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Disintegration of the Junctional Epithelium of Human Fetal Hard Palate*

Martin Matthiessen

Laboratory of electron microscopy

Helge Andersen

Laboratory of cyto- and histochemistry
Anatomy Department A, University of Copenhagen, Universitetsparken 1, Denmark

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Summary. An ultrastructural and a histochemical study of the disintegration of the human fetal palatinal junctional epithelium was carried out.

Special attention was focused both on the epithelium proper as well as on participation of the surrounding mesenchyma.

Epithelial autophagia was noticed in the form of inclusion bodies with cellular remnants as well as general cellular disintegration. The disintegration was correlated to the cellular activity of acid phosphatase and AS-esterase. The differences between human and non-human material were recorded and discussed.

In the surrounding mesenchyma, histiocytes (macrophages) were noticed participating in the epithelial disintegration, while ordinary mesenchymal cells seemed without importance.

The study of activity of alkaline phosphatase reveals that the rapidly growing ossification center of the vomer was touching the superior aspect of the epithelial junctional seams, where the epithelial disintegration starts.

Based upon the findings the following sequential steps of disintegration were discussed: 1) pressure from the outside (the vomer anlage), 2) epithelial autophagia and 3) heterophagia of epithelial remnants (invading histiocytes).

The ultrastructure and histochemistry of the so-called epithelial pearls were described.

The intercellular substance of the palatinal processes was found to consist of hyaluronic acid and of chondroitin-4- and/or -6-sulfate. The mutual ratio of the glycosaminoglucuronoglycans was discussed.

Key words: Embryology — Epithelial autophagia — Histocytochemistry — Human palate — Macrophages — Ultrastructure.

Introduction

Recently, three papers dealing with the development of the human fetal palatinal processes were published (Andersen and Matthiessen, 1966, 1967, 1968). In these papers special attention was focused on the participation of histiocytes (macrophages) in the process of disintegration of the junctional palatinal epithelium, which took place in fetuses of 38–60 mm crown-rump length (CRL). Meanwhile, it is still unsettled if histiocytes are the primary factor in the disintegration or if other additional factors are operating. Andersen and Matthiessen (1967) discussed the possibility that pressure from the rapidly growing ossification cetner of the vomer may be such a factor, since the disintegration starts in the junctional

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epithelium between the nasal septum and the fused palatinal processes, after which it spreads to the more orally situated parts of the junctional midline seam. No distinct cellular changes were recorded in the junctional epithelium prior to the disintegration.

In an attempt to clarify the sequential steps in the disintegrative process further investigation of the junctional epithelium and surrounding mesenchyma was carried out both from an electron microscopical as well as from a histochemical point of view.

Material

The material was supplied by legal abortion and comprises a total of 16 human fetuses ranging from 20 to 99 mm in CRL [20, 22, 28, 38 mm (2), 39 mm (2), 41, 43 mm (2), 44, 52, 59, 61, 92, and 99 mm], all measurements being made on the unfixed fetuses. The post-mortem interval preceding fixation was, with a few exceptions, less than 30 min. According to Andersen (1970), an interval of up to 1 hr is acceptable when using the histochemical methods performed in this study. The material [39 mm (2), 43, 44, 99 mm CRL] intended for electron microscopy was fixed within 10 min after removal of the fetus from the uterine cavity.

Methods

Fixations and Preparation

For electron microscopy the midline of the palates was removed immediately following the abortion and the specimens were cut into small pieces while immersed in the fixative. Fixation was carried out in icecold glutaraldehyde (2.5% in 0.1 M sodium cacodylate, pH = 7.4) for 45–120 min. Following a thorough wash in the buffer, the specimens were postfixed 1–2 hrs in icecold 1.33% OsO_4 buffered with 0.1 M sodium cacodylate, pH = 7.4. The specimens were then dehydrated in graded ethanol and embedded in Epon 812 omitting the use of propylene oxide to avoid extraction of lipids (Buschman and Taylor, 1971).

Other specimens were fixed in 1.33% icecold OsO_4 in 0.067 M collidinbuffer (pH = 7.4) for 1–2 hrs, after which they were dehydrated in graded ethanol. The specimens were then transferred to propylene oxide for 30 min and through graded mixtures of propylene oxide and Epon 812 to the embedment in Epon 812.

One micron survey sections stained with toluidine blue formed the basis of the selection of areas for thin sectioning. The thin sections (600–800 Å), were picked up on grids coated with parlodion and carbon and double contrasted with uranyl acetate and lead citrate.

For the histochemical study the heads of the fetuses were quickly removed as were the lower jaws in order to facilitate the diffusion of fixatives into the palatinal tissue. Icecold fixation was performed with the following fixatives: 1) Ethanol-formalin-acetic acid (pH = 3.7) (Lillie, 1965) was used for general morphology, for the preservation (possibly fixation) of glycogen, and for the fixation of heteroglycans, glycoproteins and nucleoproteins. This fixative mixture is well suited for preparation of fetal material (Andersen, 1964). 2) Icecold formol-calcium (Pearse, 1968). For paraffin sections a fixation time of 24 hrs was used. For the investigation of hydrolytic enzyme activities a fixation time of 5 min was used in the case of alkaline phosphatase, and 4 hrs in the case of acid phosphatase and naphthol-AS-esterase (Casselman, 1959, Andersen *et al.*, 1970). Following fixation, the tissue intended for enzyme studies was stored in icecold gumarabic-suc-

rose until sectioned. After initial cooling in a CO_2-expansion cooler with a covering device, 4–6 micron sections were cut on a Pearse-Slee cryostat (type HR) at $\div 25°$ C.

Histochemical Methods

1. Metachromatic staining with 0.1% toluidine blue (Merck) in 30% ethanol (Kramer and Windrum, 1955), staining time: 1–10 min.

2. Metachromatic staining with 0.1% toluidine blue in Walpole buffer (pH = 1.0), staining for 1–10 min. In some sections a preliminary oxidative deamination with ninhydrin was performed to prevent the competitive effect of proteins in the metachromatic staining of sections at low pH-values (Andersen and Ehlers, 1967).

3. Alcian blue (G. T. Gurr), (0.05%)—critical electrolyte concentration method (Scott and Dorling, 1965, Scott and Stockwell, 1967, Scott *et al.*, 1968).

