Archs oral Biol. Vol. 23, pp. 39 to 44. Pergamon Press 1978. Printed in Great Britain.

THE ULTRASTRUCTURE OF TISSUES AT THE EPITHELIAL–MESENCHYMAL INTERFACE IN DEVELOPING RAT INCISORS

H. J. ORAMS

Department of Dental Medicine and Surgery, University of Melbourne, School of Dental Science, 711 Elizabeth Street, Melbourne 3000, Australia

Summary—The ultrastructure of the basal lamina of the inner enamel epithelium and pre-ameloblast layer together with the adjoining tissues concerned in matrix formation was studied using ruthenium red and phosphotungstic acid staining. An intimate relationship was observed between the banded collagen fibres of the matrix and the fine fibrils, basal lamina, inner enamel epithelium and pre-ameloblasts. Many collagen fibres appeared to reach the basal lamina and an occasional fibre crossed the basal lamina to penetrate a pre-ameloblast; the membrane bound fibre was intracellular but not intracytoplasmic. The close association between pre-ameloblasts, basal lamina, fine fibrils, banded collagen and proteoglycan-like material suggests that the pre-ameloblasts may contribute to matrix synthesis prior to the formation of enamel matrix.

INTRODUCTION

The initiation of matrix formation in developing teeth is a genetically determined event in which the epithelial and mesenchymal elements of the tooth germ interact in a precisely timed sequence of inductive steps. The exchange of inductive information between epithelium and mesenchyme appears to take place across a basement membrane which can be seen with light microscopy to separate the inner enamel epithelium and ameloblast layer from the dental papilla. Huxley (1853) acknowledged the possible role of this membrane in tooth formation by calling it the membrana preformativa whilst Williams (1896) called it an ameloblastic membrane. Tomes (1923) doubted its existence but Orban (1944) described the membrane as thickening just prior to dentine formation, suggesting a possible role in cell induction and matrix formation.

Electron microscopy has resolved a basement membrane adjacent to inner enamel epithelium which is not analogous to that seen with the light microscope. Takuma (1967) described this membrane as an electron-dense line, approximately 25 nm thick, parallel to the distal plasma membrane of the inner enamel epithelium and separated from it by an electron-lucent band also approximately 25 nm wide. The membrane has been called a separating membrane (Nylen and Scott, 1958; Takuma, 1967), a basement membrane (Rönnholm, 1961a; Decker, 1963) and a dense lamella (Pannese, 1962). It is best referred to as the basal lamina to avoid confusion with the basement membrane of light microscopy. The total structure of basal lamina plus zone of fine fibrils which run perpendicular to the lamina is of sufficient width (1000 to 2000 nm) to be readily resolved by light microscopy and is now accepted as the basement membrane or membrana preformativa of earlier histologists. The basal lamina breaks up and disappears as predentine is mineralized (Quigley, 1959; Kallenbach, 1971).

Previous work (Orams, 1974) showed that ruthenium red is selectively localised on this basal lamina in ultra-thin sections of the formative ends of rat incisor teeth, resulting in an electron-dense line approximately 100 to 150 nm wide along the base of the inner enamel epithelial cells. This affinity for ruthenium red indicates a high concentration of protein polysaccharide complexes in the basal lamina.

My aim was to examine further the ultrastructure of the tissues at the epithelial–mesenchymal interface which are concerned in early matrix formation.

MATERIALS AND METHODS

Incisor teeth were taken from fifty 1–2 day old Sprague–Dawley rats of both sexes, bisected and the cervical halves retained for embedding in Spurr's resin for ultra-thin sectioning and examination with a Phillips EM 300 at 60 V. Two-thirds of this material was stained either with ruthenium red (RR) according to the method of Luft (1971) or with phosphotungstic acid (PTA). PTA is selective for protein polysaccharide complexes whilst RR, which is a cation, complexes with these anionic substances and couples them with osmic acid to form electron-dense material at sites of concentration. The remaining third was used as a control and left unstained. All material was fixed by immersion as described below.

