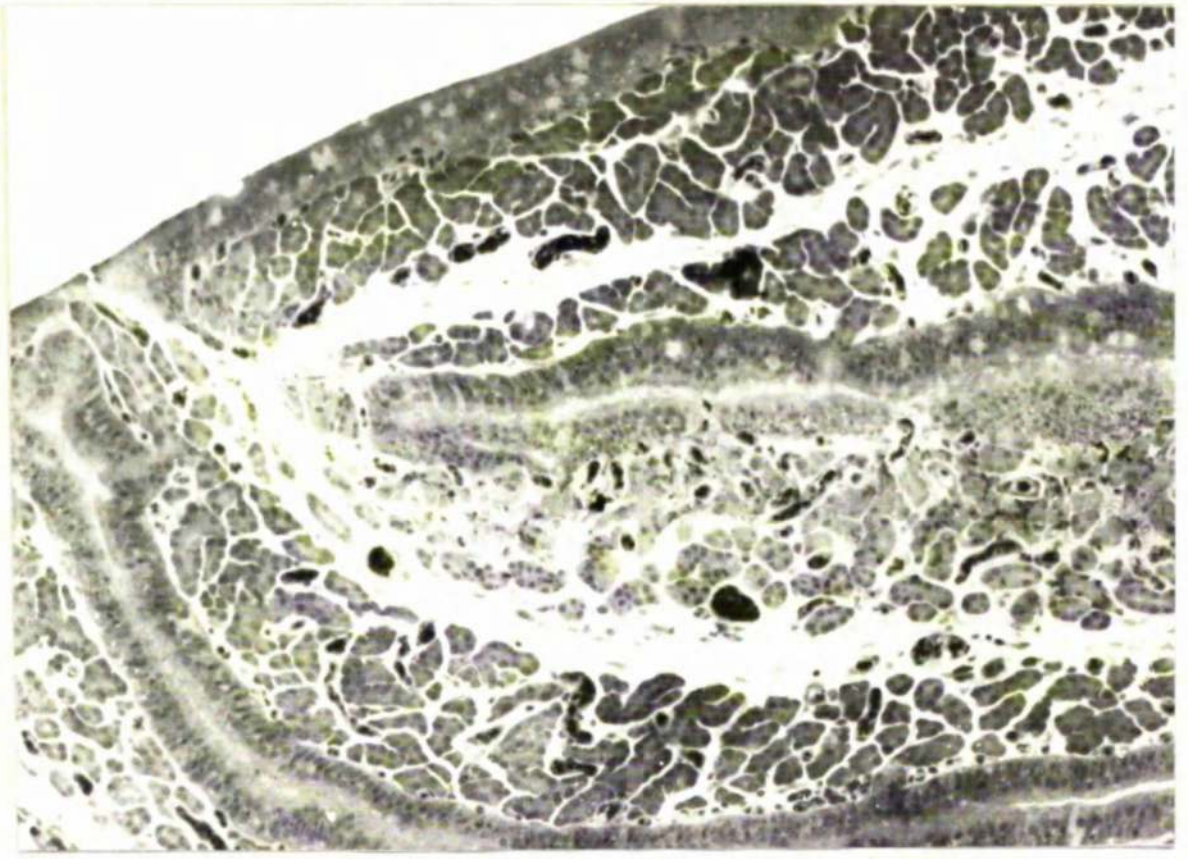


Fig 56. Shell gland pouch, epon embedded under vacuum. Toluidine blue. x90

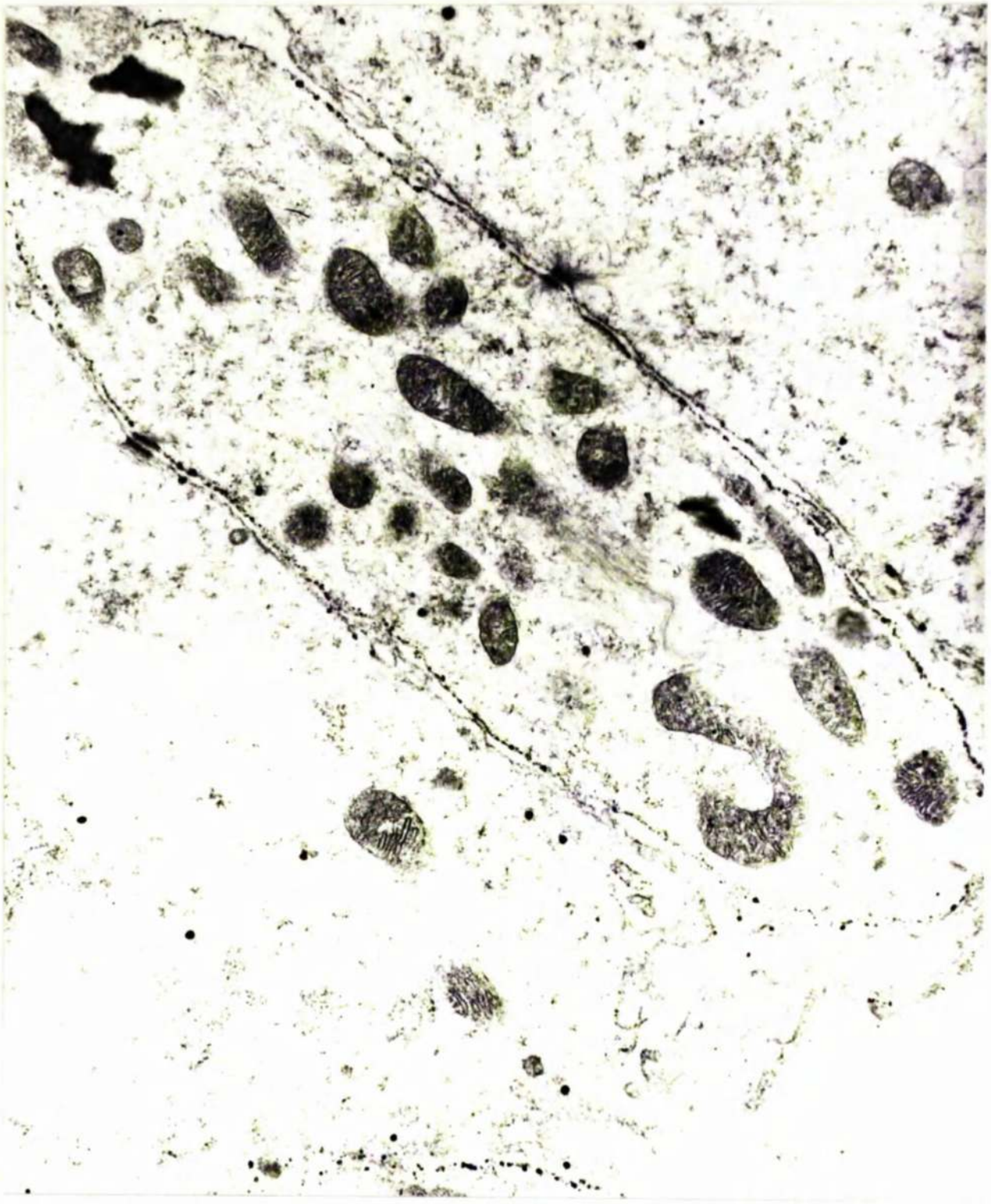


Fig 57. Mitochondrial cell from the tubular shell gland, the crystals occur mainly on the lateral cell membranes, but can also be seen inside many of the mitochondria. x27,000

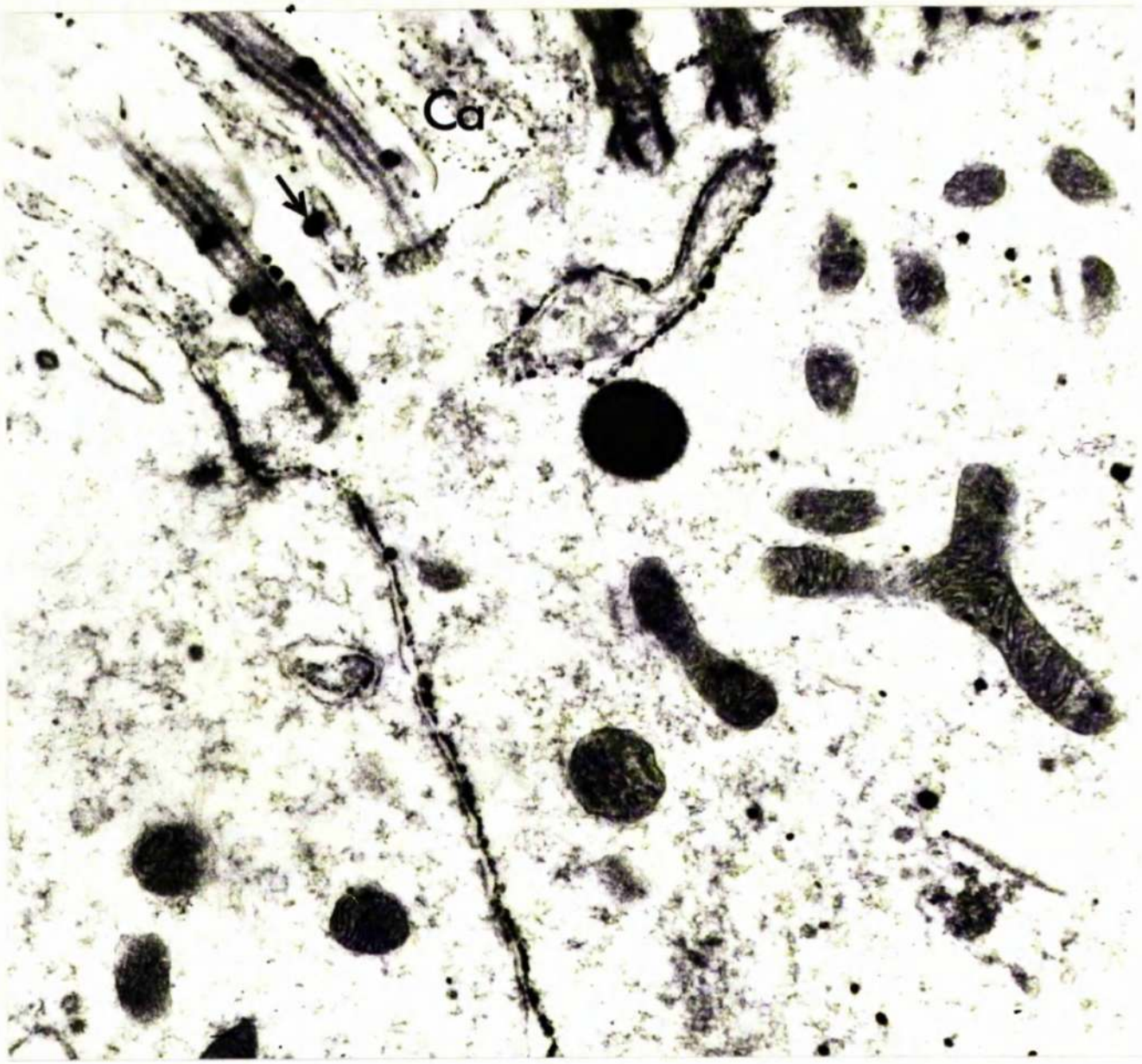


Fig 58. Part of a ciliated surface cell from the tubular shell gland. The large deposits (->) are thought to be sodium pyroantimonate. The smaller crystals over the microvilli, in the mitochondria and along the lateral cell membrane are calcium pyroantimonate (Ca). x36,000

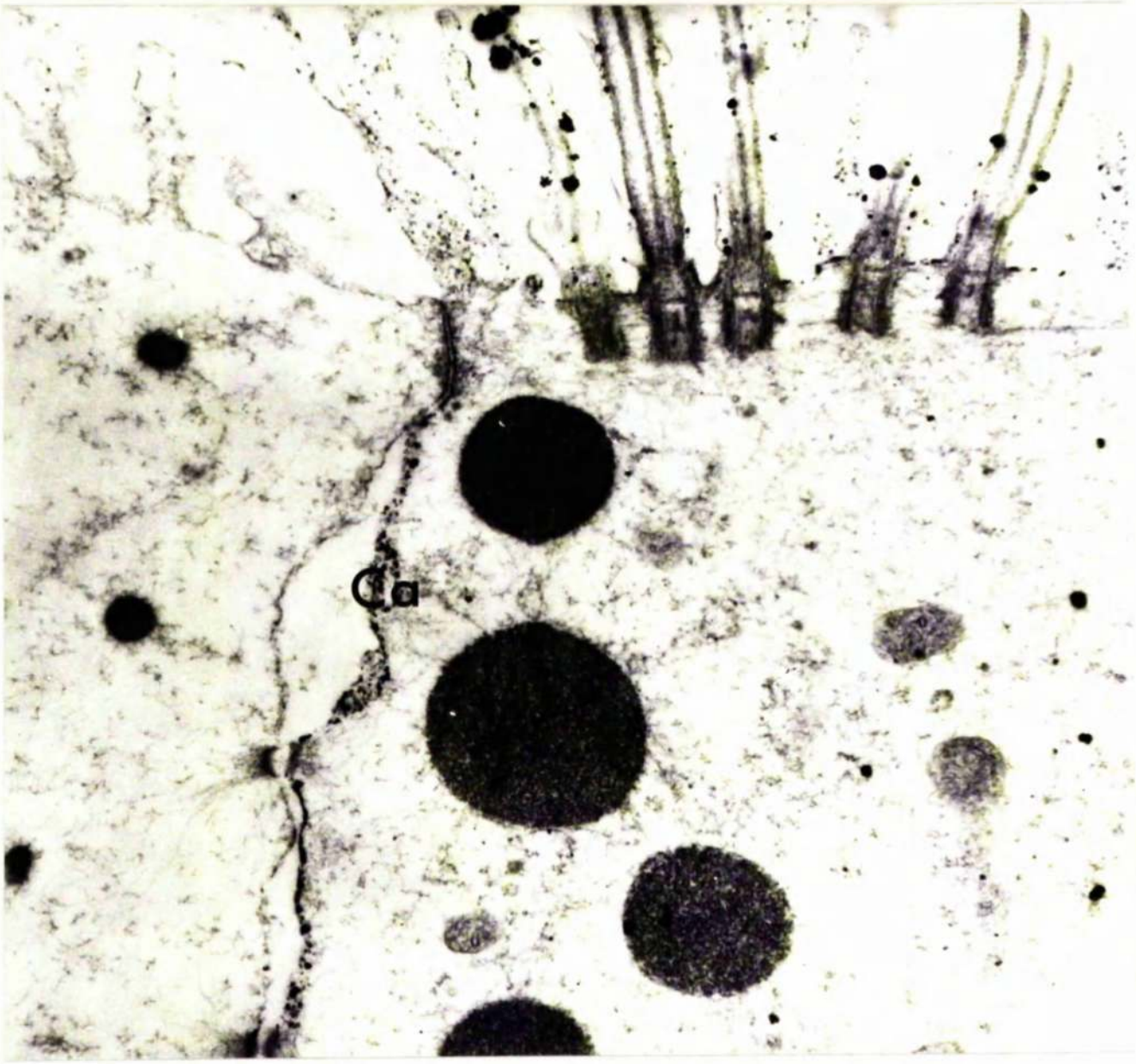


Fig 59. Ciliated cell from shell gland pouch region showing calcium pyroantimonate associated with lateral cell membrane, microvilli and cilia. Egg in isthmus. x38,000
Calcium pyroantimonate (Ca).