4. Alcian blue (0.5%) in 3% acetic acid (pH = 3.0) (Mowry, 1960).

5. Alcian blue (0.1%) adjusted to pH = 1.0 by HCl. Staining time 30 min.

6. Periodic acid-Schiff. This method was used with de Tomasi's and with Barger's and de Lamater's Schiff reagents (Pearse, 1968). Periodic acid was used as 0.5% and 1% aqueous solutions. Prior to the treatment with periodic acid, some sections were coated with celloidin in order to avoid loss of glycogen due to the periodic acid. As a control, maltase digestion (Merck) was performed (Pearse, 1960).

7. Bennett's 1-(4-chloromercuriphenylazo)-2-naphtol (Mercury Orange) method for sulphydryl groups (Bennett and Watts, 1958).

8. Gallocyanin-chromalum staining for RNA (Pearse, 1968).

9. The naphthol-AS-BI-phosphate method for alkaline phosphatase using Fast red violet LB as diazonium salt (Burstone, 1958, 1962) at room temperature.

10. The naphthol-AS-BI-phosphate method for acid phosphatase (Burstone, 1958, 1962). Same diazonium salt as above. Incubation at 37° C.

11. The naphtol-AS-acetate method for esterase (Burstone, 1958, 1962). Fast red RC as diazonium salt. Incubation at room temperature.

By the use of several series of sections it was possible to determine the incubation time required for the visual, initial activity of the named enzymes in the different cells of the sections (Andersen *et al.*, 1970). This eliminates the more problematic use of the symbols + and ++ for "maximal" activity. Furthermore, the series of sections limited the error caused by unavoidable changes in thickness of the frozen sections.

Results

The appearance and elevation of the palatinal processes and their fusion with the nasal septum has recently been described by Andersen and Matthiessen (1967). The fusion is essentially completed in the primordium of the hard palate at 36–38 mm CRL.

Histochemistry

Before and during the fusion the epithelium of the palatinal processes shows mitotic activity. The cells have a high content of glycogen, a moderate content of

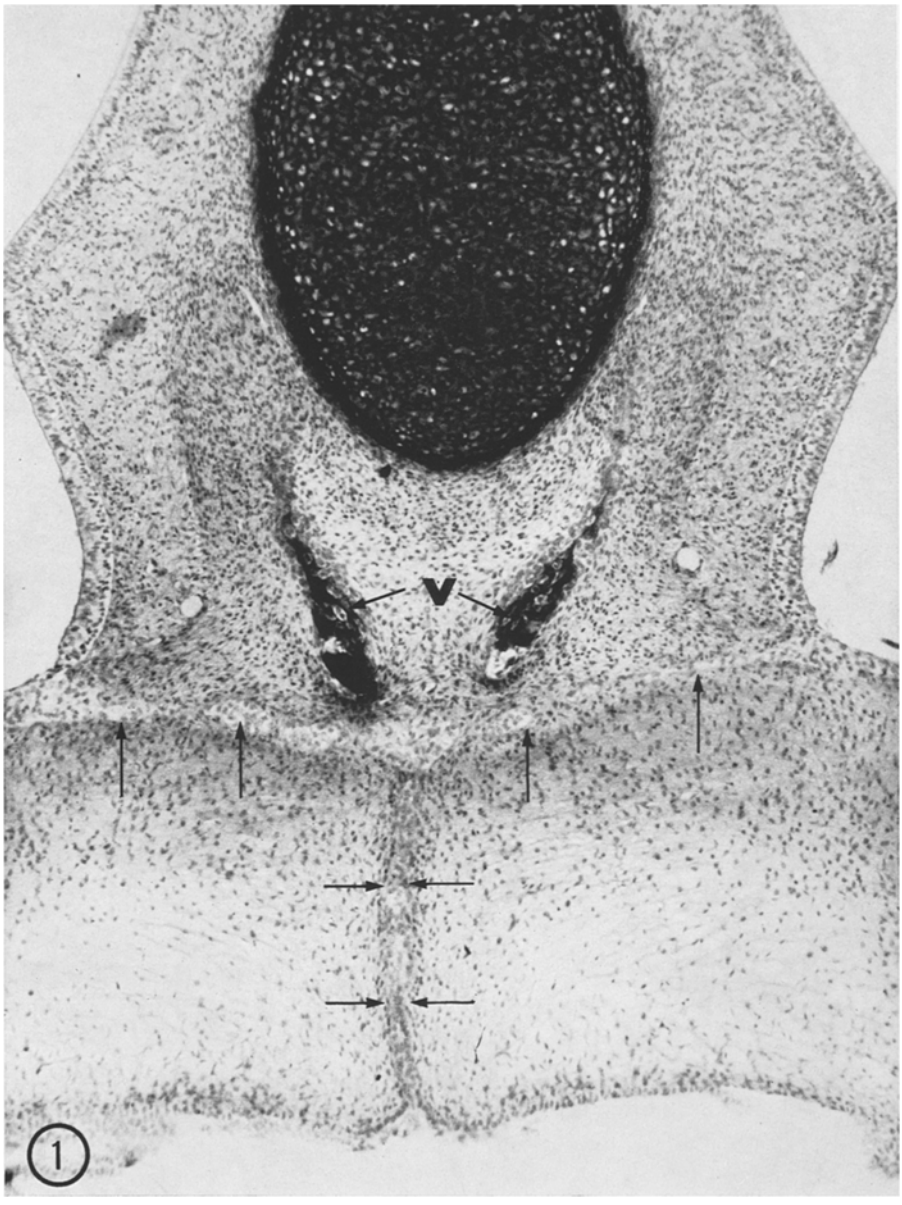


Fig. 1. Transverse section from a fetus of 43 mm CRL showing the epithelial T-formed junctional seam (arrows). *v* endesmal ossification center of the vomer. ×100

RNA, and show an uniform cytoplasmic activity of acid phosphatase and AS-esterase after 10 min of incubation. No activity of alkaline phosphatase is observed. A thin surface cell coat is noticed on each side of the nasal septum by staining with Alcian blue at pH = 3.0 and by staining with Alcian blue in the

presence of low $MgCl_2$ concentration (0.2 M). On the lower aspect of the nasal septum no definite cell coat is seen. The same is the case for the palatinal epithelium.

After the fusion transverse sections show the junctional epithelium in the form of a T (Fig. 1). The width of the epithelial seam amounts to 3–4 cells.

Prior to the fusion of the nasal septum with the palatinal processes, small cytoplasmic basophilic corpuscles are seen in some epithelial cells of the lower aspects of the nasal septum covering the vomer anlage. At this stage of development (CRL = 38 mm) similar corpuscles are not seen in the junctional epithelium between the two palatinal processes.

