Developmental aspects of secondary palate formation

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SUMMARY

Research on development of the secondary palate has, in the past, dealt primarily with morphological aspects of shelf elevation and fusion. The many factors thought to be involved in palatal elevation, such as fetal neuromuscular activity and growth of the cranial base and mandible, as well as production of extracellular matrix and contractile elements in the palate, are mostly based on gross, light microscopic, morphometric or histochemical observations. Recently, more biochemical procedures have been utilized to describe palatal shelf elevation. Although these studies strongly suggest that palatal extracellular matrix plays a major role in shelf movement, interpretation of these data remains difficult owing to the complexity of tissue interactions involved in craniofacial development. Shelf elevation does not appear to involve a single motive factor, but rather a coordinated interaction of all of the above-mentioned developmental events. Further analysis of mechanisms of shelf elevation requires development of new, and refinement of existing, in vitro procedures. A system that enables one to examine shelf elevation in vitro would allow more meaningful analysis of the relative importance of the various components in shelf movement.

Much more is known about fusion of the palatal shelves, owing in large part to in vitro studies. Fusion of the apposing shelves, both in vivo and in vitro, is dependent upon adhesion and cell death of the midline epithelial cells. Adhesion between apposing epithelial surfaces appears to involve epithelial cell surface macromolecules. Further analysis of palatal epithelial adhesion should be directed towards characterization of those cell surface components responsible for this adhesive interaction.

Midline epithelial cells cease DNA synthesis 24–36 h before shelf elevation and contact, become active in the synthesis of cell surface glycoproteins, and subsequently manifest morphological signs of necrosis. Death of the midline epithelial cells is thought to involve a programmed, lysosomal-mediated autolysis. Information regarding the appearance, distribution and quantitation of epithelial hydrolytic enzymes is needed.

The control mechanisms which regulate adhesiveness and cell death in the palatal epithelium are not fully understood. Although palatal epithelial–mesenchymal recombination experiments have demonstrated a close relationship between the underlying mesenchyme and the differentiating epithelium, the molecular mechanism of interaction remains unclear. Recently cyclic nucleotides have been implicated as possible mediators of palatal epithelial differentiation.

The developing secondary palate therefore offers a system whereby one can probe a variety of developmental phenomena. Cellular adhesion, programmed cell death and epithelial–mesenchymal interactions are all amenable to both morphological as well as bio-

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chemical analysis. Although research in the field of secondary palate development has been extensive, there still remain many provocative questions relating to normal development of this structure.

I. INTRODUCTION

During development, the palatal shelves of most vertebrates undergo a complex reorientation from a vertical position lateral to the tongue to a horizontal position above the tongue and fuse to form a single, continuous structure, the secondary palate. This involves four distinct stages including growth of the individual shelves, reorientation of the shelves and finally epithelial cell adhesion and autolysis.

Past research has emphasized the study of teratogens that induce cleft palate. This review is not intended to deal with all these studies, but to concentrate on normal secondary palatal development and to discuss possible areas of future investigation. Only recently has it been appreciated that a number of developmental events including morphogenetic movements, cellular adhesion, epithelial-mesenchymal interaction and programmed epithelial cell death can all be examined during palatal development. Understanding of these events, which are accessible to a variety of biochemical analyses, and occur relatively late in gestation after most organogenic interactions have been completed, may be relevant to a number of other developing tissues.

II. GROWTH AND ELEVATION OF THE PALATAL SHELVES

After formation of the first pharyngeal arch, the maxillary, frontonasal and mandibular processes form the boundaries of the oral-nasal cavity. Cells of the cranial neural crest are known to migrate into and contribute significantly to the cell population of the face, including the maxillary process (Johnston & Listgarten, 1972). It is reasonable to assume that much of the original palatal shelf mesenchyme originates from the neural crest. The palatal shelves initially appear as small outgrowths at the medial surface of the maxillary processes which subsequently grow down on either side of the tongue (Fig. 1A). At this stage, each shelf consists of mesenchymal cells surrounded by extracellular matrix and covered by a two- to three-cell layered epithelium (Fig. 2).

The palatal shelves begin movements which will bring them from a vertical position on either side of the tongue into a horizontal position, superior to the tongue (Fig. 1B). These movements occur rather late in gestation, well after most organogenic processes have been completed, both in man (7th week of gestation (Fulton, 1957)) and in the rat (day 16 of a 21-day gestational period (Coleman, 1965)). Early investigators envisaged the shelves rotating, as if on a hinge, from a vertical to a horizontal position (Peter, 1924; Lazzaro, 1940) and recently, Walker & Ross (1972) have supported this concept.