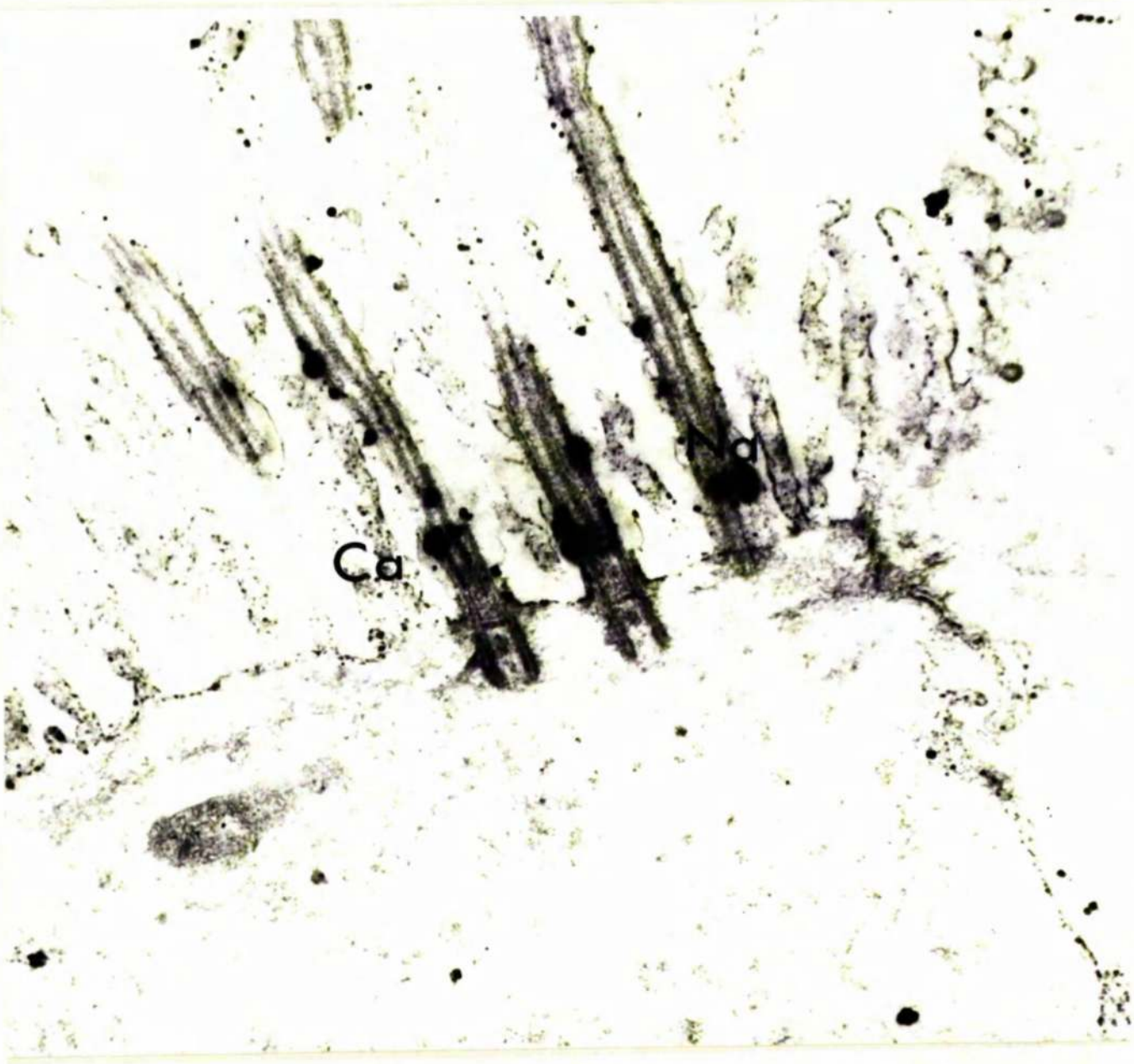


Fig 60. Cilia and microvilli lining surface epithelial cells of shell gland pouch. Crystalline deposits of calcium and sodium pyroantimonate are present. Calcifying egg in shell gland. pouch. x38,000
Calcium pyroantimonate (Ca), sodium pyroantimonate (Na).

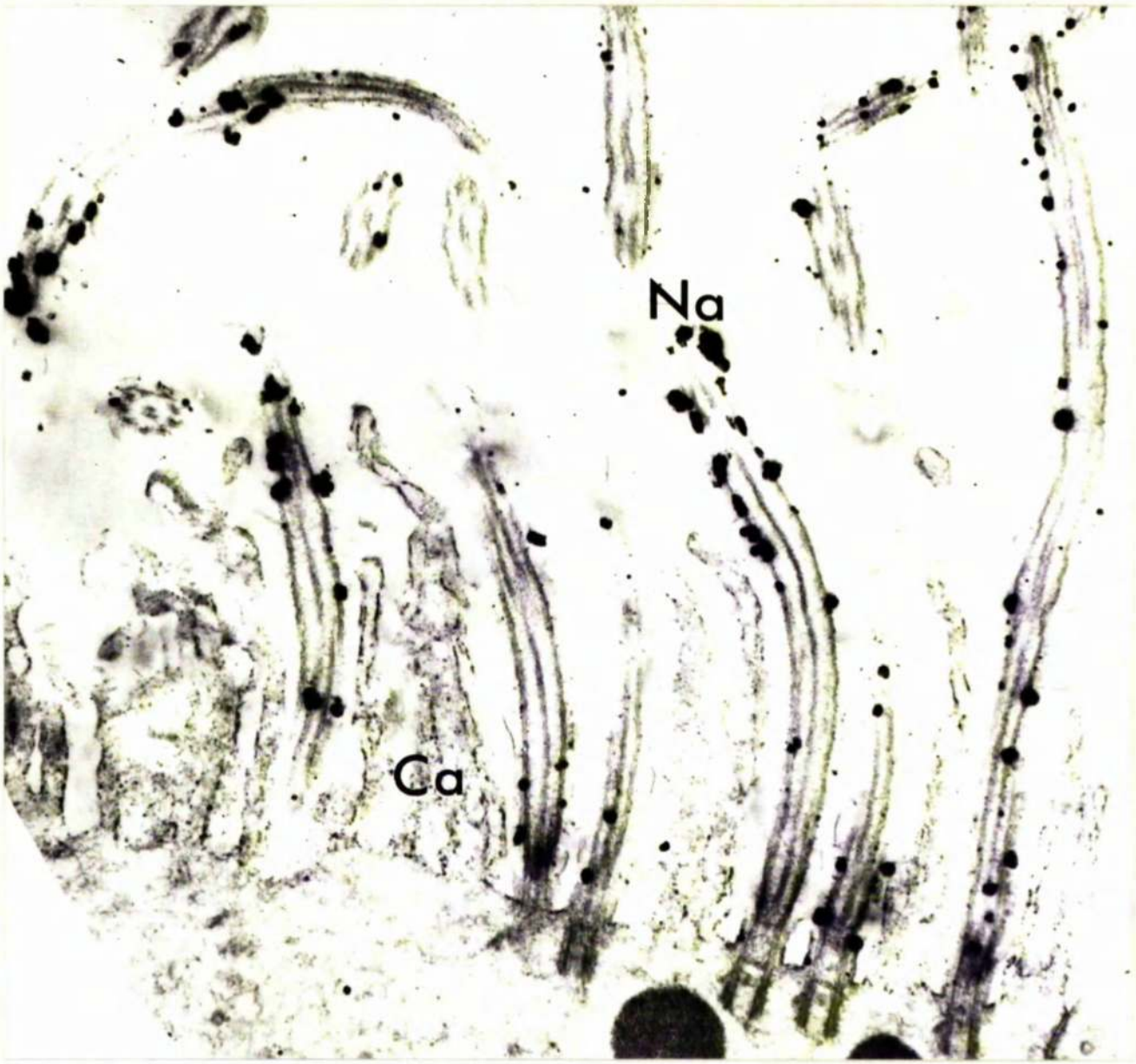


Fig 61. Cilia and microvilli on surface cells of shell gland pouch. Calcium pyroantimonate (Ca) and sodium pyroantimonate (Na). x36,000

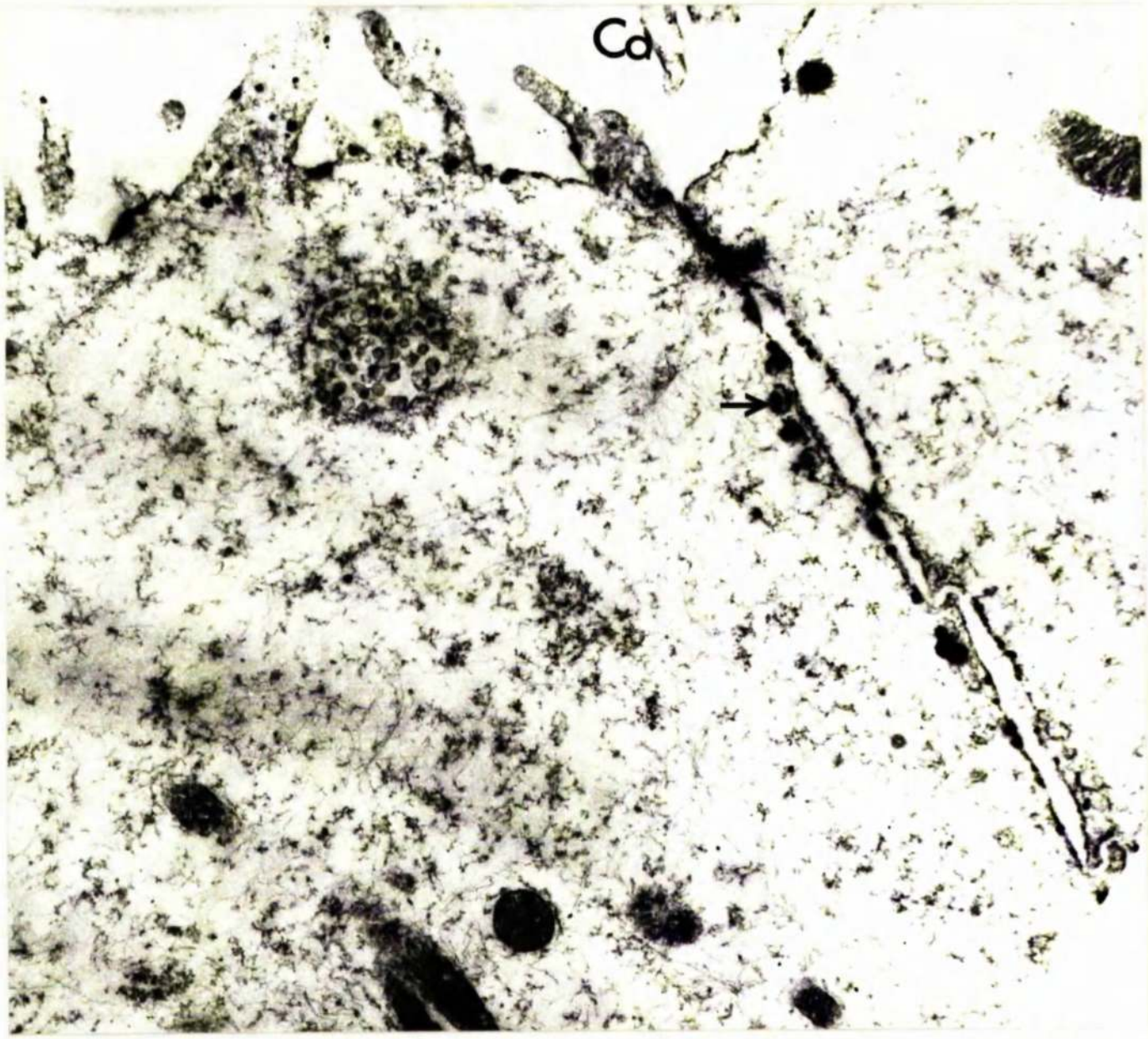


Fig 62. Part of non ciliated cell from the shell gland pouch showing largecrystals of sodium pyroantimonate (–>) along the lateral cell membrane and smaller crystals of calcium over the microvilli. x36,000 Calcium pyroantimonate (Ca).

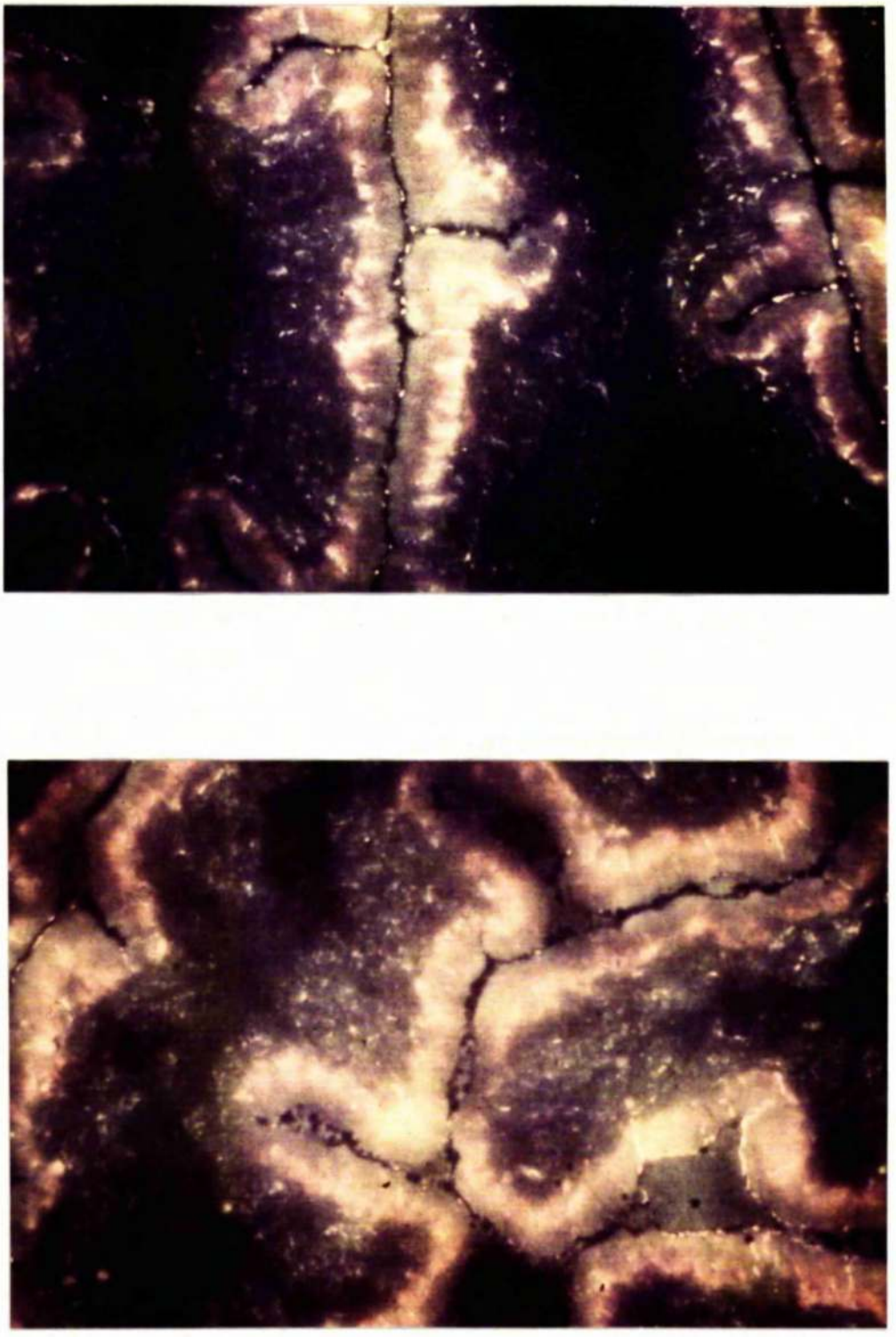


Fig 63. Quail shell gland pouch showing autofluorescence of surface epithelial cells. The nuclei in the underlying connective tissue also fluoresce. Calcifying egg in shell gland. x200

CHELATION AND ELECTRON DIFFRACTION

CHELATION

Perfusion with E.G.T.A. caused some tissue disruption, dilatation of endoplasmic reticulum and swelling of mitochondria. Despite these adverse effects, this agent did remove virtually all the small electron dense deposits from the surface epithelium, leaving only the larger masses adhering to the microvilli and basement membrane. The larger masses are thought to be sodium pyroantimonate on account of their resistance to chelation. Figs. 58, 60, 62.