When the fusion occurs between the nasal septum and the two palatinal processes, the activity of alkaline phosphatase reveals that the preosteoblasts of the anlage of the vomer touch the upper aspects of the horizontal part of the T (Fig. 2).

Simultaneously with the vascularisation of the palatinal processes during the early stages of development, a new type of cell is seen among the ordinary mesenchymal cells in the palatinal processes. These cells have an ample, often vacuolized cytoplasm with delicate, PAS-positive maltase resistant granules and show a marked nodular, cytoplasmic activity of acid phosphatase and AS-esterase, within 5 min of incubation (Figs. 3 and 4). Similar cells are noticed in the nasal septum.

After the process of fusion these cells are seen close to the junctional seams (Fig. 4). In that position large, round, basophilic corpuscles are often seen in their cytoplasma.

Staining with toluidine blue at a pH-value >4.0 shows pronounced metachromasia in the palatinal mesenchyma. However, no metachromasia is noticed at pH = 1.0, even after oxidative deamination with ninhydrin. Staining according to the Alcian blue—critical electrolyte concentration method shows strong alcianophilia in the mesenchyma at low concentrations of $MgCl_2$ (0.2 M), but at 0.6 M $MgCl_2$ the alcianophilia is extremely reduced, especially in the orally situated regions. On the other hand the chondrogenous center in the walls of the nasal cavity still shows distinct alcianophilia (disappears at 0.7 M $MgCl_2$). Staining with 0.1 % Alcian blue at pH = 1.0 gives a very faint reaction in the mesenchyma, while the mesenchyma shows a rather strong alcianophilia at pH = 3.0.

In the cells of the junctional seams nodular basophilic corpuscles appear most pronounced in the horizontal part and pari passu with the appearance of the corpuscles some of the cells demonstrate a more coarse nodular activity of acid phosphatase and AS-esterase (Fig. 3). Compared to the activity seen within 5 min of incubation in the above mentioned mesenchymal cells, the activity on the epithelial cells is moderate and appears after 10 min of incubation.

In the following stages of development lacunar formations are seen in the junctional epithelium containing the mesenchymal cell type with the strong activity of acid phosphatase (Fig. 4) and AS-esterase.

The lacunar formations are first noticed in the horizontal part of the junctional T, which are then converted to isolated islets of epithelial cells. Gradually, the lacunar formation includes the midline seam. These islets usually disappear, but in some cases islets persist in the orally situated part of the midline seam. Here

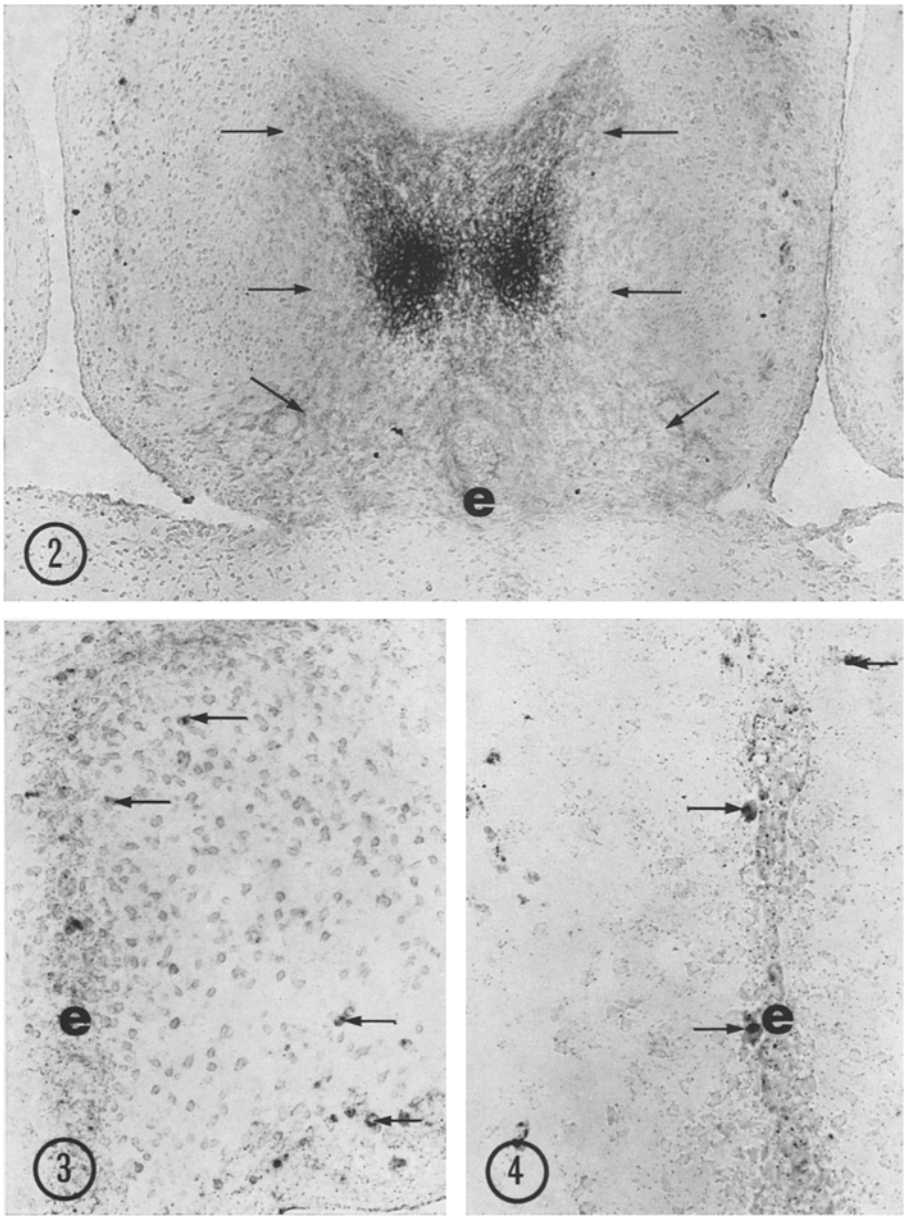


Fig. 2. Alkaline phosphatase activity in the osteoblasts and preosteoblasts of the vomer anlage (arrows). *e* horizontal part of the junctional epithelial seam. ×90

Fig. 3. AS-esterase activity in histiocytes (some marked with arrows) and in the junctional seam (*e*). ×200

Fig. 4. High activity of acid phosphatase in histiocytes (some marked with arrows) and weak activity in the junctional midline seam (*e*). ×200

they often undergo considerable growth, which converts them to concentric laminated structures or so-called pearls.