This interpretation is dependent on the location along the anterior-posterior length of the shelf, since movement occurs in different fashions in different
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Fig. 1. Schematic illustration of frontal sections taken through the anterior hard palate of the fetal rat at various times during gestation. (A) Day 15 (±8 h) – the palatal shelves have grown from the maxillary processes and assume a position on either side of the tongue. (B) Day 16 (±8 h) – the shelves have rotated to a position above the tongue, and medial-edge epithelial surfaces will make contact. (C) Day 17 (±8 h) – the medial-edge and nasal epithelial surfaces have adhered, and begun autolysis with some mesenchymal penetration of the epithelial seam (dotted line). Legend: N, nasal epithelium; O, oral epithelium; M, medial-edge epithelium.

Fig. 2. Photomicrograph of a single, vertically orientated mouse palatal shelf (PS) just prior to elevation. T, Tongue; M, maxilla. × 250.

regions. Coleman (1965) has observed in the rat fetus that rostrally (anteriorly) the palatal shelves elevate via rotation, as implied in Fig. 1A and 1B, while caudally (posteriorly) shelf elevation proceeds by means of a ‘remodeling’ of the vertical shelf. Remodeling presumably involves a shift of the palatal mesenchyme resulting in a medially directed protrusion (Fig. 3) which meets the opposite shelf in the midline above the tongue. Similar observations in other species have been reported (Walker & Fraser, 1956; Larsson, 1962; Kochhar & Johnson, 1965; Harris, 1967; Greene & Kochhar, 1973b).

(a) Coordinated tissue movements during shelf elevation

After growth from the maxillary processes, the palatal shelves are confronted with a major obstacle between them, the growing tongue (Fig. 1A). The tongue
has long been thought actively to withdraw from between the shelves prior to palatal shelf elevation. Several of the intrinsic muscles of the tongue have differentiated into fibers, responsive to both direct electrical as well as hypoglossal nerve nucleus stimulation, at least 4 h prior to palatal shelf elevation (Wragg, Smith & Boden, 1972). Recently, acetylcholinesterase activity was demonstrated at developing motor end plates in the tongue of fetal mice prior to shelf elevation (Holt, 1974). The myoneural apparatus of the tongue is therefore functional at the time of palatal shelf elevation, suggesting that the tongue has the capacity for voluntary movement at this time. The close synchrony between the onset of tongue movements and shelf elevation suggests a relationship between these two phenomena, but it is unlikely that such movement of the tongue is directly responsible for shelf elevation. Walker & Patterson (1974a) have demonstrated that palatal shelves will elevate despite prior removal of the tongue. The obstructive influence of the tongue is further supported by recent studies on shelf movement in vitro (Brinkley Basehoar & Avery, 1974; Brinkley, Basehoar, Branch & Avery 1975), in which fetal mouse heads cultured prior to in vivo shelf elevation showed the most progress in attaining shelf elevation when the tongue was removed.

A variety of other fetal movements occur during the time of shelf elevation. The initiation of fetal mouth-opening reflexes (Humphrey, 1969) as well as ‘swallowing’ movements (Walker, 1969) is closely followed by palatal elevation. To test if these were necessary for elevation of the palatal shelves, Jacobs (1971)
administered muscle relaxants to mice prior to and during the time of palate elevation. Such treatments failed to produce cleft palate and these studies were interpreted as indicating that spontaneous or reflexogenic fetal muscular activity is not essential for normal shelf elevation. It was not, however, demonstrated that these agents actually crossed the placenta and were active in the fetus. Recently, Walker & Patterson (1974b) reported cleft palate in the offspring of pregnant mice treated with barbiturates and tranquillizers, indicating that the developing fetus is affected, possibly by direct action on the fetal neuromuscular system, by the action of various central nervous system depressing agents.

It still remains, however, to be clearly demonstrated that fetal movements play a direct role in palatal development. The evidence available to date supports the concept that a moderate downward growth of the tongue anteriorly allows elevation to proceed by rotation. In the posterior region, the tongue is situated firmly in the common oral-nasal cavity so that a moderate movement downward will not allow for rotation, thereby indirectly forcing the shelf to remodel around the tongue to a horizontal position.