ELECTRON DIFFRACTION

The ring patterns obtained from both the control and the cellular precipitate indicate that the deposit is polycrystalline. Figs. 64 & 65. The crystal lattice spacings for both are given in Table 1 and accord with the spacings given in the A.S.T.M. index for calcium pyroantimonate. Fig. 66, shows the selected area diffraction pattern for the control thallous chloride.

T A B L E 1

P Y R O A N T I M O N A T E

TISSUE CRYSTALS		PREPARED PYROANTIMONATE	
constant Thallous Chloride 40.4		constant Graphite 42.5	
Diameter	d Å	Diameter	d Å
11.2	3.6	10.1	4.20
12.8	3.15	11.0	3.86
13.7	2.94	11.9	3.57
15.8	2.55	12.8	3.32
18.1	2.23	13.6	3.13
19.2		13.9	3.06
22.2	1.82	15.5	2.74
23.5		16.7	2.54
26.1	1.54	18.5	2.29
		20.3	2.09
		21.9	1.94
34.5	1.17	22.1	1.92
38	1.06	23.1	1.84
		25.6	1.66
		27.9	1.32
		37	1.15
		40	1.05

dÅ spacings only should be compared, thus, tissue crystal dÅ 3.15 ≡ prepared pyroantimonate dÅ 3.13.

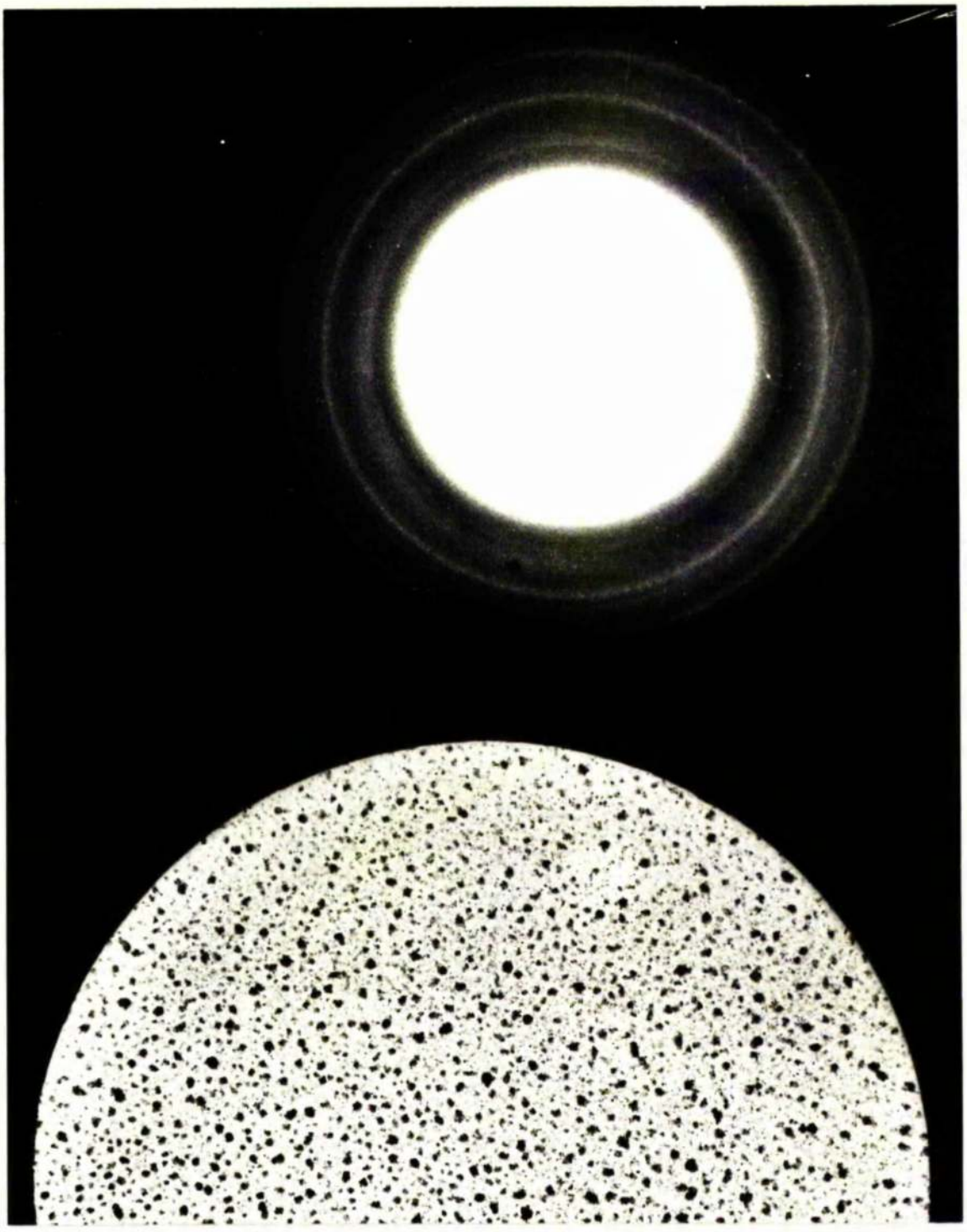


Fig 64. Diffraction pattern together with prepared precipitate of calcium pyroantimonate.

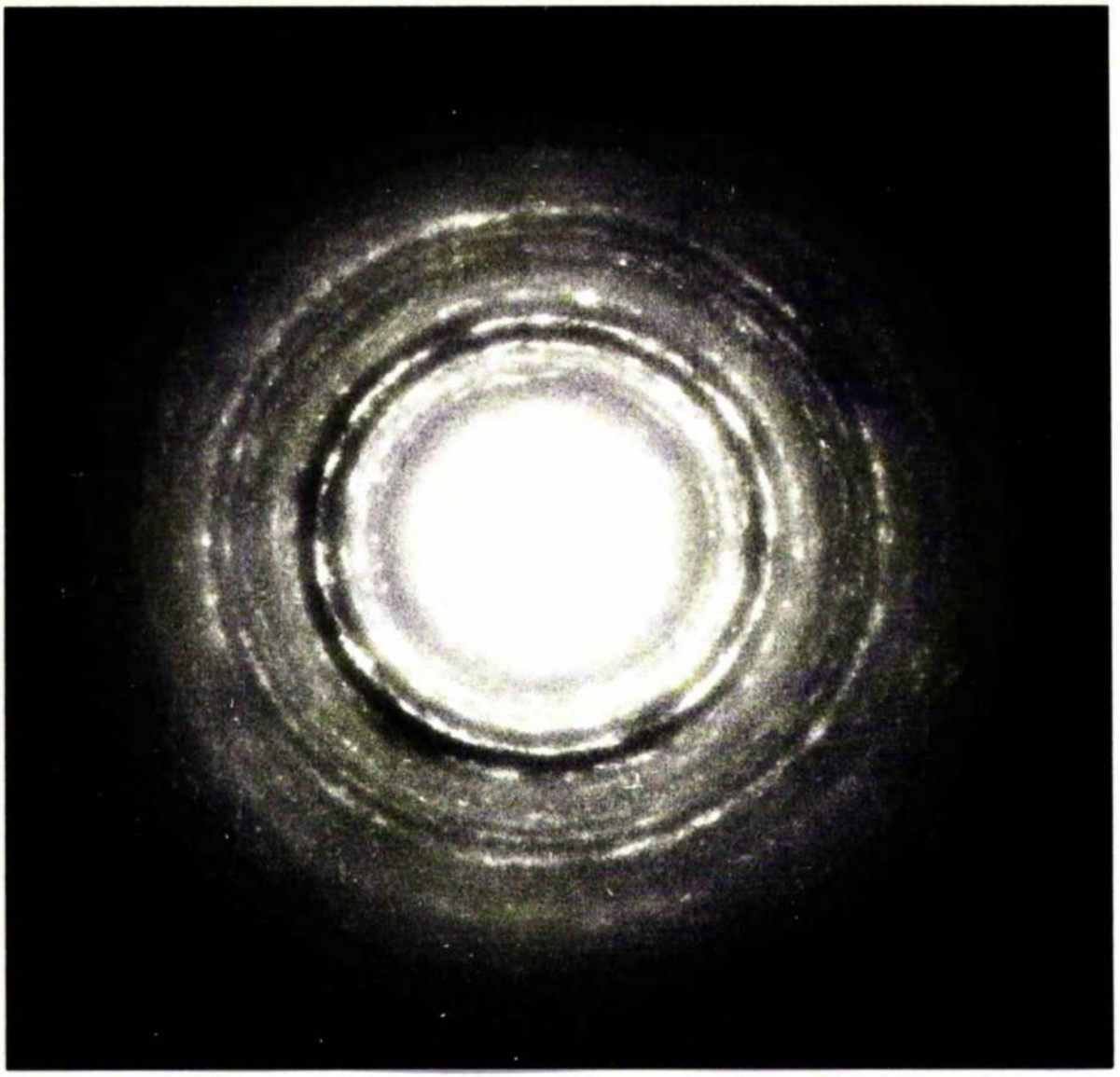


Fig 65. Calcium pyroantimonate diffraction pattern of tissue precipitate.

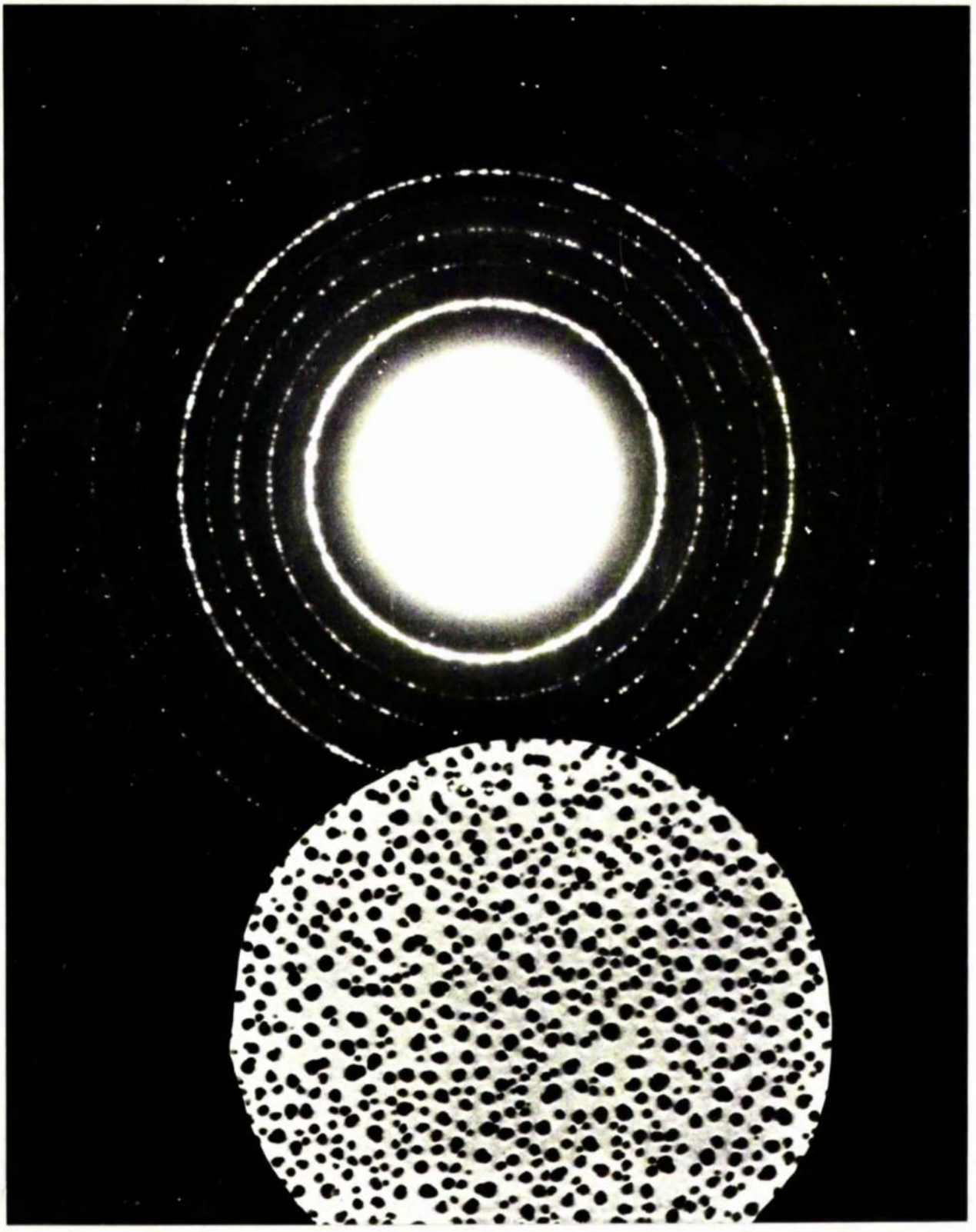


Fig 66. Selected area diffraction pattern for thallous chloride.