In the peripheral part of the pearls the cells often show mitosis and contain glycogen and RNA. In the more central part of the pearl the cells are loaded with glycogen and form concentric lamellae in which dense nuclei are seen. With mercury orange staining for SH-groups, the laminated pearls only show reaction in the peripheral cell-layer and at the borderline between the concentrically arranged central cells. In strength the reaction corresponds to the reaction seen in the ordinary oral and nasal epithelium.

Another feature of the epithelial pearls is the condensation of collagen fibrils just beneath the basement membrane. This is not seen beneath the basement membrane of the early junctional epithelium.

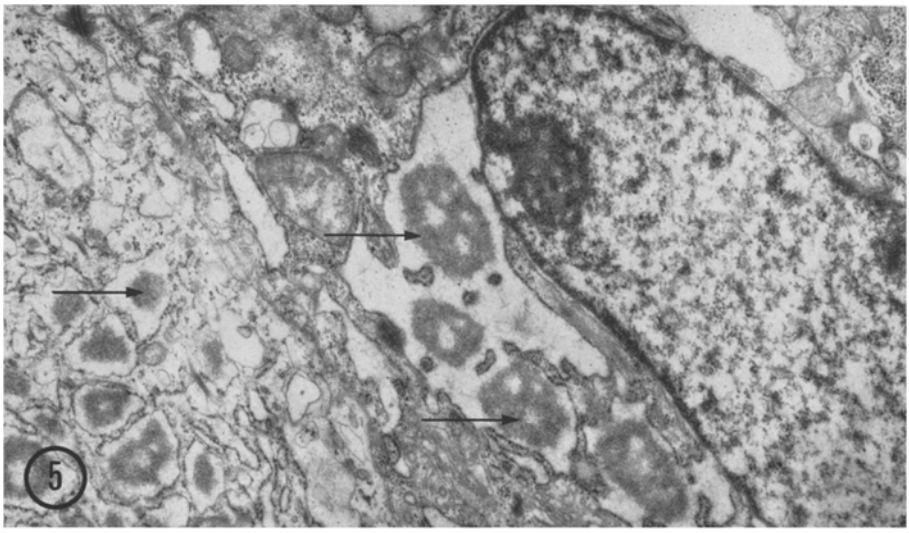
Electron Microscopy

The description that follows deals with the fate of the midline part of the junctional seam.

At the 39 mm CRL-stage the palatinal oral epithelium is bilaminar. The basal cell layer shows round nuclei with nucleoli. The cytoplasm contains mitochondria situated perinuclearly, and supranuclearly a moderate developed Golgi complex is seen. Only a few profiles of a rough-surfaced endoplasmic reticulum are noticed, while polyribosomes and small dark lysosome-like bodies are found scattered in the cytoplasm. Ample glycogen is seen supranuclearly as well as infranuclearly. The borderline between the epithelium and mesenchyma is even. The basal plasma membrane is unfolded, and weakly developed half desmosomes are seen. Laterally, the cells show interdigitations while the plasma membrane facing the surface cell layer shows several desmosomes. The superficial cell layer is flattened parallel to the surface. The nucleus is somewhat condensed and the supranuclear part of the cytoplasma contains few mitochondria and a small Golgi complex. The cells contain large amounts of glycogen. Microvilli are present at the surface.

At the 39 mm CRL-stage the midline junctional seam shows cells with a general morphology in accordance with the cells described above, while other cells show different variations in ultrastruture. Some of the latter are extremely loaded with glycogen, while others show a rough-surfaced endoplasmic reticulum, which is extremely distended with a flocculated dark material (Fig. 5), and the cells also show a well developed Golgi complex. Other cells show varying sizes of membrane limited inclusion bodies containing glycogen, lipids, remnants of distended rough-surfaced endoplasmic reticulum and of mitochondria as well as tonofibrils (Fig. 6). Finally some cells show varying degree of disintegration and shrinkage including the nucleus which shows pyknosis (Figs. 7 and 8). At 39 mm CRL the basal plasma membrane only shows weakly developed half desmosomes and, beneath the lamina basalis, only a few collagen fibrils are seen. At the 44 mm stage half desmosomes are well developed and there is a considerable increase of collagen fibrils beneath the lamina basalis.

In the surrounding mesenchyma two different types of cells are noticed. One of them is an ordinary mesenchymal cell with slender cytoplasmic projections. The nucleus is rounded with several nucleoli, and the cytoplasm contains profiles of rough-surfaced endoplasmic reticulum among which scattered polyribosomes



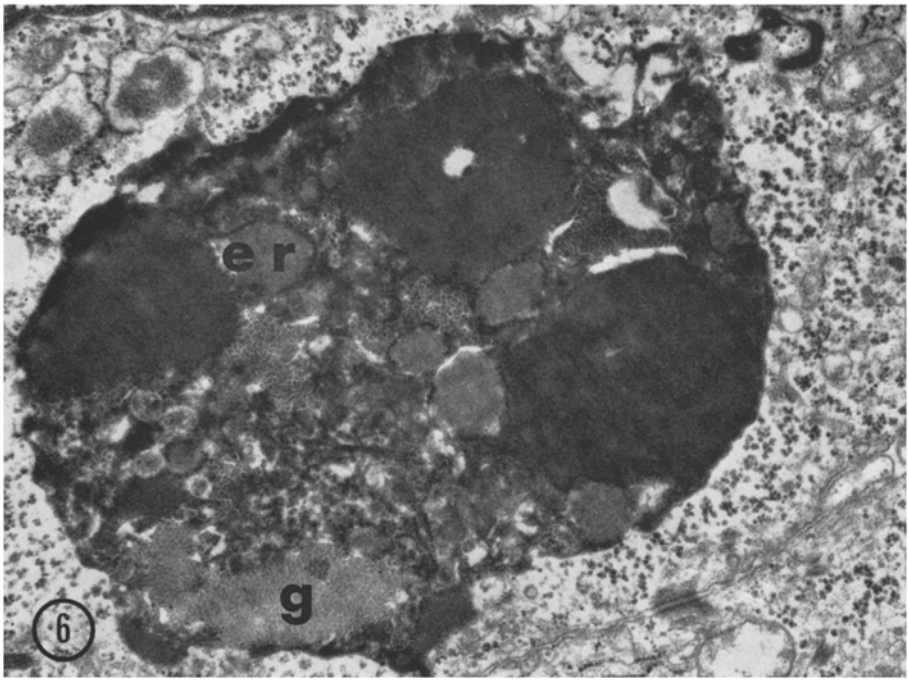


Fig. 5. Epithelial midline seam-cells with distended rough-surfaced endoplasmic reticulum with a flocculated dark material (arrows). ×10500