Growth and straightening of the cranial base has also been proposed as playing a role in palatal elevation. In rodent fetuses, the flexed cartilagenous cranial base straightens prior to and during shelf elevation and various authors have suggested that this straightening is necessary for shelf elevation (Harris, 1964, 1967; Verrusio, 1970; Wragg, Klein, Steinvorth & Warpeha, 1970; Smiley, Hart & Dixon, 1971; Hart, Smiley & Dixon, 1972; Larsson, 1972; Long, Larsson & Lohmander, 1973; Taylor & Harris, 1973). This movement, combined with growth of the lower jaw (Harris, 1967; Humphrey, 1971) results in an increased vertical dimension of the anterior one-half of the oral cavity. Lifting of the anterior portion of the cranial base could facilitate the rotation of the anterior region of the palatal shelves (Harris, 1967). However, measurements of cranial base changes are usually derived from fixed, embedded material, subject to shrinkage and other artifacts inherent in tissue preparation. A study of cranial base changes on frozen sections should prove interesting. The role of the cranial base in shelf elevation remains controversial, as elevation in vitro does not require an intact cranial base (Brinkley et al. 1975).

Mandibular growth has also been implicated in palatal elevation (Zeiler, Weinstein & Gibson, 1964). The forward growth of the mandible presumably carries the base of the tongue from between the shelves posteriorly, and downward growth of the mandible creates room above the tongue anteriorly for the shelves to elevate. The evidence, however, for a differential mandibular growth spurt prior to shelf elevation is presently inconclusive (Zeiler et al. 1964; Hart, Smiley & Dixon, 1969).

The preceding discussion (IIa) points out the fact that the role these external structures play in palatal elevation is complex and as yet unclear. It seems reasonable that a highly coordinated interaction of many of these structures
will prove to be necessary for elevation, but at the present state of our knowledge, it is impossible to say which of these factors is most important.

(b) Role of cellular proliferation and extracellular matrix

An 'internal shelf force' developing from within the shelf was proposed by Walker & Fraser (1956) to explain elevation of the palatal shelves. While it is conceivable that rapid proliferation of mesenchymal cells in certain areas of the palate could induce movement, Walker & Fraser (1956) and Hughes, Furstman & Berdick (1967) found no evidence of an unusually high mitotic rate in the shelf mesenchyme at the time of elevation. Jelinik & Dostal (1973, 1974) injected colchicine into the amniotic fluid of the mouse fetus at various times prior to as well as during elevation and subsequently counted metaphase figures in the mesenchymal cells. They found that the peak of proliferation in the mesenchyme preceded elevation by 24 to 48 h. Similar conclusions have been reached by measuring the accumulation of DNA with time in the rat palate (Hassell, Pratt & King, 1974).

It was thought that even after elevation, the shelves had to grow extensively towards each other to make contact. However, recent studies using freeze-dried (Wragg, Diewert & Klein, 1972) or freeze-sectioned material (Greene & Kochhar, 1973a) have shown that the palatal shelves are in contact along at least part of their medial edges immediately upon elevation. This is most evident in the anterior region of the shelves where elevation occurs by rotation. Growth does not appear to play a direct role in elevation of the shelves, although a critical examination of mesenchymal growth rates in different regions of the shelf both prior to and during elevation is needed.

Walker & Fraser (1956) postulated that an elastic fiber network in the palatal mesenchyme provided the necessary force for elevation, although it has since been demonstrated that no such network is seen in the palate at the time of elevation (Stark & Ehrman, 1958; Loey, 1962; Frommer, 1968; Frommer & Monroe, 1969). Attention thus turned to the role of other extracellular matrix components in elevation, since the matrix is a prominent constituent of the shelf mesenchyme and a variety of teratogens alter the synthesis or metabolism of its components.

Prior to and during elevation the palatal shelves actively synthesize sulfated proteoglycans (see discussion below) (Larsson, Bostrom & Carlsloo, 1959; Larsson, 1961; Walker, 1961) and there is also a rapid accumulation of extracellular metachromatic material in the palatal mesenchyme (Larsson, 1961; Jacobs, 1962). This metachromatic material was degradable by testicular hyaluronidase (Anderson & Mathiessen, 1967) and based on the known specificity of this enzyme was presumed to be chondroitin sulfate and/or hyaluronic acid. Pratt, Goggins, Wilk & King (1973) labeled the glycosaminoglycans synthesized by palatal shelves at the time of elevation in vivo and also in vitro with [3H]-glucosamine and [35S]-sulfate. Subsequently the tissue was treated
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with papain to remove the protein portions and the various polysaccharide species were resolved by chromatography on DEAE-cellulose. Tissue levels were found to reflect synthetic activity and showed that hyaluronic acid accounted for 65% of the total glycosaminoglycans and sulfated glycosaminoglycans accounted for the remainder.