NEUTRON ACTIVATION ANALYSIS

NEUTRON ACTIVATION ANALYSIS

Egg No.	PPM Antimony
1	250
2	200
3	250
4	210
Control	160

The sample eggs do not show any great deviation from the control, thus showing that pyroantimonate does not transfer itself.

CHEMISTRY OF PORPHYRINS

DEPTH OF PIGMENT LAYER

The pigment layer is most obvious in Figs. 67 & 68, i.e. Black Headed Gull and quail. In the former the pigment layer is overlain with cuticle, while in the latter there appears to be no cuticular layer. The slightly darker band under the pigment in Fig. 68 may be the result of pigment diffusion but is more likely due to the opacity of the shell.

Measurements of the pigment layers show that in the gull the layer is approximately 1.2mm thick, while in the quail it is 2.1mm thick. In view of the intimate relationship between pigment and calcium carbonate in the White Leghorn and Brown Ranger it is not possible to measure the depth of the pigment layer. Figs. 69, 70.

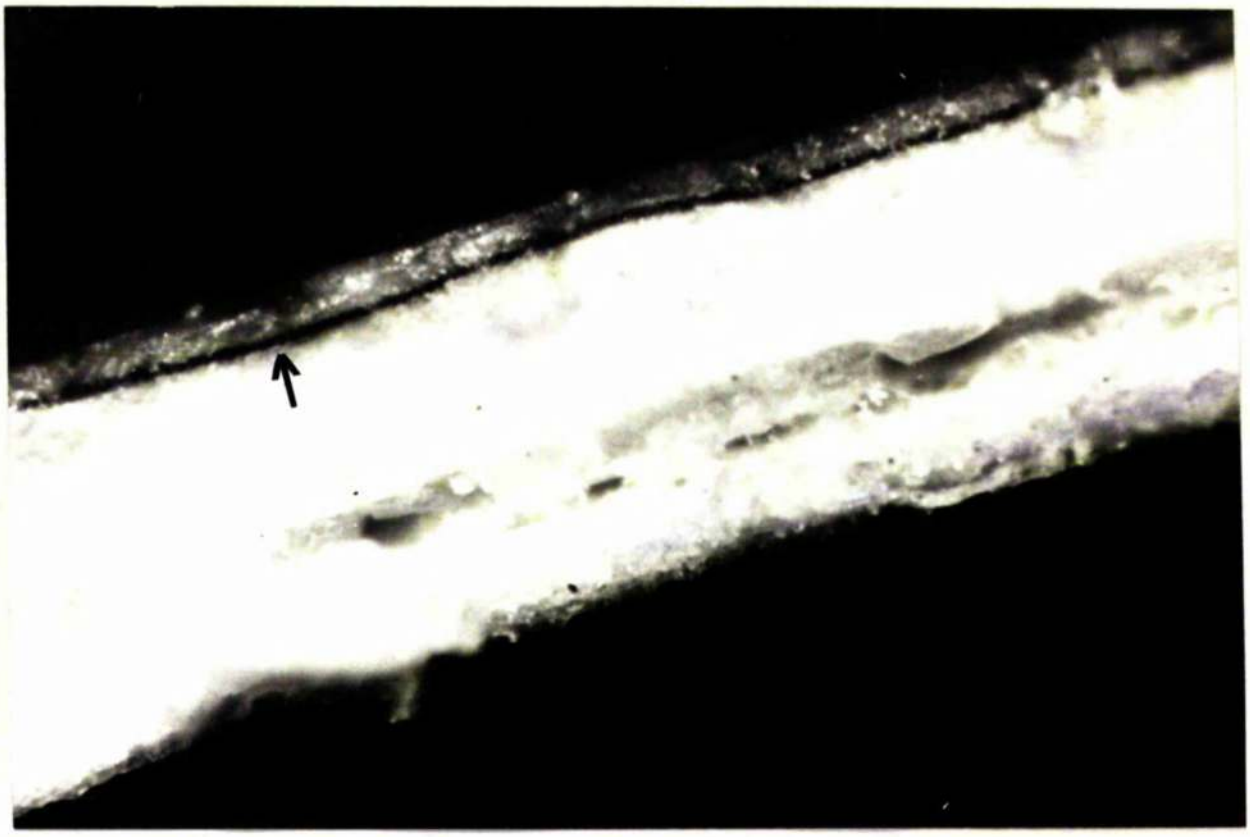


Fig 67. Shell from Black Headed Gull showing dense pigment layer (—>) beneath the cuticle. x200

Fig 68. Quail shell showing dense pigment layer ->. x200

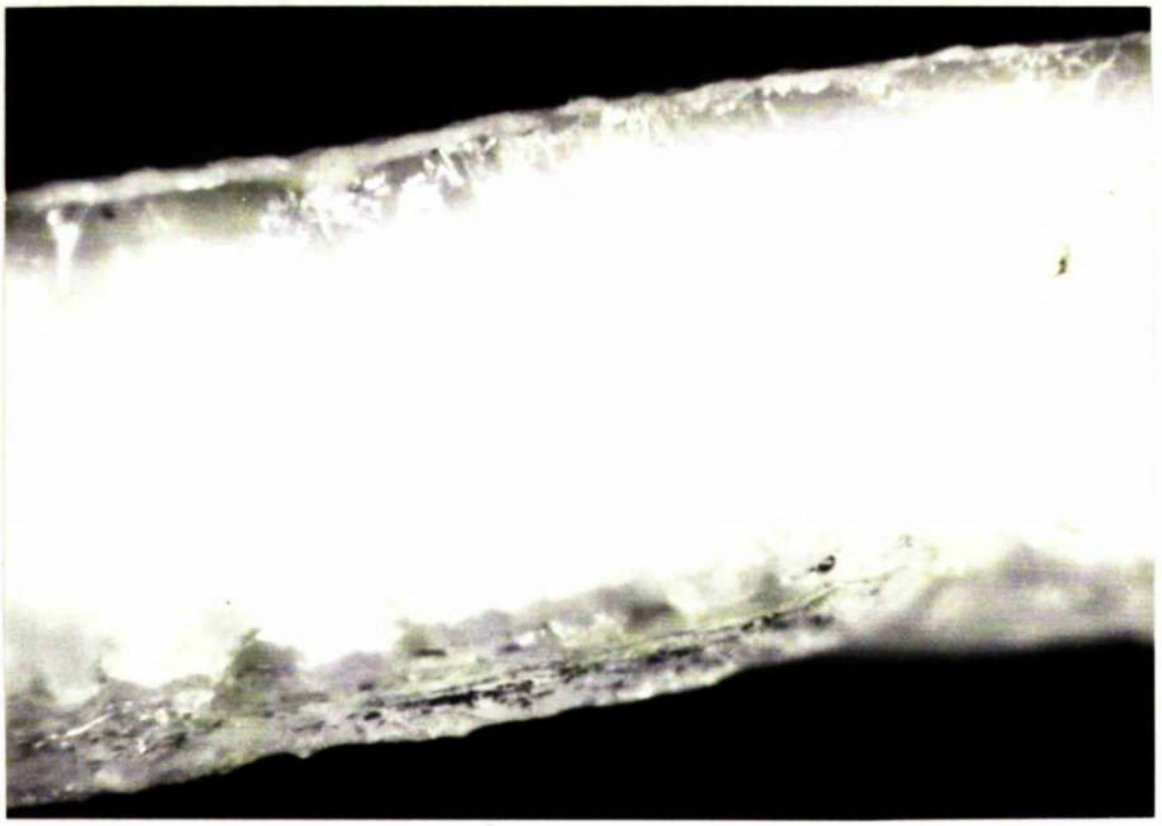


Fig 69. Shell from White Leghorn. No pigment layer can be detected. x200

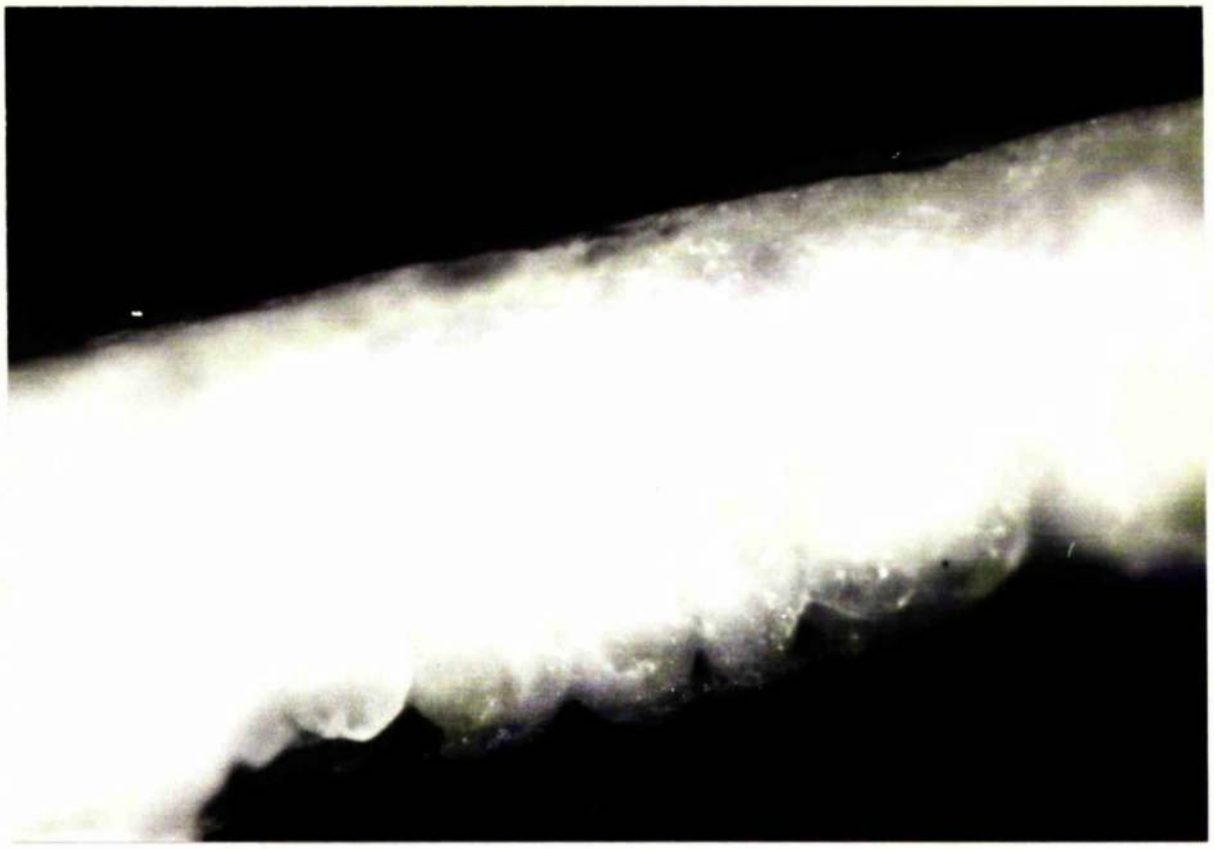


Fig 70. Shell from Brown Ranger, pigment layer is not readily discernible. x200

INFRA RED ANALYSIS

Graphs 1 & 2 show the spectra (4,000 - 625cm^{-1}) for the sublimed pigment (cuticular and shell). Graph 3 shows the spectrum for quail pigment ground with potassium bromide and Graph 4 is the spectrum for the shell minus pigment + KBr.

The spectra for sublimed pigment are almost identical and show absorption for indol NH (3,300cm^{-1}), CH-saturated (2950cm^{-1} and 2920 cm^{-1}) conjugated C-O (1665cm^{-1}) and CH-saturated at (1450 cm^{-1}).

The additional peaks in Graph 3 at 1430cm^{-1} and 870cm^{-1} are due to contamination from the egg shell, Graph 4. The peak at 1520cm^{-1} is probably due to the presence of water while the absorption at 1050cm^{-1} can be attributed to the presence of C-O.

The small shoulder at 1770cm^{-1} observed only in the spectra of sublimed pigment may be the result of degradation during evaporation.

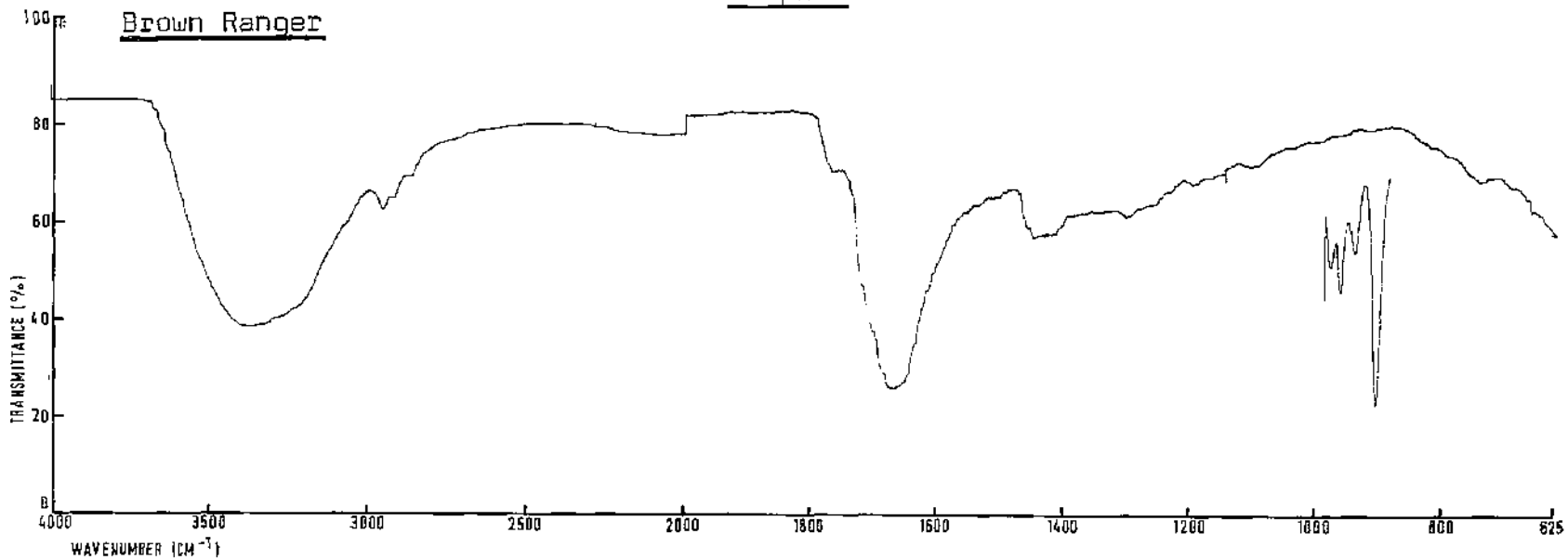
These figures are presented in Tables 2 & 3.

Only the peaks caused by contamination from the shell are given in table 3 (shell + KBr).