Fig. 6. Epithelial cell containing an autophagic vacuole with glycogen (*g*) and distended rough-surfaced endoplasmic reticulum (*er*). ×24800

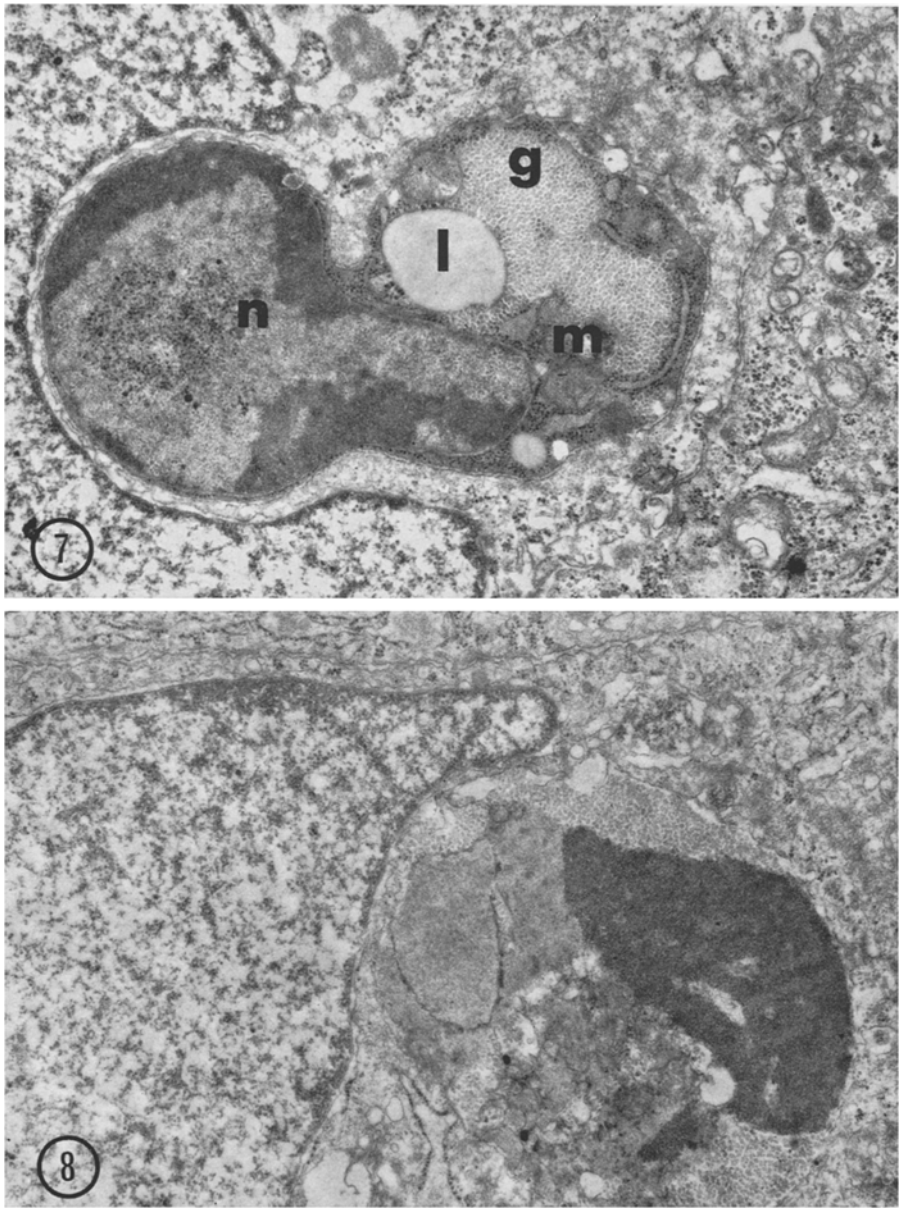
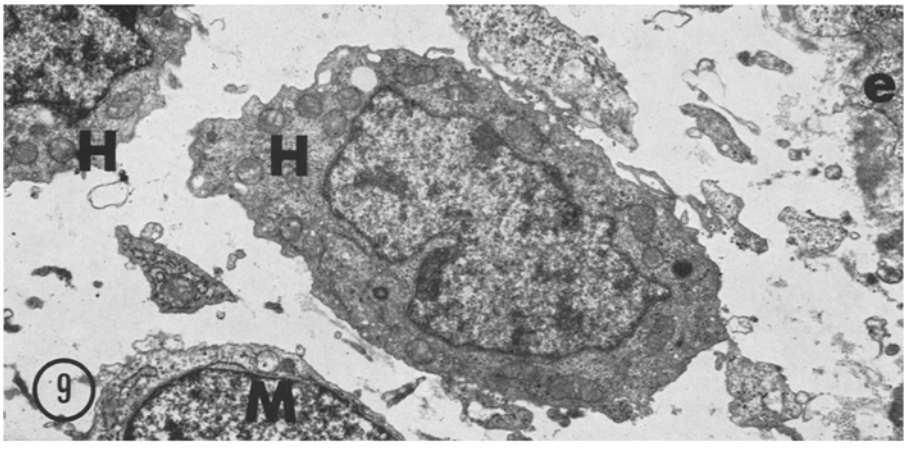


Fig. 7. Disintegrating epithelial cell with nuclear pyknosis (*n*). *g* glycogen, *l* lipid droplet, *m* mitochondrion. The localization of the disintegrating cell in a nuclear indentation may either represent an epithelial heterophagia or a neighbouring epithelial cell extending partially around the disintegrating cell, contributing to its translocation. ×20400