Hyaluronate is an extremely hydrated high molecular weight unbranched polysaccharide (Laurent, 1970), and its production and accumulation is often associated with swelling of embryonic tissues and morphogenetic movements (Toole, Jackson & Gross, 1972; Pratt, Larsen & Johnston, 1975). Prior to elevation, the presumptive oral half of the palatal mesenchyme contains larger extracellular spaces than the presumptive nasal portion of the shelf (Anderson & Mathiesson, 1967). The accumulation of hyaluronate may be especially high in this region of the shelf. A number of teratogens, which induce cleft palate by inhibiting or delaying palatal elevation, alter glycosaminoglycan metabolism in the palate (cortisone, Larsson, 1962; chlorcyclizine, Wilk, Steffek & King, 1970; diazo-oxo-nor Leucine, DON, Pratt, Coggins, Wilk & King 1973). These studies suggest that glycosaminoglycans, especially hyaluronate, play an important role in shelf elevation.

Another component of the palatal shelf mesenchyme which may play a role in elevation is collagen. Pratt & King (1971) found a significant increase in the amount of palatal collagen, estimated to be 4–6% of the total palatal protein, at the time of shelf elevation (day 15–16 in the rat). β-aminopropionitrile (BAPN) appears to inhibit shelf elevation and cause cleft palate by preventing the crosslinking of collagen in the palate as well as the whole embryo (Steffek, Verrusio & Watkins, 1972; Wilk, King, Horigan & Steffek, 1972; Pratt & King, 1972a, b). BAPN specifically inactivates lysyl oxidase which ordinarily oxidizes lysine residues in collagen to aldehydes that react to form the actual crosslinks (Pinnel & Martin, 1968). The teratogenic action of BAPN implies that collagen fibers play a structural role in shelf elevation since no change in synthesis or deposition is noted and presumably only the tensile strength of collagen fibers is affected.

Collagen fibrils have been observed in the palate of the mouse (Smiley & Dixon, 1968; Smiley, 1970). In the palate of the rat, collagen fibrils are observed throughout the mesenchyme, but are particularly abundant in the area adjacent to the basal lamina of the presumptive oral epithelium. Here, the fibres are oriented uniformly along the anterior (rostral) to posterior (caudal) axis of the shelf (Hassell & Orkin, 1974). This location in the palate suggests that these fibers may serve as attachment or anchorage sites by which a force could be transmitted to the rest of the mesenchyme during elevation.

Although the components of the palatal matrix have thus far been considered individually, it is likely that they form functional macromolecular complexes. In other systems, specific interaction between collagen and sulfated GAG (Toole & Lowther, 1968; Lowther, Toole & Herrington, 1970; Greenwald, Schwartz &
Cantor, 1975) and also between hyaluronate and proteoglycans (Hardingham & Muir, 1972) have been observed. The hyaluronate–proteoglycan interaction was subsequently shown to be essential in the organization of proteoglycan aggregates in cartilage matrices (Hascall & Heinegard, 1974). Additionally, small molecular weight proteins were shown to be critical for stabilizing the hyaluronate–proteoglycan interaction (Gregory, 1973; Hardingham & Muir, 1974; Hascall & Heinegard, 1974; Rosenberg, Hellman & Kleinschmidt, 1975a). A model for these interactions has been discussed recently (Hascall & Heinegard, 1975; Rosenberg et al. 1975b; Wiebkin, Hardingham & Muir, 1975). Such complexes have not yet been defined in other tissues but if they occur in palatal shelves they could be of structural importance during elevation.

Rapid movements in biological systems are usually brought about by contractile elements. The contractile nature of wound granulation tissue has recently been demonstrated (Gabbiani, Majno & Ryan; 1973), and actin and myosin have been reported in a variety of tissues and cells other than muscle (Pollard & Weiheing, 1974). Lessard, Wee & Zimmerman (1974) have shown that actin and myosin are synthesized in the mouse palate at the time of shelf movement. A calcium-dependent ATPase, presumably myosin, has been observed in the posterior area of the palate and it has been suggested that this represents smooth muscle which along with striated muscle in the extreme posterior soft palate, might be involved in shelf elevation (Babiarz, Allenspach & Zimmerman, 1975).

The relative importance of the extrinsic, as well as various intrinsic, factors involved in palatal shelf elevation can be further clarified by the development of new and the refinement of existing in vitro methods. The in vitro procedures of Brinkley et al. (1975) and of Walker & Patterson (1974a) may offer techniques by which the process of shelf elevation may be directly observed.

The most widely held concept of palatal elevation supports a direct and active involvement of the palatal shelves in their own morphogenetic movement. This view, however, does not rule out the possible involvement of various external factors previously discussed.