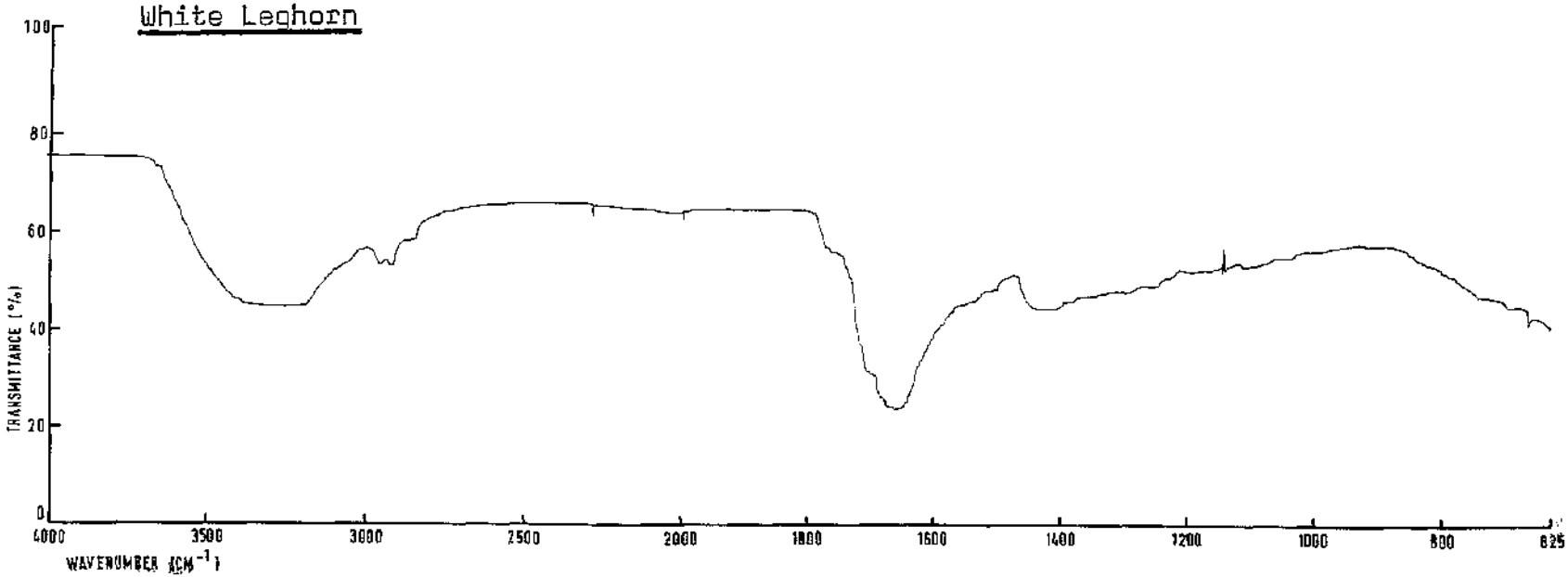
The spectra are consistent with the characteristic absorption frequencies of porphyrins and in particular protoporphyrin.

Graph 1

Brown Ranger



White Leghorn

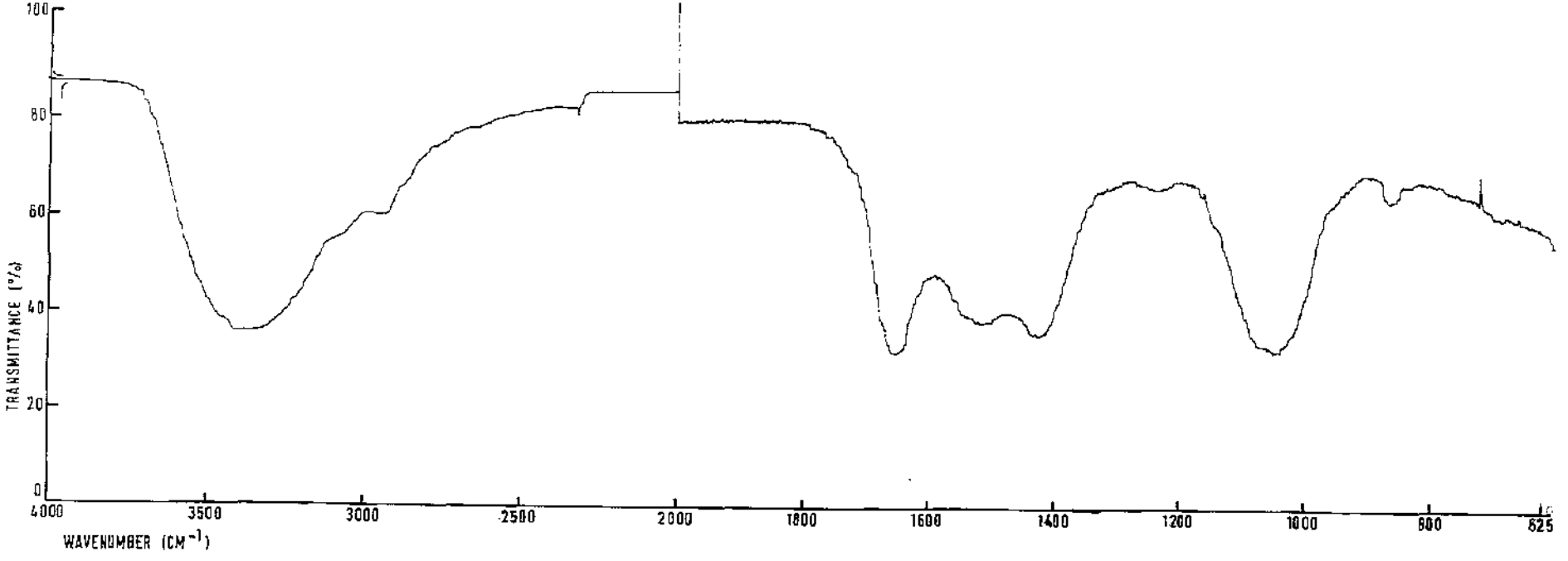


Infra Red Spectra

Graph 2

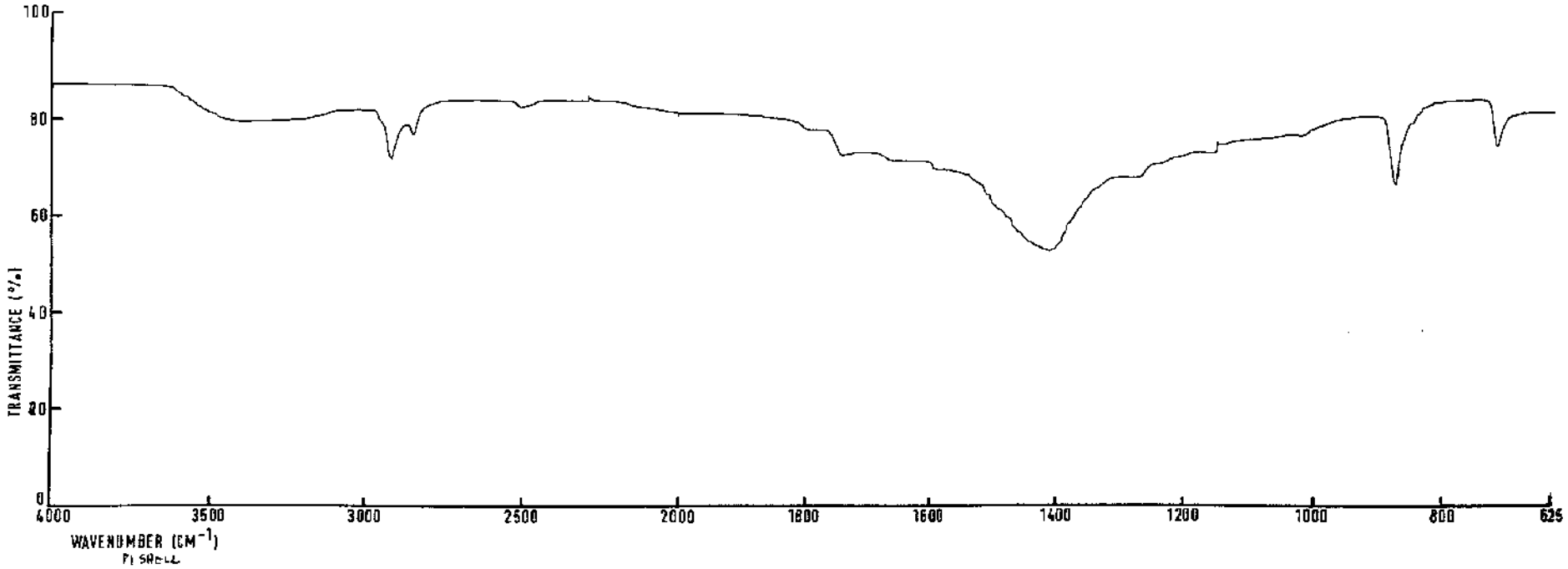
Infra Red Spectra

Graph 3



Infra Red Spectrum for Quail Pigment + KBr

Graph 4



Infra Red Spectrum

Shell + KBr

TABLE 2

SUBLIMED SHELL

Peak values cm^{-1}

Quail	White Leghorn
3200	3300
2945	2950
2920	2920
1770	1770
1670	1660
1450	1450

Black Headed Gull	Brown Ranger
3250	3300
2950	2960
2920	2920
1770	1770
1660	1665
1450	1450

T A B L E 3

Quail pigment + KBr	Shell + KBr
3400	
2950	
1660	
1520	
1430	1420
1050	
870	870

X-RAY FLUORESCENCE

None of the metal ions tested for, i.e. lead, cobalt, zinc, iron, calcium and magnesium, were present in the sublimed pigment.

ULTRASTRUCTURAL ANALYSIS OF PIGMENT CRYSTALS

In all the samples examined, i.e. sublimed crushed and pure protoporphyrin, the dominant crystals were long and needle-shaped. Figs. 71 & 72. The black bands, buckling bands could be due to the thermal effect of the beam or the manner in which the crystals are lying on the substrate on top of each other thus producing a strain in the crystal.

Electron diffraction studies on each of the crystalline specimens produced similar patterns. Figs. 73, 74, 75.

Fig 71. Sublimed crystals of protoporphyrin from the shell of the Quail. x80,000

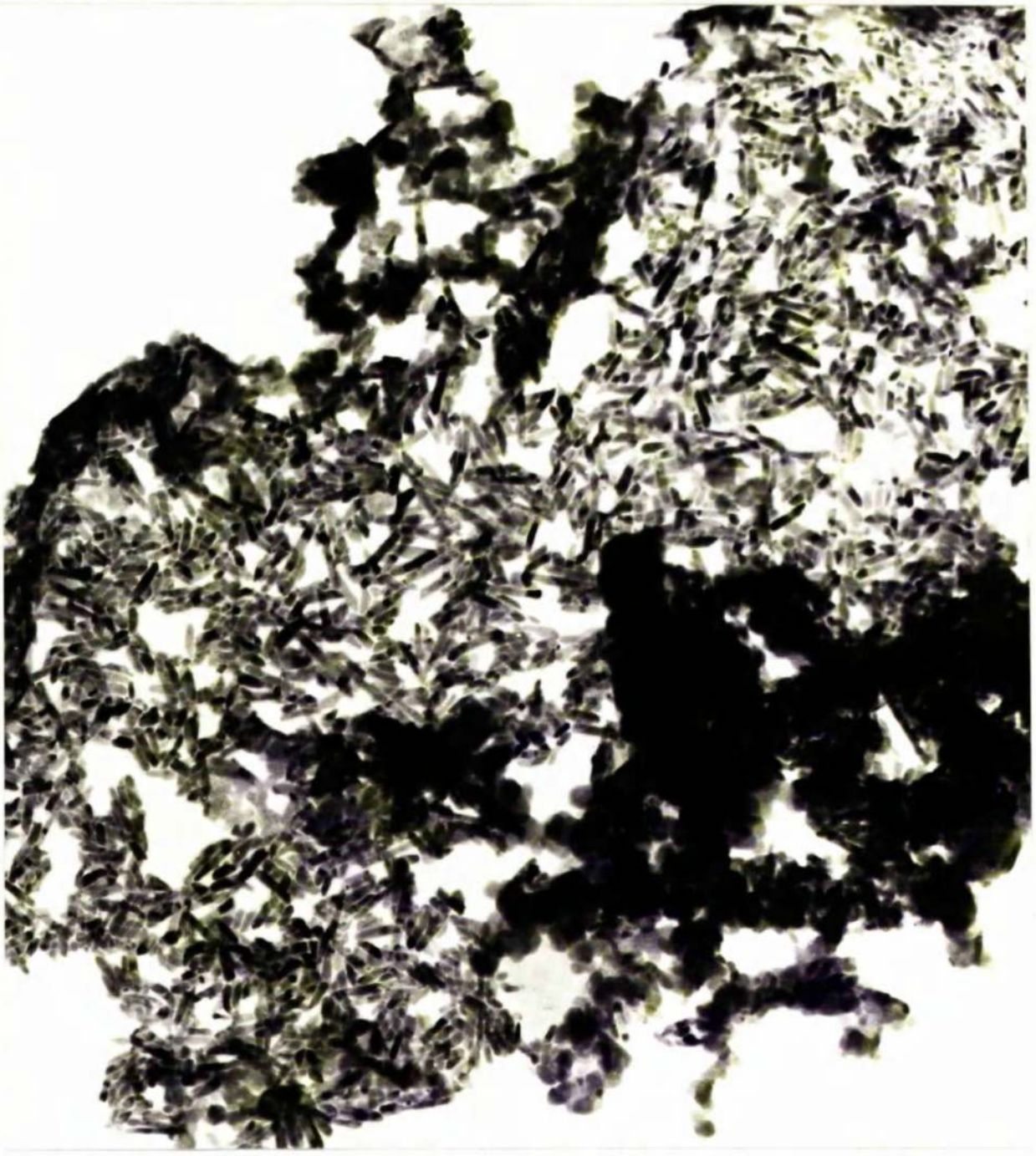


Fig 72. Crystals of protoporphyrin prepared by crushing the shell of the Black Headed Gull. x60,000