Fig. 8. Represents a more advanced stage than shown in Fig. 7. ×20400



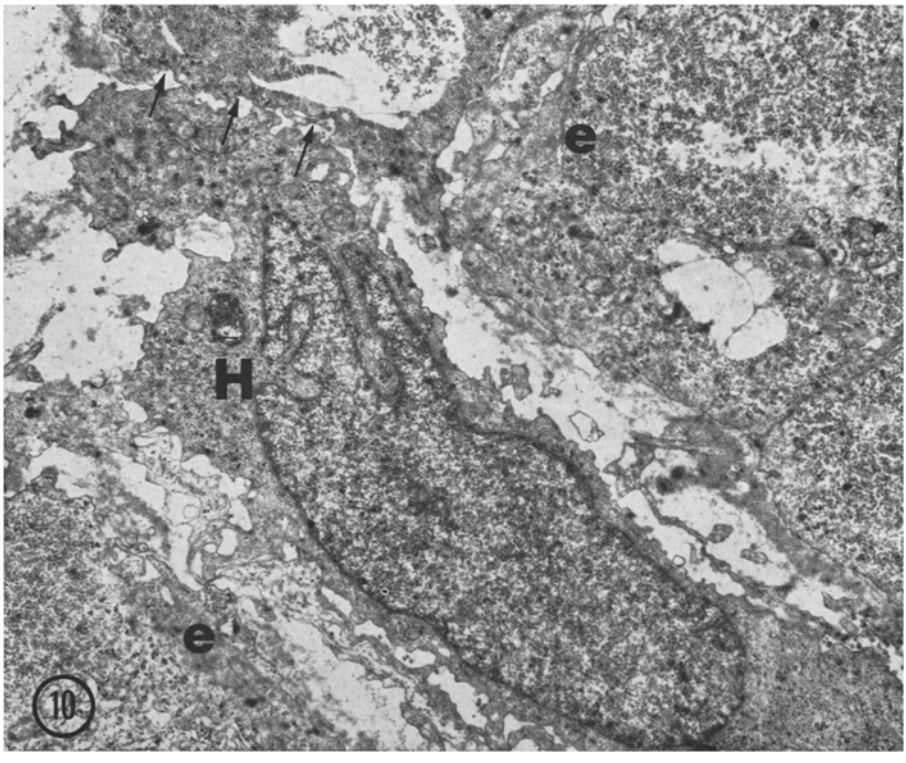


Fig. 9. Histiocytes (*H*) and an ordinary mesenchymal cell (*M*) close to the midline epithelial seam (e). ×8300

Fig. 10. Histiocyte (*H*) in lacunar formation of the midline epithelial seam (*e*). The basal lamina is not visible in the area of contact (arrows) between histiocyte and epithelium. ×11000

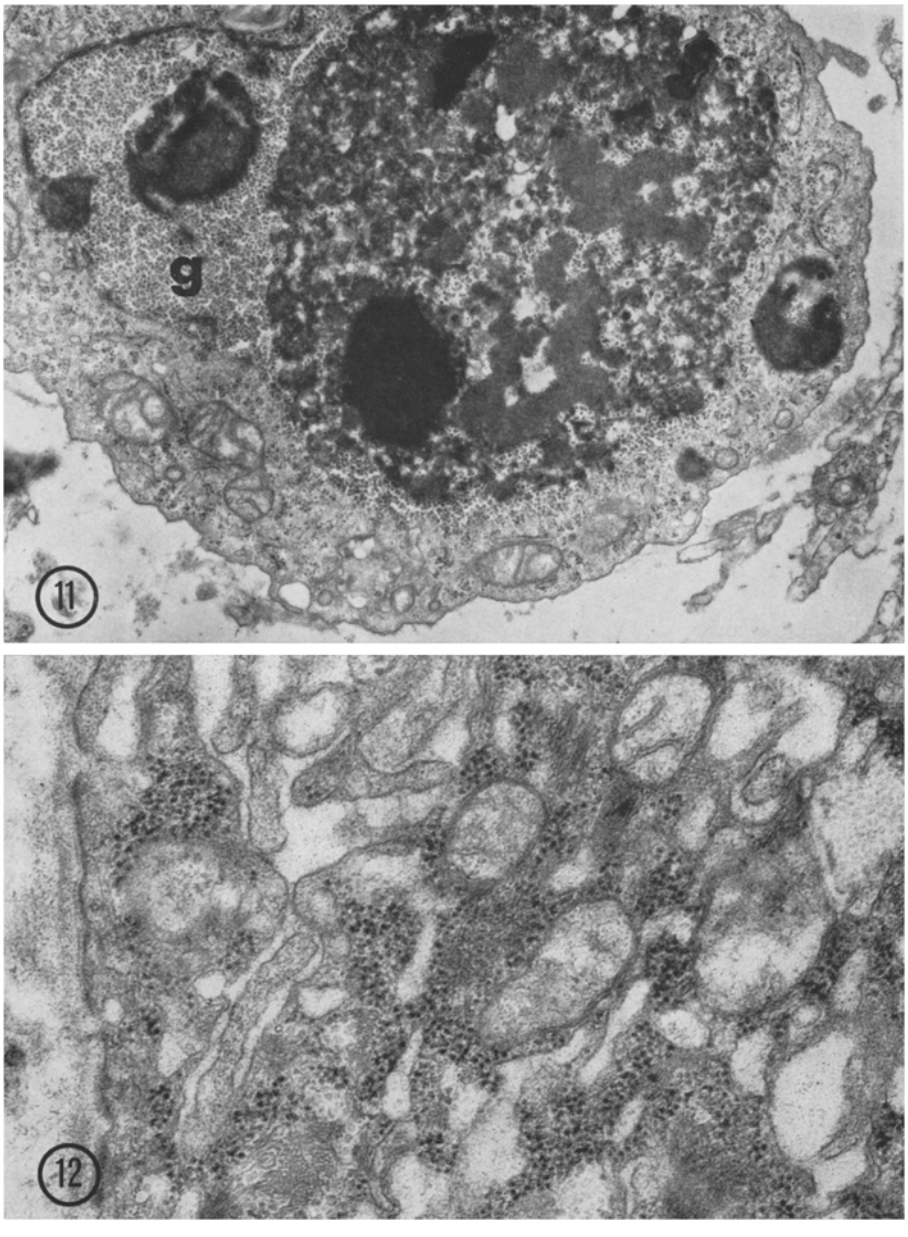


Fig. 11. Histiocyte with inclusion body containing disintegrated epithelial remnants especially characterized by the appearance of glycogen (*g*), which is not a normal constituent of the histiocytes. ×20400

Fig. 12. The peripheral sector of an epithelial pearl from a fetus of 99 mm CRL. ×44400