For the RR procedure, dissected teeth were directly collected into 2.5 per cent v/v glutaraldyhyde in 0.1 M sodium cacodylate buffer containing 1.0 mM RR and fixed for one hour. After rinsing three times in 0.1 M sodium cacodylate buffer, the teeth were post-fixed in osmic acid—RR solution (2 per cent w/v OsO_4 in 0.1 M sodium cacodylate buffer pH 7.2 plus 1.0 mM RR) for three hours at room temperature.

Control teeth and those for PTA staining were similarly treated but without RR. The teeth were then thoroughly washed in sodium cacodylate buffer, dehydrated, orientated and embedded in Spurr's medium.

During dehydration, 1 per cent PTA was added for those teeth which were to be stained by this method and all RR material was blocked-stained with uranyl acetate. An LKB Pyramitome with target marker was used to select areas in the formative regions of the matrix for ultra-thin sectioning with an LKB Ultrotome III. Some sections were further grid-stained with PTA, uranyl acetate and lead citrate. All electron micrographs were made on 35 mm Kodak Recordak film.

RESULTS

In the selected areas examined, the inner enamel epithelial cells had differentiated to tall columnar cells 10 to 12 μm long and 1 to 2 μm wide with oval nuclei situated at the proximal ends. Chromatin was dispersed as a fine granular material throughout the nuclei, which contained one or two nucleoli. The cytoplasm was packed with ribosomes and contained some rough-surface endoplasmic reticulum. Mitochondria were prominent throughout the cells but were more concentrated at the proximal end.

These cells were identified as pre-ameloblasts because of their morphology and degree of differentiation. They were packed closely together with little intercellular space; desmosomes were observed attaching adjacent cell membranes near the proximal and distal ends. At their proximal ends, the cells rested on a basal lamina which separated them from the underlying mesenchymal tissues. At this stage in development, predentine matrix was forming but no recognisable enamel matrix was present.

RR-treated material showed intense staining of the basal lamina of the pre-ameloblasts, all plasma membranes and forming matrix, indicating a high concentration of protein polysaccharide complexes (Fig. 1). Early matrix between pre-ameloblasts and odontoblasts on the labial aspect of the incisor was composed of numerous dense-staining dots or RR-positive material distributed in a web of lighter staining fine cross-linking fibrils (Fig. 1). The diameter of the dots ranged from 25 nm to 50 nm but the cross-linking fibrils were as fine as 5 to 10 nm in many areas. There were few recognisable collagen fibres in this early matrix but odontoblast processes extended into close proximity to the densely stained basal lamina of the pre-ameloblasts (Fig. 2). In both RR and PTA-stained material, the basal lamina of the pre-ameloblasts and of the inner enamel epithelium was resolved into two laminae: a dark lamina composed of granular material, 50 to 100 nm wide, separated from the plasma membrane by a lucent lamina also 50 to 100 nm wide (Figs. 3 and 4). Numerous fine non-striated 10 to 15 nm thick fibrils streamed away from the basal lamina perpendicularly into the matrix to be interconnected or crossed by similarly stained more randomly orientated fine fibrils (Figs. 3 and 4). In the RR-stained material, darker staining dots were distributed at intervals over these fibrils and appeared in many instances to be junctions of cross-linking.

The fine fibrils appeared to cross the lucent zone and be in contact with the plasma membrane whilst in the opposite direction they passed into the matrix. As matrix formation progressed, the banded collagen fibres increased in numbers and many ran parallel to the fine fibrils, some becoming continuous with the fibrils whilst others contacted the dense lamina (Figs. 4 and 5). These collagen fibres which were forming in the early matrix were distinctly banded and could be traced down between the odontoblasts to the sub-odontoblast layer (Fig. 6). In the RR-stained material, dark staining dots were observed on and in close association with banded collagen fibres as well as in the matrix, but distribution of the dots was random and bore little or no relationship to the periodicity of the banding. Fine fibrils were observed inter-linking the dots and collagen fibres to each other.

An occasional collagen fibre crossed the basal lamina and intruded into a pre-ameloblast, apparently pushing the plasma membrane before it so that the fibre appeared to be lying intra-cellularly but still separated from the cytoplasm by plasma membrane (Figs. 7–9). Serial sections confirmed that the fibres were intracellular but not intracytoplasmic. Careful identification of cell boundaries excluded the possibility that the fibres had intruded between cells.