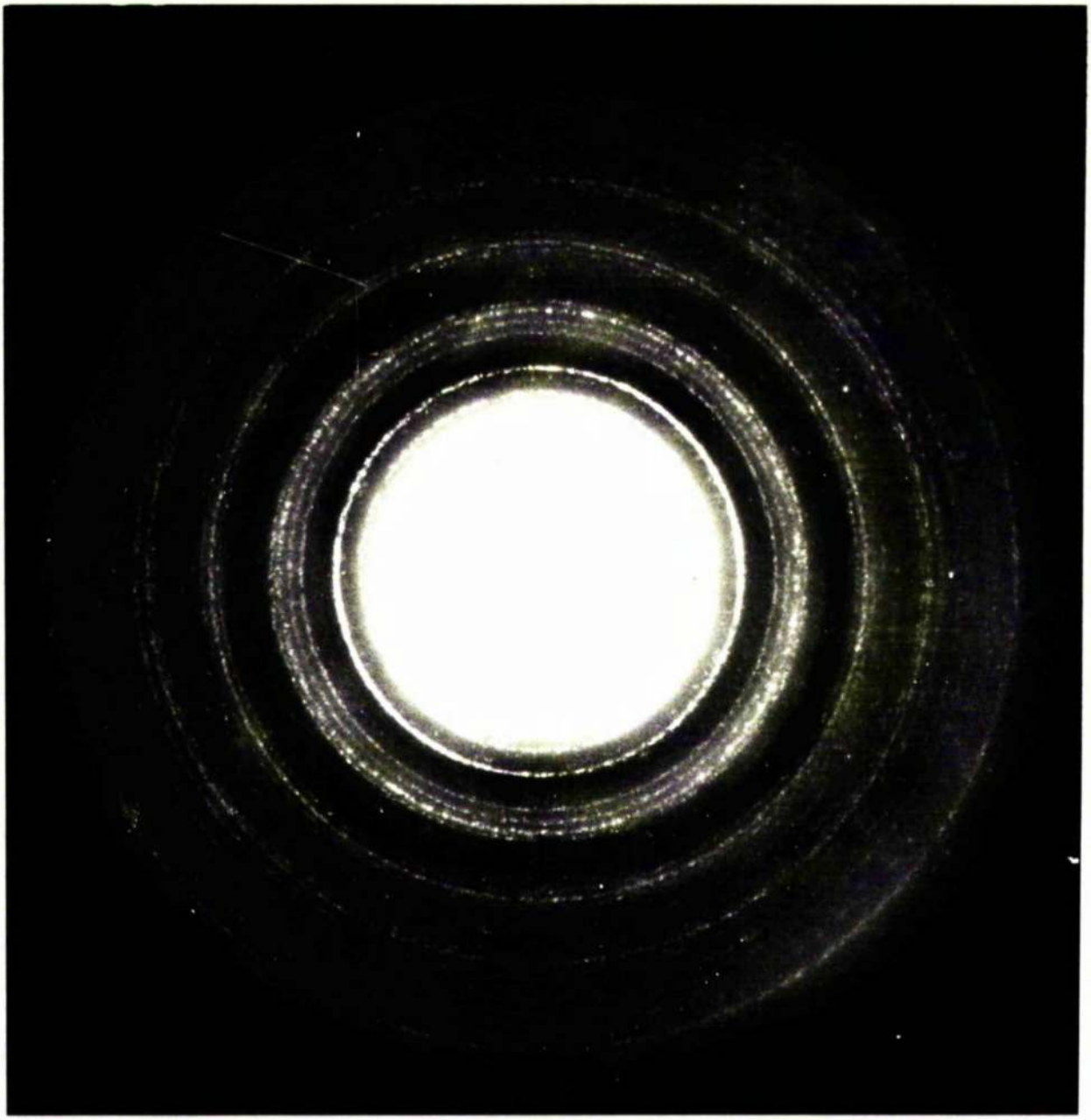


Fig 73. Electron diffraction pattern of sublimed crystals of protoporphyrin from the Quail shell.

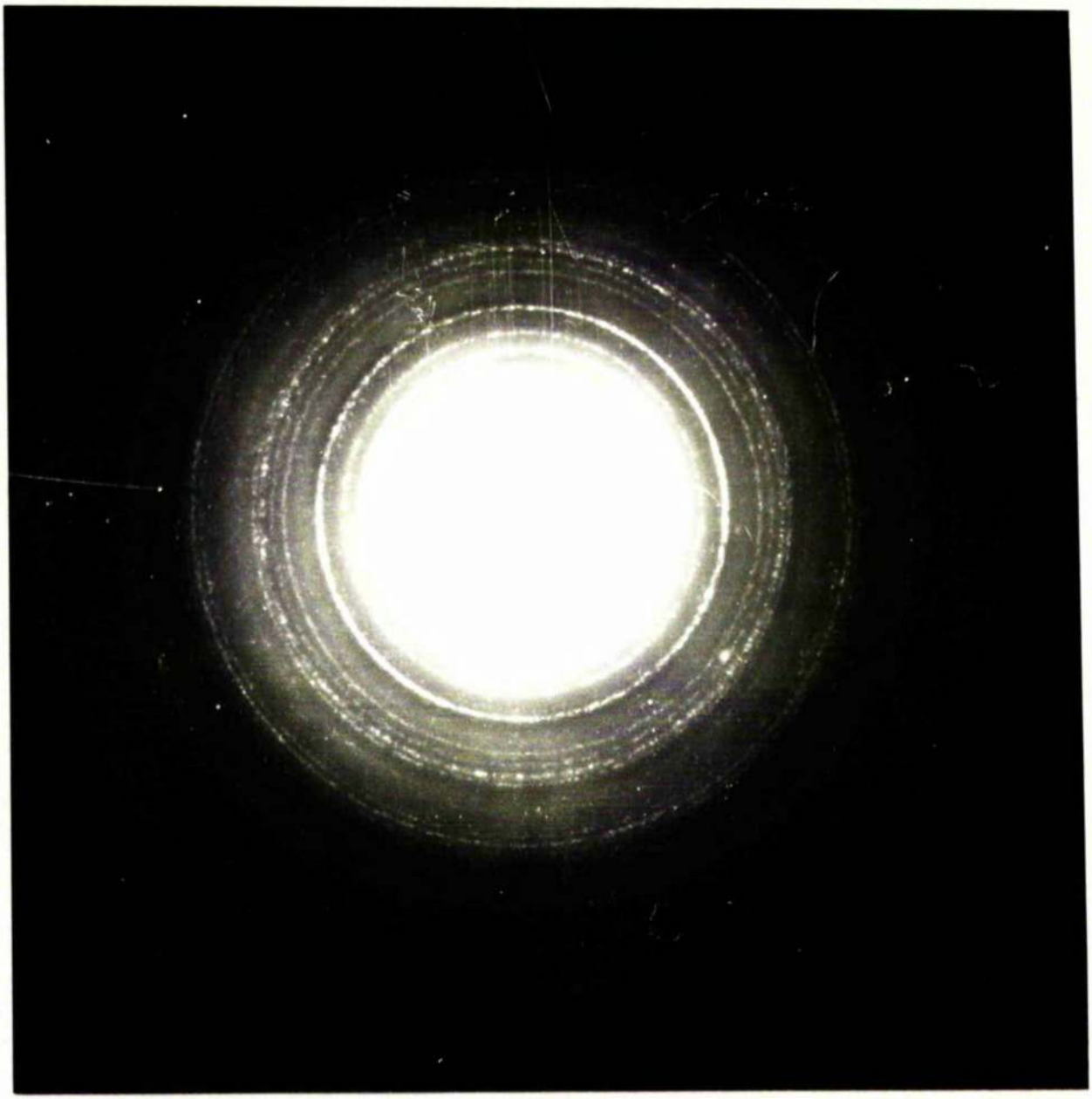


Fig 74. Electron diffraction pattern of crushed protoporphyrin from the shell of the Black Headed Gull.

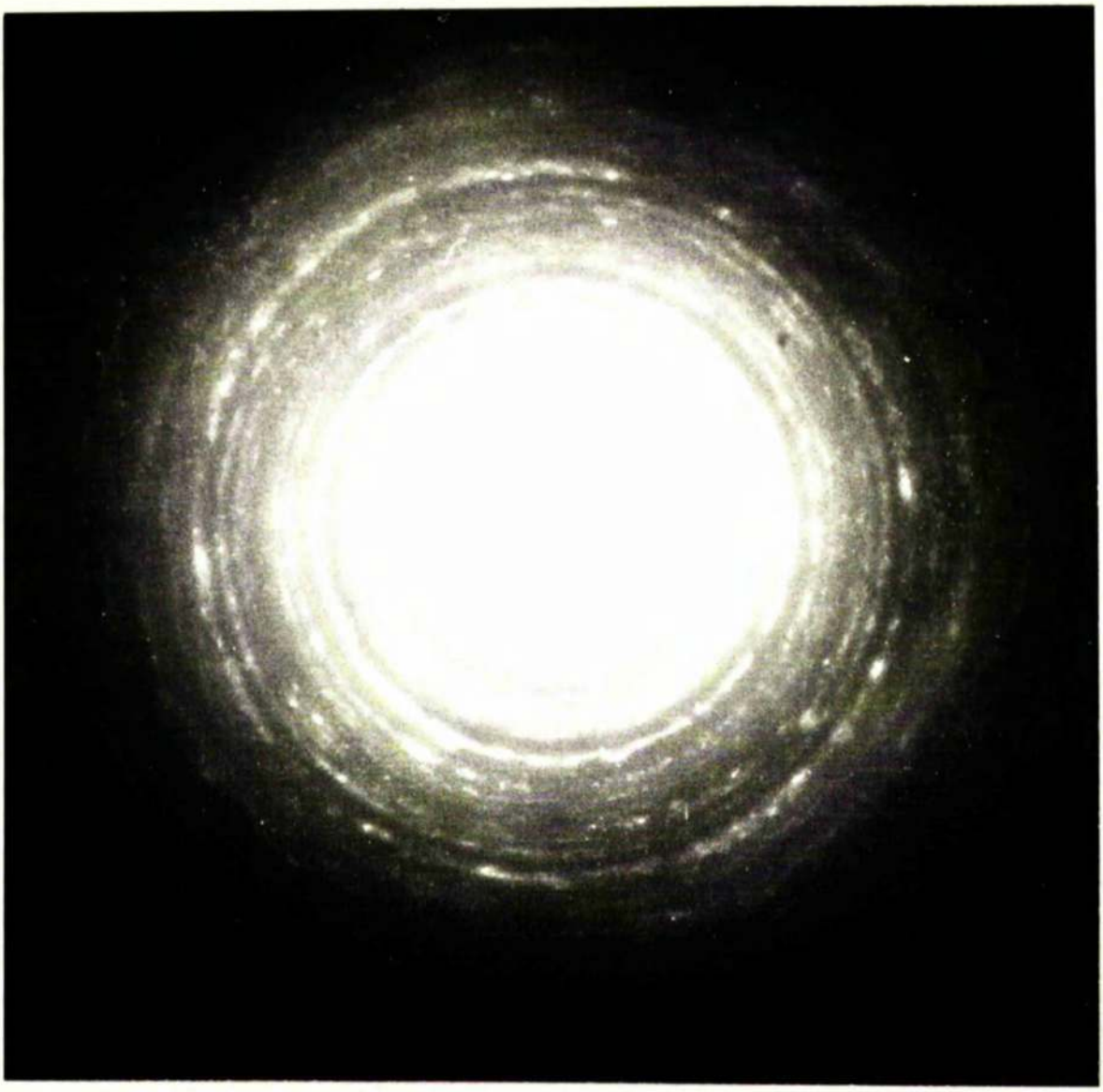


Fig 75. Electron diffraction pattern of pure protoporphyrin.

B I O C H E M I S T R Y

ACID PHOSPHATASE FLUCTUATIONS

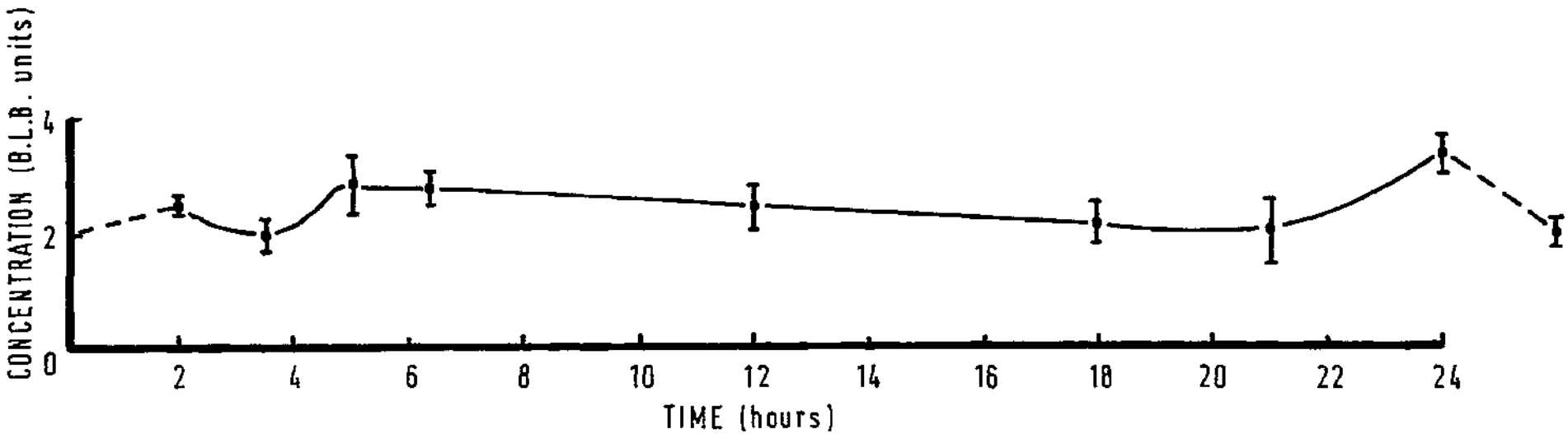
Plasma acid phosphatase

Acid phosphatase activity in the plasma reached a peak 2.89 ± 0.523 B.L.B. units with an egg in the isthmus. During the major period of shell formation activity was minimal, but reached a peak 3.10 ± 0.25 B.L.B. units when shell calcification was completed. Thereafter the level of activity dropped to the pre-ovulatory value of 2 ± 0.29 B.L.B. units. Graph 5.