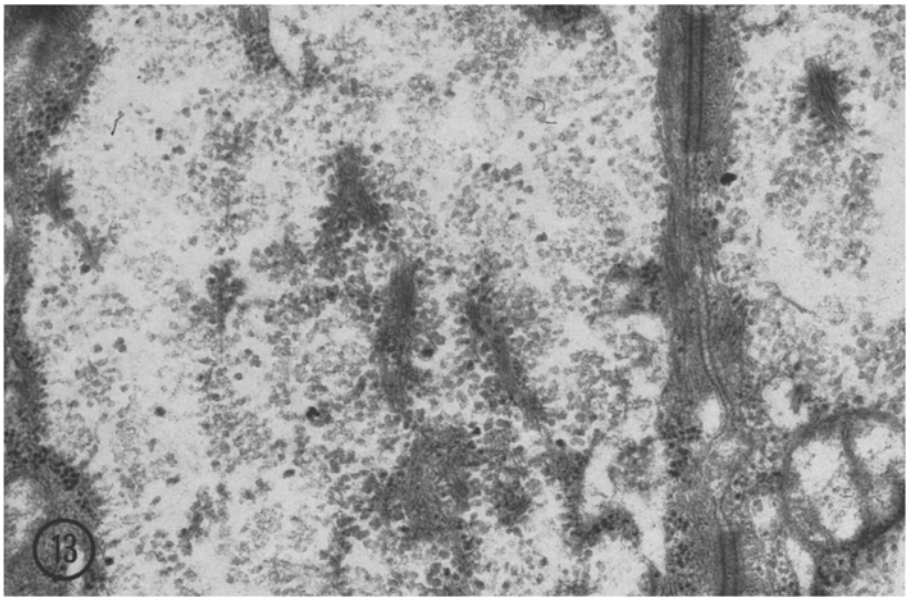


Fig. 13. More centrally situated sector of an epithelial pearl from a fetus of 99 mm CRL. ×37000

are noticed. The mitochondria are rounded or slightly elongated and are found in a moderate number only. The cell contains a well developed Golgi complex and some coated vesicles.

The other type of cell shows an indented nucleus and a highly irregular cellular outline. The abundant cytoplasm is dark and contains many mitochondria as well as numerous polyribosomes, but only few profiles of a rough-surfaced endoplasmic reticulum. The cytoplasm contains several membrane bound dense granules (0.1–0.3 μ) and a well developed Golgi complex. Centrioles are occasionally seen (Fig. 9). The two types of mesenchymal cells seen in the electron microscope are correlated to the two types seen with the light microscope in the thin survey sections.

When the above mentioned changes appear in the midline junctional epithelial seam, the latter type of cell often adjoins the seam. When the contact is very close the basal lamina has disappeared in the area of contact (Fig. 10). Then, the cell often contains membrane limited inclusion bodies of an appearance very similar to those described above for the epithelial cells (Fig. 11). Although the contents of the bodies seems more disintegrated and irregular in outline, one can still recognize glycogen, bundles of tonofibrils, and remnants of desmosomes. In the cytoplasm surrounding the bodies, lysosome-like dark granules are noticed. Only occasionally are the inclusion bodies seen in the cytoplasm of the ordinary mesenchymal cells.

The ultrastructure of an epithelial islet or pearl from a fetus of 99 mm CRL shows that the cells in the peripheral layer are highly interdigitating and connected by numerous desmosomes (Fig. 12). The cytoplasm is dark and contains

glycogen, several mitochondria and a well developed Golgi complex as well as bundles of tonofibrils. The cells are attached to the lamina basalis by numerous tightspaced half desmosomes. The remaining part of the pearl consists of concentrically arranged cells, which also show numerous interdigitations and well developed desmosomes. The tonofibrils are arranged in the peripheral part of the cells (Fig. 13). Apart from a few mitochondria the cytoplasm is loaded with glycogen. No keratohyaline granules are observed in the cells.

Discussion

The fact that the junctional epithelium shows membrane bound inclusion bodies with remnants of organelles, as well as nuclear pyknosis, seems to indicate that the junctional epithelial seam exhibits autophagia leading to partial as well as total cellular disintegration as soon as the junctions occur. This is further supported by the appearance of a relatively high activity of both acid phosphatase and AS-esterase in some cells, in which the final reaction product is seen as coarse cytoplasmic granules in contrast to the fine granules in the adjacent cells.

A special feature is the highly developed and distended rough-surfaced endoplasmic reticulum, with a floccular material, which is not seen in the epithelium outside the junctional seam, (e.g. the oral epithelium and the transition between the midline junctional seam and the oral epithelium). It is difficult to interpret the significance of this intracisternal material. A similar accumulation is noticed in secretory cells, in which the secretory machinery is not completely developed (Moe, 1971), as well as in chondrocytes of developing cartilage (Goel, 1970).

It is of interest that in the pre-fusional stages no distinct epithelial surface cell-coat was observed corresponding to the prospective sites of junctions but only on the lateral aspects of the nasal septum. Recently a distinct surface coat of the luminal cells in the early Rathke's pouch was noticed (Andersen *et al.*, 1971) and its possible role in preventing an establishment of cellular contacts between the walls of the pouch was discussed.

The cell noticed among the ordinary mesenchymal cells exhibits the characteristics of a histiocyte (macrophage) and is undoubtedly the same cell as recently described by Andersen and Matthiessen (1966) and Andersen (1970). This cell appears in the palatinal processes simultaneously with the vascularization of the palatinal processes. In the following stages of development, this cell escorts the distribution of the vessels toward the midline epithelial seam (Andersen and Matthiessen, 1967). The same type of cell is noticed in the nasal septum. When the cell is seen in close contact with the epithelial midline seam, it often contains large membrane bound inclusion bodies with a content which appears to be of an epithelial origin. It is likely then that the histiocytes or macrophages participate in the disintegration of the junctional epithelium. This is further supported by the fact that the lamina basalis seems to be absent at those places, where the histiocytes are immediately adjacent to the epithelium. It is difficult to settle whether the histiocytes only attack the epithelial cells already showing autophagia or other epithelial cells of the seams.

As the histiocytes contain more cytosomes (secondary lysosomes) than the epithelial cells and show a strong activity of acid phosphatase and AS-esterase

(based upon the incubation time needed for the visual initial activity) it is likely that the final lysis of the junctional epithelium takes place in the histiocytes.

Whether the ordinary mesenchymal cells participate in the epithelial disintegration is difficult to explain. Only occasionally are cellular remnants observed in an ordinary mesenchymal cell and it only shows a few cytosomes and a weak cytoplasmic activity of acid phosphatase and AS-esterase. Possibly, it is only a temporary engulfing of extruded epithelial cytosegresomes.

Farbman (1968) noticed phagocytosis of remnants of seam cells by nearby connective tissue phagocytes. However, Farbman made no reference to the exact nature of the phagocytes.

Smiley and Dixon (1968) described a fibroblastic migration into the epithelial seam, which however is never seen in the present study. Furthermore they suggested a phagocyte function of some of the epithelial cells following disintegration of neighbouring epithelial cells, which is in accordance to the study made by Michaels *et al.* (1971) on cell death in epidermis of the external gills of *Rana pipiens*. On the other hand, they stated that macrophages participate in the removal of dying cells. A phagocytotic function of epithelial cells has not been observed with certainty in the present study (Fig. 7).