Examination of the basal lamina of the pre-ameloblasts at higher resolution (up to $\times$ 203,000) showed that the dark lamina and fine fibrils were composed of similarly stained particulate matter of two orders of size: large particles 10 to 15 nm in overall dimension and smaller dense staining particles 1 nm overall dimension (Fig. 10). Small membrane-bound vesicles approximately 100 to 200 nm in diameter were observed both in the distal ends of the pre-ameloblasts and in the forming matrix, many in close proximity to the basal lamina (Figs. 1, 3 and 11).

In more mature matrix with abundant collagen, the lamina densa was no longer apparent and the plasma membrane of the basal ends of the pre-ameloblasts was obscure and extensively infolded. However, a band of fine fibrils, approximately 1 μm wide and similar to that already described persisted between the pre-ameloblasts and the collagenous matrix.

DISCUSSION

The intimate association between basal lamina, fine fibrils and forming collagen, the presence of membrane-bound vesicles in close proximity to the basal lamina, and the similarity in structure and staining characteristics between the basal lamina and fine fibrils suggests that the pre-ameloblasts contribute to matrix synthesis prior to the appearance of recognizable enamel matrix. Although pre-ameloblasts are not fully differentiated cells and do not contain the mass of rough-surface endoplasmic reticulum seen in secreting ameloblasts, they do contain sufficient rough-surface endoplasmic reticulum and other organelles to be consistent with a capability to synthesize and secrete protein. Cell differentiation is a gradual process and, once organelles appear, it is reasonable to suppose that they commence to function. The membrane-bound vesicles observed in the distal ends of pre-ameloblasts and in the forming predentine matrix in close proximity to the basal lamina are similar in size, morphology and staining characteristics to ameloblastic bodies which are believed to be secretion granules of enamel (Frank and Nalbandian, 1967). The appearance of these vesicles in the

predentine matrix close to the basal lamina suggests that secretion is already taking place prior to overt enamel formation. The nature of such secretion is obscure but Weinstock (1972) demonstrated the biosynthesis of enamel proteoglycans by fully differentiated ameloblasts and it seems reasonable to suggest the possibility of an earlier secretion of proteoglycans into the forming predentine matrix, perhaps to embed and cross-link the forming collagen fibres.

Other evidence substantiates the presence of proteoglycans and related substances within predentine matrix. For example, the RR-positive dots and fine fibrils I have described are similar in morphology, staining characteristics and size range to those observed in the matrix of epiphyseal cartilage and identified as proteoglycans (Eisenstein *et al.*, 1971; Thyberg *et al.*, 1973; Silbermann and Frommer, 1974). Nagai *et al.* (1974) observed RR-positive granules linked by fine filaments in the dentine and predentine of developing rat molars and noted that the granule count correlated with the concentration of acid mucopolysaccharide in the dentine and predentine. These granules and fine filaments were of similar order of size to those I describe. Biochemical analysis (Jones and Leaver, 1971) has shown chondroitin -4-sulphate as the major glycosaminoglycan in dentine with chondroitin -6-sulphate, hyaluronic acid, dermatan sulphate and a non-sulphated galactosaminoglycan as minor constituents. Holbrook and Leaver (1976) identified phosphoprotein in dentine together with glycoproteins different from those in other mineralized tissues.

The presence of banded collagen fibres within preameloblasts is unexpected but a similar finding was reported by Ten Cate and Porter (1975) in respect of fibroblasts of periodontal ligament. They interpreted the presence of banded collagen fibres within membrane-bound compartments in fibroblasts as due to a process of degradation and not synthesis, in which fibroblasts were actively remodelling the collagen fibres during tooth formation and eruption. Pre-ameloblasts may be engaged in a similar role in matrix remodelling. The fact that only a few fibres were seen within cells would be consistent with this speculation because it is unlikely that much collagen would be produced during matrix synthesis and preameloblasts perhaps only remove an occasional fibre that protrudes beyond the main mass.