Shell gland fluid acid phosphatase

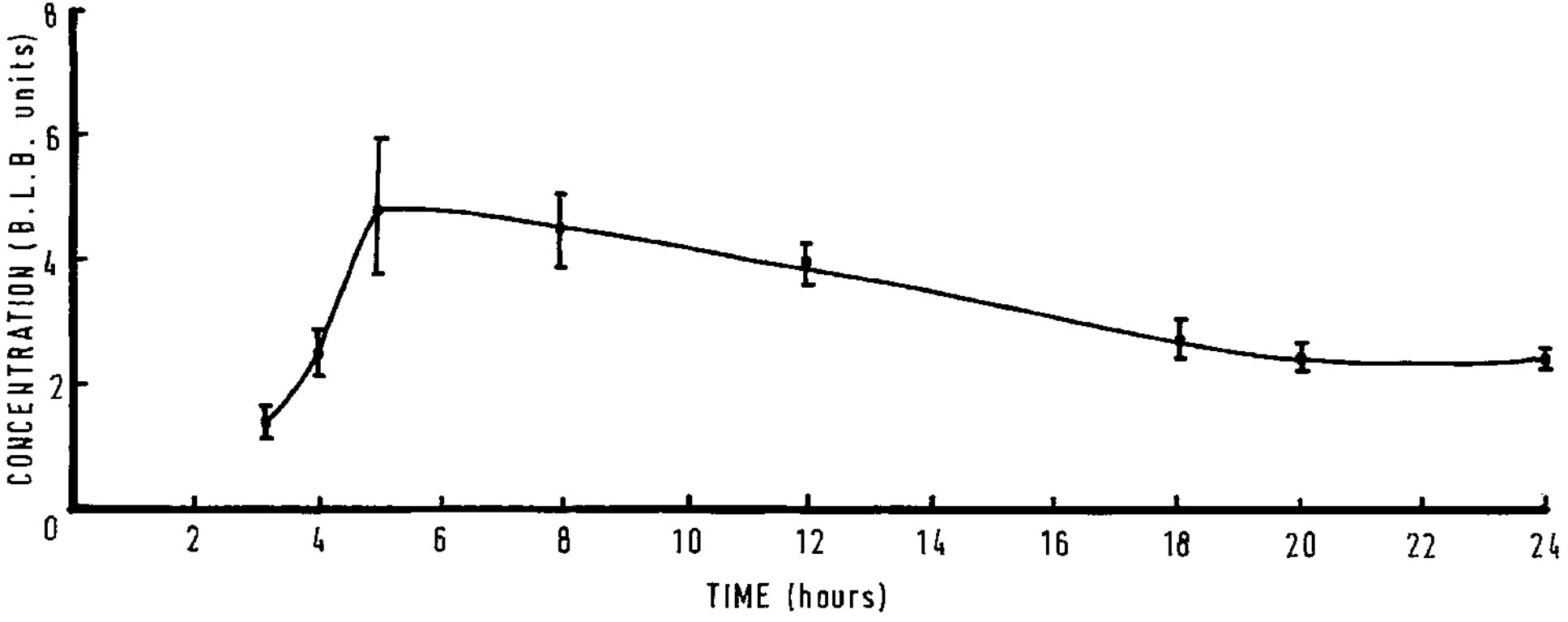
The graph for shell gland fluid phosphatase activity shows a steep rise to reach a maximum of 4.9 ± 1.24 B.L.B. units with an egg in the isthmus. This corresponds to the peak noted in relation to plasma acid phosphatase. Thereafter activity decreased to reach a minimum level at oviposition. Graph 6. See also Table 4.

Graph 5



Plasma Acid Phosphatase Activity

Graph 6



Shell Gland Fluid — Acid Phosphatase Activity

TABLE 4

Time in laying cycle (hrs)	Plasma acid phosphatase	Time in laying cycle (hrs)	Shell gland fluid acid phosphatase
2	2.5 ± 0.113	3	1.4 ± 0.243
3.5	1.9 ± 0.265	4	2.38 ± 0.313
5	2.89 ± 0.523	5	4.9 ± 1.24
6.25	2.7 ± 0.289	8	4.56 ± 0.522
12	2.32 ± 0.406	12	3.9 ± 0.204
18	2.12 ± 0.376	18	2.99 ± 0.387
21	1.83 ± 0.573	20	2.51 ± 0.272
24	3.10 ± 0.25	24	2.44 ± 0.161
Ovulation	2 ± 0.29		

ANALYSIS OF TISSUE PORPHYRINS

The values for uroporphyrin, coproporphyrin and protoporphyrin in the isthmus, shell gland (pouch region) and shell gland/vagina are given in tables 5, 6, 7.

On average a threefold increase in mg porphyrin/gm tissue takes place during calcification. In every case the concentration of protoporphyrin is greatest in the shell gland.

TABLE 5

QUAIL

UROPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0	0
Shell Gland	0	0
Shell Gland/Vagina	0	0

COPROPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0.016	0
Shell Gland	1.18	0.38
Shell Gland/Vagina	0	0

PROTOPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0.823	0.222
Shell Gland	4.765	1.765
Shell Gland/Vagina	0	0

TABLE 6

BROWN RANGER

UROPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0	0
Shell Gland	0.066	0
Shell Gland/Vagina	0.016	0

COPROPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0	0
Shell Gland	.021	.06
Shell Gland/Vagina	.007	0

PROTOPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0.078	0.025
Shell Gland	2.51	0.89
Shell Gland/Vagina	1.74	0.61

TABLE 7

WHITE LEGHORN

UROPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0	0
Shell Gland	0	0
Shell Gland/Vagina	0	0

COPROPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0.064	0.022
Shell Gland	1.5	0.49
Shell Gland/Vagina	0.032	0

PROTOPORPHYRIN

Tissue	Egg in shell gland	Oviduct empty
	mg/g wet wt	mg/g wet wt
Isthmus	0.19	0.06
Shell Gland	2.12	1.001
Shell Gland/Vagina	.646	0.206

D I S C U S S I O N

Since one of the aims of this thesis is to attempt to elucidate some of the functions of the cells lining the isthmus and shell gland the discussion will be on a functional basis. Where possible each process will be discussed in turn, although it is apparent from the results that some of these occur simultaneously, e.g. plumping and thinning of thick white; calcification and porphyrin deposition to some extent.

Shell membrane formation

The developing egg spends approximately 70 minutes in the isthmus. During this time it acquires two soft membranes, a thick outer membrane and a thin inner membrane which together weigh 143mg and are approximately 70μ thick (Simkiss & Taylor 1971); Masshoff and Stolpmann (1961) suggested that the fibres which intertwine to form the membranes have a central core covered by a mantle of different chemical composition. Simons & Wiertz (1963) have identified the core as ovokeratin. The presence of hydroxyproline and hydroxylysine (Balch & Cooke 1970) however suggests that it is more collagenous in composition. Balch and Cooke (1970) consider the mantle to be glycoprotein complex.

The structure of the membrane fibres is reflected in the material filling some of the gland lumena. Initially however the secretory material is not discretely organised into core and mantle; how this reorganisation comes about is a matter of conjecture. It might be explained by a cross linking of adjacent functional groups on the protein molecules forming an irregular polymer in the centre leaving the less dense carbohydrate sheath on the outside. The material filling the gland lumena originates from the numerous intracellular granules of variable electron density, which according to Khairallah (1966) are a complex of protein and carbohydrate. The present histochemical findings lend support to this view and suggest furthermore that additional carbohydrate derives from the surface epithelial non ciliated cells which contain less secretory material succeeding the passage of the egg. This corroborates the findings of Robinson *et al* (1968).

Since these granules have been observed at all levels of the isthmus, it must be assumed that the entire region is involved in membrane formation. This concurs with the findings of Draper *et al* (1972) and refutes the earlier suggestion of

of/
Leonard (1968) that the shell membranes are secreted specifically by the upper isthmus. The results of Johnston et al (1963) must also be modified in the light of these recent findings, since the granules which they found in the gland cells of the "isthmo-uterine junction" and which Simkiss (1968) implicated in the initiation of crystal formation occur at all levels of the isthmus. The variance in electron density possibly reflects different stages in the formation of one or at most two types of mature protein granule. The present results relating to mamillae formation which will be discussed subsequently suggest that the gland cells play no role in the calcification mechanism.

The present findings are in agreement with those of Khairallah (1966) and Draper et al (1972) as regards the presence of intracisternal granules in the isthmian gland cells, however there is some disagreement as regards their temporal and spatial distribution. According to Khairallah they only occur with an egg in the isthmus, while Draper et al (1972) suggest that they are a constant feature of all the gland cells. In this study intracisternal granules

granules/
were observed in only a proportion of the gland cells but were found at all levels of the isthmus at all stages of the laying cycle.

Granules of this type have also been observed in cells of the exocrine pancreas of the dog (Ichikawa 1965), guinea pig (Palade 1956), chick pigmented retina (Stanka 1971) and more recently in preameloblasts of rat incisors (Kallenbach 1972) and have been interpreted as being the protein precursors of mature zymogen granules (Palade 1956).

One might ask, why only some protein secreting cells should exhibit this phenomenon and not others. Kallenbach (1972) has developed the "protein precursor" theory of Palade (1956) further suggesting that the lumen of the RER in preameloblasts is one of the storage sites for enamel protein and that interference with the transport between RER and Golgi apparatus is one mechanism contributing to this storage phenomenon. As evidence of this block in enamel protein synthesis he remarks upon the vesiculation of the Golgi apparatus in cells with intracisternal granules.

The smooth surfaced vesicles observed in the isthmian gland cells represent according to Draper et al (1972)

(1972)/
continuity between the RER and the Golgi apparatus. Since in the present study vesiculation was only apparent in the Type 2 cell it is feasible that in these cells there is a lack of establishment between the RER and Golgi. Certainly cells of this type rarely contain the more flocculent less electron dense material considered to originate from the Golgi; they do contain secretion granules however; it must be assumed therefore that the initiation of the blocking phenomenon is independent of the state of secretory activity.

If the concept proposed by Kallenbach (1972) is correct then these isthmian gland cells will eventually enter a secretion stage which will unblock protein transport between RER and Golgi, giving rise to the other cell type observed in this region and presumably also to various intermediate stages.

Before the membranes are laid down the albumin is surrounded by a thick envelop of acidic sulphated mucopoly-saccharide secreted by the lower magnum (Solomon 1971). Since the distance between the egg and the membrane fibres in the gland lumena which are not released until an egg is

is/
present, is greater than 100Å, ionic attraction between the albumin and the basic protein in the gland cells cannot account for the release of the latter. It is more likely that the local pressure exerted by the egg on the isthmus squeezes the protein up the tubular glands and out onto the surface of the oviduct; at this level, i.e. less than 100Å, ionic attraction can occur. Light end bonding between the acid and base groups of the albumin and membrane fibres would account for the fairly weak cohesion between these two layers.

Mamillae production

According to Simkiss & Taylor (1971) "the shell is formed from centres of crystallization on the outer surface of the shell membranes". These "centres" are the so-called mamillae, which comprise a protein core surrounded by calcite crystals.

The close structural association between the outer shell membrane and the mamillary core has been reported on several occasions, Tyler & Simkiss (1959), Simons & Wiertz (1963). Robinson & King (1968) extended these observations suggesting that the core represents a specialised form of membrane like

like/
material with disulphide bonds occurring predominantly within the molecular chain rather than between chains.

More recently Cooke & Balch (1970) analysed the chemical composition of the outer shell membrane and mammillary cores and reported the presence of considerably more carbohydrate in the latter.

The present histochemical analyses of the tubular shell gland show that the gland cells in addition to their content of glycogen granules, also reported by Davidson & Draper (1969), contain PAS positive granules. Unlike the isthmian granules however these granules are consistently negative to tests for the presence of protein. It would appear therefore that the gland cells in this region function primarily in the production of carbohydrate. This finding is confirmed also at ultrastructural level; the granules in the tubular shell gland are similar in electron density to the paler granules in the isthmus, which according to Khairallah (1966) represent the carbohydrate moiety of the membrane fibres.

In the light of these findings it is suggested that the tubular shell gland is primarily responsible for the formation

formation/
of the mamillary cores.

The calcite mantle which surrounds the core is generally thought to be produced in the isthmus. This concept has arisen through the work of a number of investigators including Taylor & Hertelendy (1960) and Schraer & Schraer (1965). The latter calculated that during the passage of the egg through the isthmus the concentration of calcium fell from 77mg Ca/g ash to 38mg Ca/g ash and suggested that the nucleation of the crystal seeds around the mamillary cores occurred while the egg was still in the isthmus. However since these authors failed to specify the source of their material the distinct possibility exists that they were measuring calcium levels in the tubular shell gland.

According to Suzuki et al (1959 & 1960) sulphotransferase is present in the isthmus and is associated with the sulphation of carbohydrates present in the true shell (Simkiss & Tyler 1957). However as pointed out by Baker & Balch (1962) the sulphate present in the shell matrix is present as chondroitin sulphate which is not an integral part of mamillary cores and cannot therefore be implicated in the initiation of crystallization.

crystallization./

More recently Robinson et al (1968) observed microcrystals on the outer surface of eggs removed from the isthmus. However the position of the egg within the oviduct before it reaches the shell gland pouch, can be readily altered by a number of extraneous factors such as handling, their findings must therefore be treated with some reserve.

The electron diffraction data presented in this thesis suggests that the isthmus plays no part in calcification since crystals of calcium pyroantimonate were never detected within the cells lining this region. The surface epithelial cells lining the tubular portion of the shell gland on the other hand, including the mitochondrial cells, accumulated crystals at all stages of the laying cycle. It would appear therefore that these cells are the ones primarily involved in the production of the mamillary mantle.

Formation of outer thin white

At oviposition the normal egg consists of the oocyte suspended by chalazae in a mass of albumen, the whole surrounded by two soft shell membranes and a calcite shell. The albumen comprises four distinct layers, viz. the inner chalaziferous layer, a second thin layer, a third thick layer and an outer thin layer. Although most of these layers are secreted by the lower infundibulum and magnum; separation into distinct layers is not apparent until the egg enters the shell gland. Satisfactory explanations have been provided to explain the method of formation of the first three layers (Asmundson 1931 : Scott & Huang 1941). However, the origin of the outer thin white is less well understood although several theories have been proposed. According to Sturkie (1965) the outer thin white is formed by the addition of plumping fluid to the egg after it enters the shell gland. This theory was extended by Leonard (1968) who suggested that the final albumen layer was not merely the result of the addition of fluid, but was the end point in a process of gel-hydration during which the thick white absorbed a large volume of water, swelling as it did before cohesion between the molecules was broken down. Both of these theories are entirely dependent upon physical phenomena,

phenomena,/
however in the light of recent experiments it would appear that the thinning process is the result of the interaction of both physical and chemical forces.

Thick white differs from thin in its high content of ovomucin (Brooks & Hale 1961). Analysis of this glycoprotein has shown it to be a complex of two fractions:

(1) a homogeneous glycoprotein designated α-ovomucin and

(2) a carbohydrate rich fraction designated β-ovomucin (Robinson & Monsey 1971).

In a subsequent paper Robinson & Monsey (1972) reported that the outer thin white in oviposited egg increased in volume as a result of the breakdown of the β-ovomucin fraction. It is assumed that this "breakdown" is enzymatic rather than mechanical although the authors do not expand upon their use of the term.

Two hydrolytic enzymes, namely α-mannosidase and β-n-acetyl glucosaminodase both capable of hydrolysing glycoprotein and thereby reducing its viscosity have been identified in hen egg white (Lush & Conchie 1966). These enzymes are known to occur in high concentration in lysosomes (Du Praw 1968). In the present investigation and in the work of Nevalainen (1969)

Nevalainen (1969)/
lysosomes identified by their content of acid phosphatase were localised in the gland cells of the shell gland pouch region. In this study maximum numbers occurred during the isthmian phase of egg development. Their release from the cells during the first four hours of calcification when egg plumping is known to occur was followed by a corresponding rise in the concentration of shell gland fluid acid phosphatase. The fluctuating plasma concentrations of acid phosphatase which agree with the findings of Taylor et al (1965) as regards peak levels may reflect a state of equilibrium between the plasma and shell gland fluid or they may be, as suggested by these authors, associated with bone cell activity.

Although there is no direct evidence that the hydrolytic enzymes found in egg white originate from lysosomes in the shell gland it is possible that this is their tissue of origin and that their presence in the plumping fluid contributes to the partial liquefaction of the β-ovomucin component and hence to the lower concentration of total ovomucin in the outer thin white.