The fate of the junctional epithelial seams has recently been described using a non-human material (Pourtois, 1966; Hughes *et al.*, 1967; Mato *et al.*, 1967; Angelici and Pourtois, 1968; De Angelis and Nalbandian, 1968; Farbman, 1968; Smiley and Dixon, 1968). Common to most of the above mentioned studies is the finding of an excessive epithelial autolysis only, while Hughes *et al.* (1967) and Angelici and Pourtois (1968) make reference to the surrounding histiocytes in accordance with the studies made by Andersen and Matthiessen (1966, 1967).

By comparing the studies on the non-human material with the present study, it is evident that pronounced differences exist. The disintegration processes in the non-human material takes place very rapidly compared to the human material, which must be related to the difference in prenatal lifetime. The same must be applied to the lack of a distended rough-surfaced endoplasmic reticulum in the non-human material and to the occurence of half desmosomes in the human material of the present study.

The observation made by Angelici and Pourtois (1968) that the non-human material shows no epithelial activity of acid phosphatase in the prefusional stage of the palatinal processes but, activity only corresponding to the junctional seam, is in disagreement to the observations made by Andersen and Matthiessen (1967) for human material. The disagreement may be related to enzyme-inactivation caused by the prolonged fixation (over-night) performed by Angelici and Pourtois. Both in the previous and in the present study only a fixation time of 3–4 hrs was used, which according to Casselman (1959) preserved 70% of the activity of acid phosphatase. While the authors mentioned above mainly look for a direct epithelial morphological evidence on which they can base a reason for the evoked cell death, the present authors (1967) focused the attention to the pressure from the rapidly growing endesmal ossification center of the vomer. This is supported by the present study in which preosteoblasts with a high activity of alkaline phosphatase are noticed adjacent the superior aspect of the horizontal part of the junctional T. The fact that the disintegration of the junctional T starts in the

horizontal part, from which it spreads to the superior part of the midline seam, further supports this hypothesis.

It seems reasonable to assume that the following sequence may exist: 1) pressure from the outside, 2) epithelial autophagia and 3) heterophagia of epithelial remnants by invading histiocytes (macrophages).

A similar sequence has recently been assumed in connection with the involution of the mammary glands from rats (Helminen and Ericsson, 1968).

Moreover this is in keeping with the fact that non-disintegrated epithelial islets or pearls may persist in the oral (lower) part of the midline seam. These pearls often show a hypertrophy and are converted to laminated structures, which have been considered as cornified (Wood and Kraus, 1962; Kitimura, 1966) in human fetal material. This is not supported by the present study. The lamellae were proved to consist of cytoplasmic sheets containing glycogen and peripherally situated tonofibrils. Moreover no keratohyaline granules were noticed and the mercury orange method for SH-groups was of the same strength as in the ordinary oral epithelium. The fate of the pearls has been discussed recently (Andersen and Matthiessen, 1967).

In the previous paper (Andersen and Matthiessen, 1967) it was concluded on the basis of digestibility with testicular hyaluronidase and the pronounced decrease in metachromasia by staining at pH < 4.0 that the intercellular substance of the palatinal mesenchyma was predominantly hyaluronic acid. This conclusion is further supported by the present study. On the basis of the following reactions: Pronounced metachromasia at pH-levels > 4.0, lack of metachromasia at pH $= 1.0$, strong alcianophilia at low electrolyte concentrations ($\leqq 0.2$ M $MgCl_2$) and by staining at pH $= 3.0$, weak alcianophilia at pH $= 1.0$ as well as in the presence of higher electrolyte concentration (0.6 M $MgCl_2$), the digestibility with testicular hyaluronidase, it must be concluded that the glycosaminoglucuronoglycans in question are hyaluronic acid and chondroitin-4- and/or -6-sulfate.

The ratio between the named glycosaminoglucuronoglycans is difficult to assert. Pronounced metachromasia at pH-values > 4.0 and decrease and absence of metachromasia at low pH-values strongly indicates the presence of polycarboxylates (e.g. hyaluronic acid), since these carboxyl groups are largely undissociated at pH-levels $\leqq 3.0$–4.0. However, at low pH-levels pre-existing organic cations belonging to proteins may compete with the dye-cations and thereby obscure the presence of polycarboxy-sulfates (e.g. chondroitinsulfates). Accordingly, it is of interest, that preliminary oxidative deamination of proteins by ninhydrin has no effect on the lack of metachromasia noticed in the present study at low pH-values, while elsewhere this procedure shows a pronounced effect on protein-competition (Andersen and Ehlers, 1967). Strong alcianophilia in the presence of low electrolyte concentrations ($\leqq 0.2$ M) as well as the faint alcianophilia at higher electrolyte concentrations (0.6 M) support the prevalence of a polycarboxylate. As discussed in detail by Andersen *et al.* (1971), the present authors prefer to extend the CEC to 0.2 M $MgCl_2$ for hyaluronic acid, when dealing with tissue.

On the other hand, faint alcianophilia at pH $= 1.0$ and at 0.6 M $MgCl_2$ as well as the digestibility with testicular hyaluronidase indicate the presence of chondroitin-4- and/or -6-sulfate. The reason why the sulfate half ester group of

the chondroitinsulfates does not cause metachromasia at low pH-levels may be due to a low concentration of chondroitinsulfates or to too widely spaced anion-groups, since according to Sylvén (1958) and Bergeron and Singer (1958) the intercharge distance must be about 5 Å er less to yield metachromasia. Furthermore, variations in the degree of sulfation occur and have been observed in chondroitinsulfates of rapidly growing cartilage (Meyer, 1969). Probably, the presence of chondroitinsulfates may be related to the extracellular assembly of collagen. It has been pointed out that chondroitin-4-sulfate is associated with the architecture of mediumsized, and chondroitin-6-fulfate with the finest, collagen fibres (Meyer, 1969). In the present study we noticed only fine bundles of collagen fibrils, which so far indicates the existence of chondroitin-6-sulfate at the expense of chondroitin-4-sulfate.

The role of glycosaminoglucuronoglycans (hyaluronic acid) in the elevation of the vertical palatinal processes was discussed by Andersen and Matthiessen (1967). The "water binding" of the hyaluronic acid may be related to its osmotic effect (Mathews, 1967).

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Dr. M. Matthiessen
Laboratory of Electronmicroscopy
Anatomy Department A, University
Universitetsparken 1
Copenhagen (Denmark)