On the other hand, the possibility of synthesis of some collagen fibres by pre-ameloblasts cannot be excluded. Although the fibres were intracellular, they were extra-cytoplasmic, i.e. they protruded into the cell but did not breach the plasma membrane, which appeared to be pushed ahead of the fibre. This is consistent with the accepted view that in collagen synthesis banded fibres are assembled outside the plasma membrane from tropocollagen units synthesized within the cell. Collagen synthesis is no longer regarded as the exclusive preserve of mesenchymal cells and its synthesis by epithelial cells has been demonstrated (Trelstad, 1971; Carlson, 1973). Trelstad and Slavkin (1974) have shown that isolated tooth germ epithelium may produce collagen *in vitro*.

Another possibility is that the intimate relationship between tissues at the epithelial–mesenchymal interface is concerned with the inductive events during tooth formation. Slavkin (1972) has made a strong case for the role of membrane-bound RNA-containing vesicles as chemical messengers in the epithelial–mesenchymal interaction during odontogenesis, but this does not take account of the fine fibrils which cross the basal lamina to reach the plasma membrane on the epithelial side and which appear to become continuous with collagen fibres on the mesenchymal side. These fibres are part of early matrix formation but the question arises whether their contact with the plasma membrane of pre-ameloblasts is a manifestation of synthesis by these cells or of an inductive role by them. The synthesis hypothesis seems easier to accept than the inductive one.

Acknowledgements—I wish to thank Professor Peter Reade, Chairman, Department of Dental Medicine and Surgery for his advice in the preparation of this paper, Miss Deborah Seward for technical assistance in electron microscopy and Mr. Tadeus Dobrostansky and Miss Anne Williams for photographic assistance. The work was supported by the National Health and Medical Research Council (Australia)—Grant No. 233152.

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Plate 1.

Fig. 1. Matrix composed of electron-dense dots cross-linked by fine fibrils. Formative end of rat incisor stained with ruthenium red. Pa = pre-ameloblast, Bl = basal lamina, M = matrix, Od = odontoblast, D = dot of RR-positive material, Cf = cross-linking fibrils, Vs = vesicle. × 21,170

Fig. 2. Odontoblast process extending to basal lamina. Formative end of rat incisor stained with phosphotungstic acid. Pa = pre-ameloblast, Od = odontoblast process, Bl = basal lamina. × 21,170.

Fig. 3. Basal lamina composed of dense lamina and lucent lamina. Fine fibrils stream away perpendicularly to basal lamina. Formative end of rat incisor stained with phosphotungstic acid. Pa = pre-ameloblast, Vs = vesicle, Ll = lucent lamina, Dl = dark lamina, f = fine fibrils, Od = odontoblast. × 10,585.

Fig. 4. Collagen fibres merging with fine fibrils close to basal lamina. Formative end of rat incisor stained with phosphotungstic acid. Pa = pre-ameloblast, Ll = lucent lamina, Dl = dark lamina, C = collagen fibre, f = fine fibril. × 21,170.

Fig. 5. Fine fibrils perpendicular to basal lamina and merging with collagen fibres. Some collagen fibres reach the basal lamina. Formative end of rat incisor stained with phosphotungstic acid. Pa = pre-ameloblast, Bl = basal lamina, f = fibril, C = collagen fibre. × 21,170.

Fig. 6. Collagen fibres in the sub-odontoblast layer passing between the odontoblasts to reach the matrix. Pa = pre-ameloblast, C = collagen fibres, Od = odontoblast, S = sub-odontoblast cell. × 4763.

Plate 2.

Fig. 7. Collagen fibre which has crossed basal lamina and is penetrating a pre-ameloblast. Formative end of rat incisor stained with ruthenium red. Pa = pre-ameloblast, Bl = basal lamina, M = matrix, C = collagen fibre, Pm = plasma membrane. × 21,170.

Fig. 8. Banded collagen fibre crossing lucent lamina and penetrating pre-ameloblast, stained with ruthenium red. Pa = pre-ameloblast, C = collagen, Pm = plasma membrane. × 93,600.

Fig. 9. Collagen fibre lying intra-cellularly and enclosed by plasma membrane. Stained with ruthenium red. Pa = pre-ameloblast, C = collagen fibre, Pm = plasma membrane. × 93,600.