The observed fluctuations in numbers of lysosomes and mitochondria/

mitochondria/
are not strictly relevant to the process of thin white formation but are best discussed at this point.

It has been appreciated for some time that lysosomes may play some role in cell turnover. Vacuoles (phagosomes) containing fragments of cell organelles including mitochondria have been observed in several tissues including the shell gland pouch region (Nevalainen 1969) although the author equates their presence purely with a calcium deficiency. It is thought that these vacuoles unite with lysosomes which results in the digestion of engulfed material (De Duve and Wattiaux 1966). The present investigation has shown a cyclical variation in the number of lysosomes in the shell gland pouch region in actively laying birds, the peak concentration occurring at a time when mitochondria are few in number. Although there is no direct evidence to suggest that the observed fluctuations are related, the lysosomal degradation of the gland cell mitochondria involving the hydrolytic action of acid phosphatase would account for the decrease in mitochondrial numbers preceding calcification.

It has been postulated that mitochondria may originate

(a) By division (b) Anew from microbodies

(c) From various membrane systems within the cell.

cell./

A comprehensive review of these theories is given by Lehninger (1964). The concept which is favoured is division from pre-existing mitochondria (Luck 1963). In his experiments utilising C^{14}-choline, the author showed that mitochondria grew by addition of new lecithin to the already existing framework. The mitochondria in the gland cells of the pouch region are unique as far as their form is concerned, large bizarre types predominating over the typical oval-round forms characteristic of the rest of the oviduct. An increase in mitochondrial numbers by the mechanism suggested by Luck (1963) would require growth of original form and subsequent subdivision by budding. It may be therefore that the bizarre forms observed in this region are mitochondria undergoing such a process.

The decrease in lysosomal numbers can be explained by their release into the lumena of the gland cells. This corroborates the findings of Nevalainen (1966). The increase in the number of multivesiculated bodies during the latter stages of shell formation observed in this study may be indicative of the subsequent increase in lysosomal numbers since according to Novikoff & Shin (1964), Wood (1965), Ericsson et al (1965) multivesiculated bodies are lysosome precursors.

Plumping fluid

When the egg enters the pouch portion of the shell gland, the two soft shell membranes are fairly loosely applied to the albumen. During its sojourn in this region, the egg increases in size or 'plumps' (Richardson 1935) through the addition of a watery secretion containing dissolved salts.

The precise cells involved in this transport phenomenon have not been unequivocally identified although a number of suggestions have been put forward. Richardson (1935) concluded that the uterine glands (tubular glands of the shell gland) functioned to secrete a thin fluid albumen, while Tyler (1956) less explicitly implicated the distal part of the oviduct in the production of plumping fluid. The time taken for this process is probably about six hours (Warren & Scott 1935 (a) & (b); Burmester, Scott & Card 1939; Bradfield 1951). More recently Draper (1966) suggested that egg plumping was initiated in the lower isthmus and completed in the shell gland.

Taking into consideration the relationship between the ionic composition of plumping fluid and plasma, a measurable potential difference would be expected in association with the transport of fluid from the cells to the lumen of the oviduct. Leonard (1968) detected such a difference 30-40MV in the region

region/
she calls the "lower isthmus" and mentions the presence of "typical ion secreting cells" in this region. It is not clear however to which cells she is referring. There is confusion in terminology concerning the exact position of the junction between isthmus and shell gland and it is likely that the region she calls lower isthmus is in this thesis referred to as the distal tubular shell gland.

Since the addition of this large volume of water (11.3g- Leonard 1968) is a cyclic occurrence, it might be expected that the cells responsible for its transport would show ultrastructural changes concomitant with or preceding the secretory phenomenon. In the present investigation a number of such changes were observed not only in the gland cells lining the distal portion of the tubular shell gland but also in the pouch region of the shell gland during the initial stages of calcification when plumping is known to occur. These variations, viz. pronounced folding of lateral cell membranes and the presence of large numbers of mitochondria are recognised features of ion transporting cells, for example the cells lining the proximal convoluted tubule of the kidney and the parietal cells in the fundic stomach.

In view of the structural similarity between the gland cells lining the distal tubular shell gland and those cells in the pouch region, it is suggested that the secretion of plumping fluid is not confined to the narrow region suggested by Leonard (1968) but is also a feature of the shell gland pouch.

Calcium localisation and transfer

Since the shell gland contains very little calcium at any one time, it has proved difficult in the past to localise this ion in transit. It was hoped that by combining intravenous injection of Ca^{45} with vacuum embedding, difficulties such as translocation during fixation would be surmounted. Unfortunately Ca^{45} injected as a chloride salt has the lowest eutectic point of any naturally occurring salt(-54.9°C.) The equipment used in this experiment, however would only provide a maintainable temperature of -30°C. This would explain the absence of Ca^{45} from apparently well preserved paraffin embedded material. Presumably the same explanation can be applied at ultrastructural level although the poor structural preservation of epon embedded material suggests that the embedding technique may also be at fault.

Considerably more success was encountered using pyro-antimonate as the perfusate. The results of the neutron

neutron/
activation analysis show that potassium pyroantimonate is not only an efficient precipitant, but that it prevents translocation of calcium during processing. It is possible to say with certainty therefore that the small 150Å masses represent local concentrations of calcium and not calcium pyroantimonate that has been precipitated there by chance, and that since they occur only in the surface epithelial cells, the latter are principally involved in the transfer process.

The mitochondrial sequestering of calcium has received much attention in recent years (Hohmann & Schraer 1966; Vasington & Murphy 1962; Lehninger 1966) the research being carried out on isolated mitochondrial fractions. From these investigations and the subsequent studies of Talmage (1970) it is now accepted that the uptake of calcium by mitochondria is one of the cells defense mechanisms to prevent calcium saturation, calcium occurring within the mitochondrial matrix as calcium phosphate. (Rossi & Lehninger 1964; Greenawalt et al 1964).

The shell gland is well vascularised as evidenced by its bright red colour during shell formation. Now if the transfer of calcium ions was occurring via the gland cells, the intravascular/

intravascular/
perfusion of potassium pyroantimonate should precipitate calcium within the gland cell mitochondria, at least during the first two to three hours of calcification when venous total plasma calcium values are fairly high (Hodges 1969). Only the mitochondria in the surface epithelial cells however contain crystalline deposits, thereby substantiating the hypothesis that the surface cells are the principal route of ion transfer.

The calcium necessary for shell formation comes from the diet and when needed from medullary bone under the influence of the parathyroids and ultimobranchial body. Blood calcium is not in a uniform state, however, it occurs in two fractions: a diffusible one which includes ionic calcium and calcium complexed with citrate, phosphate, etc. and a non-diffusible fraction which comprises a protein bound moiety. Analysis of arterial and shell gland venous blood with and without a calcifying egg present, suggest that both fractions contribute to shell formation indicating a state of equilibrium between them (Hunsaker & Sturkie 1961).

In the transfer of calcium from the blood across the epithelium to the egg, two basic mechanisms can be considered, namely simple diffusion and active transport. The former

former/
is dependent upon a higher concentration of calcium ions within the cell compared with the lumen of the oviduct. However in the vicinity of the egg there is likely to be a higher concentration of calcium ions - a necessary pre-requisite if deposition of calcium carbonate is to occur, so the probability of a simple diffusion process occurring across the surface epithelium can be ruled out.

Recent evidence favours active transport involving a carrier. A calcium binding protein was first localised in the intestinal mucosa of vertebrates where its formation was induced by administration of Vitamin D and its presence correlated with the cellular transfer of calcium ions. Kallfelz et al (1967) identified the protein carrier in the soluble phase of the tissue homogenate. Corradino et al (1968) have shown that the mature shell gland also contains a calcium binding protein. It has not been established, however, whether the carrier is surface epithelial in origin or whether it is to be found in the glandular mucosa. In view of its association with calcium ions and the preferential localisation of calcium in the surface epithelial cells it might be expected to share a similar spatial distribution.

Phosphate has also been implicated as a carrier

carrier/
(Ebashi & Lipmann 1962). In the present investigation free, i.e. non membrane bound acid phosphatase was localised in the surface epithelial lining the shell gland during the initial stages of shell formation. Since the prime function of a phosphatase is to hydrolyse phosphate esters it is feasible that the acid enzyme is providing a source of phosphate ions for combination with calcium, or it may be that the active transport of phosphate ions across the surface epithelium is providing the driving force for the movement of the calcium ions (Helbock et al 1966).

The reason for the discrete temporal and spatial localisation of phosphatase in the surface cells of the pouch region and not in similar cells lining the tubular shell gland which are also involved in calcium transfer is not readily understood, but may eventually be explained by a more critical look at the initial slow phase of shell deposition.

Pigment deposition

It has been established in this series of experiments that the quail, White Leghorn, Black Headed Gull and Brown Ranger can all synthesise porphyrins and that the secreted form is protoporphyrin. In view of their autofluorescence in ultra-violet light it would appear that all the cells lining the shell gland pouch and to a lesser extent, the isthmus in the quail, function in the deposition of this polycrystalline pigment. This contradicts the work of Tamura et al (1965) who reported the presence of pigment granules only in the ciliated cells of the shell gland pouch. It is not clear however whether these authors consider the ciliated cells to be responsible for the synthesis of porphyrins or merely the route of transfer.

Measurements of the thick surface pigment layer (cuticular) in the quail and Black Headed Gull suggest that if the surface cells - both ciliated and non-ciliated are responsible for the synthesis of porphyrins, they will have to secrete at least 25% of their own volume at the end of calcification when this cuticular layer is added. This is a conservative estimate since no account has been taken of the pigment present within the calcified matrix. In view of this high percentage it is

is/
highly improbable that the surface cells synthesise porphyrins and even more unlikely that the process is confined to the ciliated cells as suggested by Tamura et al (1965).

The most likely source of the pigment is the blood, in view of its powerful porphyrin synthetic capabilities (Dressel & Falk 1954; Granick & Mauzerall 1958) and since blood flow through the shell gland increases during the calcification process it is feasible that porphyrins derived from the blood are transferred to the egg across the surface epithelial cells - hence their autofluorescence in ultraviolet light.

In the biosynthesis of porphyrins the initial step involves the enzymic condensation of glycine with succinyl CoA to form δ-aminolevulinic acid (A.L.A.). This reaction requires the presence of Vit. B_6 as pyridoxal phosphate (Gibson et al 1958). A pyridoxal - PO_4 - glycine complex combines with succinyl CoA (Kikuchi et al 1958). This condensing reaction which is catalysed by the enzyme A.L.A. synthetase occurs in the mitochondria. The A.L.A. is then

then/
transferred to the cytoplasm. During the phase of shell formation when porphyrin deposition occurs the gland cells are filled with mitochondria and are therefore a rich source of porphyrin precursor. This would explain the persistence of large numbers of mitochondria throughout shell formation in cells not apparently involved in calcium transfer.

With reference to the mechanism of porphyrin transfer across the cell membrane, Cohn (1955) postulated that the porphyrins found in the Harderian gland were transported as a porphyrin/lipid complex. No evidence has been found in the present investigation to suggest a similar mechanism in the surface cells of the shell gland. Porphyrins are cyclic compounds formed by the linkage of four pyrrole rings through methylene bridges and one of their characteristic features is their formation of complexes with metal ions such as iron, magnesium and possibly calcium. In such a complex, the metal ion is co-ordinated in the central position in the molecule and if present will sublime unchanged. The results of the X-ray fluorescence analysis suggest that eggshell

eggshell/
protoporphyrin is not complexed with a metal and so the possibility of the latter acting as a carrier can be ruled out. This is an interesting feature well worth further investigation in view of the acknowledged instability of porphyrins.

Porphyrins are insoluble in aqueous solution, but it is appreciated that they are soluble in weak acidic solutions. It is therefore suggested that during shell formation, the shell gland is perfused with the acid salt of porphyrin arising from the increased blood supply at this time. In view of the high local P^H in the vicinity of the shell it is reasonable to assume that small local precipitations of porphyrin will occur by dissolution of the acid salt.

Towards the end of calcification the P^H of the shell gland becomes more alkaline (El Jack & Lake 1967). This would cause a general precipitation of porphyrin thereby giving rise to the surface pigmentation observed in the quail. The lack of surface pigmentation on the eggs of the White Leghorn and Brown Ranger is probably due to a function of the porphyrin available.

The mechanism outlined above would permit a low concentration of porphyrin within the calcite matrix as has been observed, with in some species a much higher surface concentration. The uniformity of size of the surface crystals together with their high crystalographic symmetry suggests a rapid deposition process,

process,/
which would be expected from this mechanism. The areas of uneven deposition on the surface would be random and probably a coincidence of surface calcite morphology.

Spongy matrix and cuticle

The organic matrix of the shell is a complex of chondroitin sulphate, fat and protein (Simkiss & Taylor 1971). According to Cooke & Balch (1970) the matrix is not evenly distributed throughout the thickness of the shell, but is concentrated about one third of the way into the shell. According to Breen & de Bruyn (1969) the ciliated surface cells lining the shell gland pouch are responsible for the production of the proteinaceous material for the forming egg shell. This hypothesis is based purely on morphological evidence since the granules within these cells which have been observed on many occasions (Johnston et al 1963; Nevalainen 1969; Makita et al 1973) do not stain with any of the routine histochemical tests. The results of the present investigation do not provide the definitive answer, but serve to corroborate the views expressed by Breen and de Bruyn (1969) as regards the potential of the ciliated cell for protein production.

Analyses of the polysaccharide component of the shell show that it occurs in different proportions within different

different/
parts of the matrix. This may be a reflection on the mode of crystal growth rather than the sporadic release of muco-substances from the cells lining the shell gland.

According to Breen and de Bruyn (1969) the non ciliated cells lining the shell gland pouch contain an acid mucopoly-saccharide. The present results do not confirm this finding but suggest rather that sulphated acidic mucosubstances are a feature of the non ciliated surface cells lining the tubular shell gland, this supports the earlier findings of Johnston et al (1963). It is therefore suggested that the chondroitin sulphate moiety of the spongy matrix derives from the tubular shell gland.

The cuticle which is assumed to have a protective function is deposited on the outer surface of the shell and is of variable thickness. It is composed of 90% peptide with galactose, mannose, fucose and hexosamine (Baker & Balch 1962). Cooke & Balch (1970) have since extended these earlier findings to include glucose, an unidentified pentose and sialic acid. Richardson (1935) considered the non ciliated basal cells of the shell gland pouch to be responsible for cuticle formation. Tyler & Simkiss

Tyler & Simkiss/
(1959) and Baker & Balch (1962) in support of Richardson's findings noted that prior to oviposition the basal cells were filled with secreting granules. In the present study maximal activity was recorded in these cells prior to calcification. The reason for the difference between these results and those presented by Tyler & Simkiss (1959) and Baker & Balch (1962) is not known. It is evident however from the present work and the results of Johnston et al (1963) that the non ciliated basal cells in the pouch region contain a carbohydrate component, so from their histochemical reaction they might be considered to be responsible for the formation of the cuticle.

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