Fig. 10. Basal lamina of pre-ameloblast at high magnification. Formative end of rat incisor stained with ruthenium red. Pm = plasma membrane, Ll = lucent lamina, Dl = dark lamina, f = fine fibrils, P = particulate matter. × 146,160.

Fig. 11. Membrane-bound vesicles close to basal lamina of pre-ameloblasts. Basal lamina has become indistinct. Pa = pre-ameloblast, Bl = basal lamina, Vs = vesicle, C = collagen fibre. × 21,170.

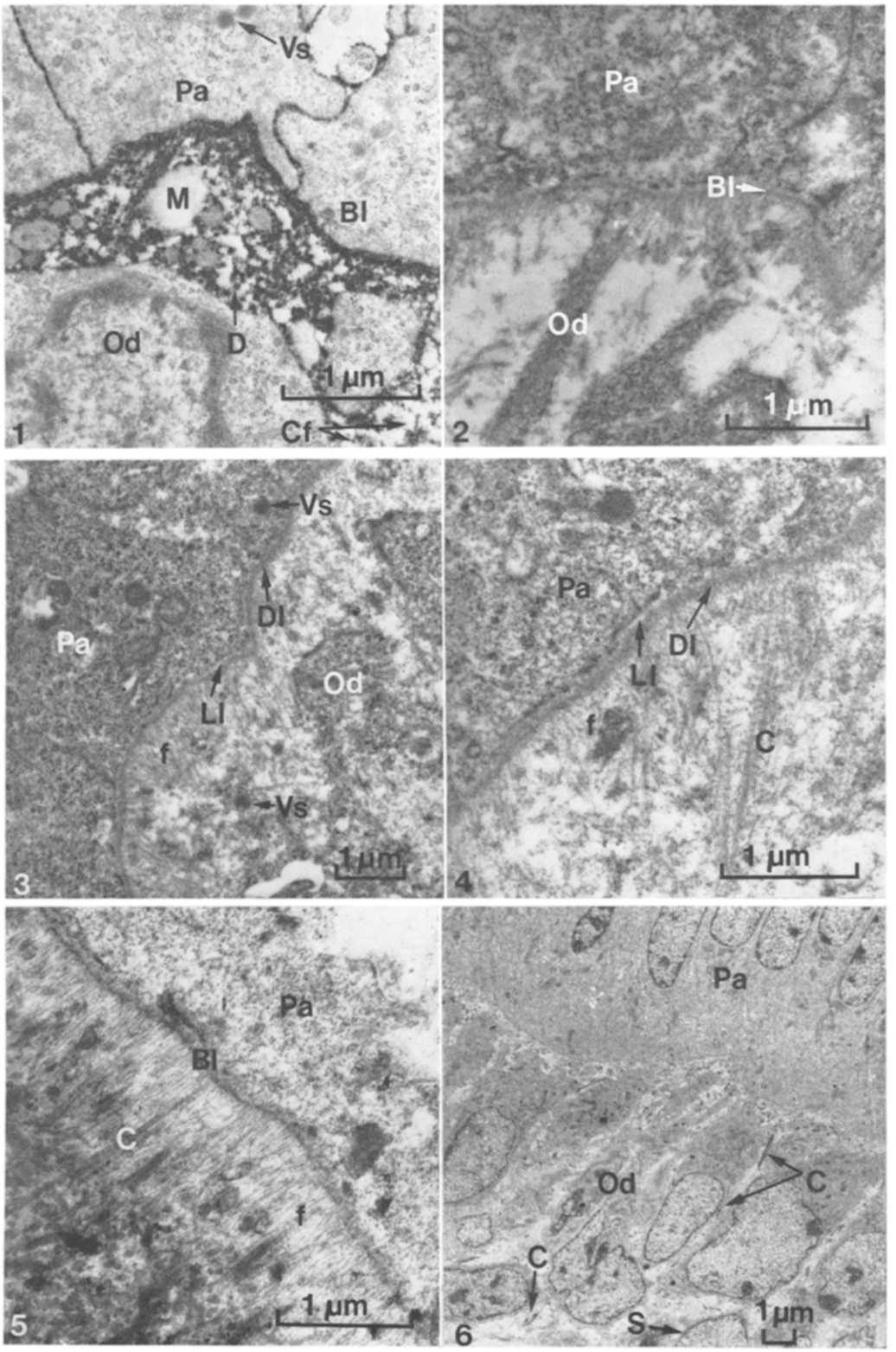
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Plate 1

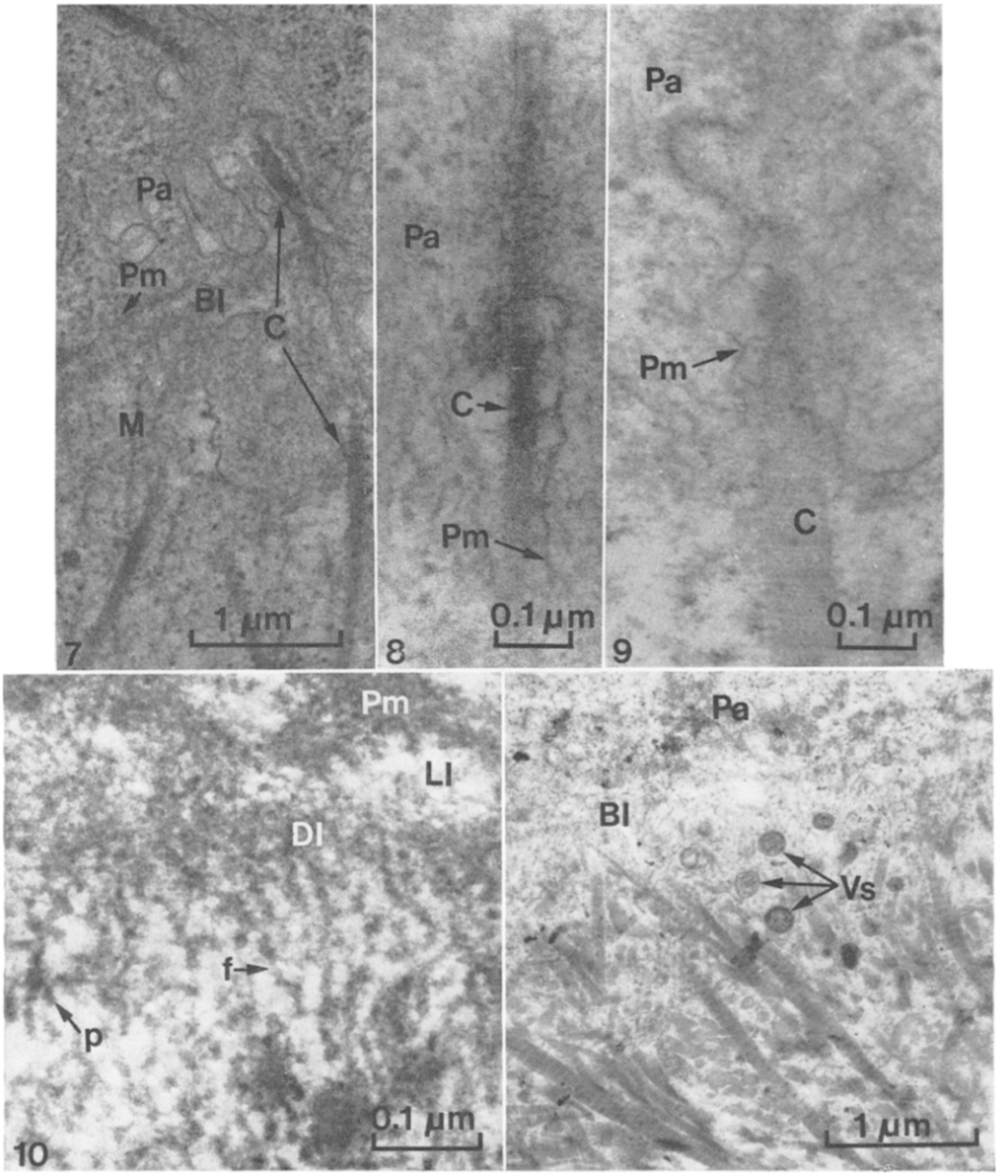
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Plate 2