### III. Fusion of the Palatal Shelves

#### (a) Epithelial cell adhesion

Electron microscopic studies of mouse (Farbman, 1968), rat (Hayward, 1969), hamster (Chaudhry & Shah, 1973) and human palatal tissue (Matthiessen & Anderson, 1972; Mato, Smiley & Dixon, 1972) have demonstrated that the fine structure of the palatal epithelium both before and during epithelial adhesion and seam formation is remarkably similar in these species. Prior to contact, the epithelium of the medial edge of the elevating palatal shelves is two to three cell layers thick with a surface squamous cell layer and underlying cuboidal cell layer(s). The epithelial–mesenchymal border exhibits an intact basal lamina
Fig. 4. Electron micrograph of a squamous epithelial cell on the surface of a mouse palatal shelf just prior to contact with the opposite shelf. Note the microvilli (MV) present at the surface. D, desmosome; G, Golgi apparatus; M, mitochondrion; RER, rough endoplasmic reticulum. × 36400.

separating the epithelial cells from the underlying mesenchymal cells (DeAngelis & Nalbandian, 1968; Smiley, 1970; Chaudhry & Shah, 1973). The surface epithelial cells exhibit prominent microvilli, as cytoplasmic extensions (Fig. 4) which flatten and subsequently disappear upon contact with the opposite palatal shelf (DeAngelis & Nalbandian, 1968; Matthiessen & Anderson, 1972; Waterman & Meller, 1974).
Once contact between the two apposing shelves has been made, a tight adhesion develops, as attempts to separate the shelves after contact produce tearing of epithelial cell membranes (Zeiler, Weinstein & Gibson, 1964; Farbman, 1968). Although desmosomes may support contiguity between apposing epithelial surfaces (DeAngelis & Nalbandian, 1968; Brusati, 1969; Chaudhry & Shah, 1973), Farbman (1968) found no evidence of desmosomes between adhering palatal epithelial surfaces in the mouse.

Much of the present emphasis on the study of palatal adhesion can be attributed to the earlier work of Pourtois (1966, 1968a, b, 1970) on epithelial cell adhesion and death during palatal development. Initial adhesion of one shelf to another may be explained by the presence of an extracellular carbohydrate-rich surface coat on the developing epithelium of the palatal shelves. Epithelial cell membranes of apposing shelves are separated by a 10–20 nm. Intercellular space of uniform width (Hayward, 1969), presumably containing surface glycoproteins. Initial adhesion mediated by cell-surface carbohydrates may serve to keep the shelves in contact until more permanent, desmosomal connections can be made. Farbman (1968) and Matthiessen & Anderson (1972) were unable, utilizing various histochemical stains, to demonstrate a surface coat, although Hayward (1969) using transmission electron microscopy described an ‘ill-defined fuzzy material’ on medial-edge epithelial cell surfaces prior to contact.

A carbohydrate-rich, surface coat has been shown to increase dramatically on the epithelial surface (medial-edge) prior to contact. This has been shown at the ultrastructural level by the binding of the plant lectin concanavalin A (CON A) (Pratt, Gibson & Hassell, 1973a; Pratt & Hassell, 1975) and by staining with ruthenium red (Greene & Kochhar, 1974). The chemical nature of this surface coat is probably complex since CON A binds to mannosyl and glucosyl residues of glycolipids and glycoproteins and ruthenium red binds to glycosaminoglycans and glycoproteins. Scanning electron microscopic observations of the medial-edge epithelial surface prior to contact in several species show the progressive appearance of a fine filamentous material (Waterman, Ross & Meller, 1973; Meller, personal communication, 1974), although this material is not observed in the human (Waterman & Meller, 1974).

Surface glycoproteins and glycosyltransferases have been implicated in cell-to-cell adhesion in a variety of systems (Roseman, 1970; McGuire, 1972). DON (diazo-oxo-norleucine), a glutamine analog, has been shown to prevent adhesion of mouse teratoma cells (Oppenheimer, 1973) and epithelial adhesion between homotypic palatal shelves (Pratt, Greene, Hassell & Greenberg, 1975). D-glucosamine reversed the action of DON on both the teratoma cells and palatal epithelial cells. Shelves cultured in the presence of DON bind much less labeled CON A at the epithelial surface than shelves cultured without DON (Greene & Pratt, 1975). These data suggest that amino sugars are incorporated into macromolecules which are necessary for intercellular adhesion. The observation (Pourtois, 1970, 1972) that adhesion of palatal shelves in vitro was not
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inhibited by proteolytic enzymes could be explained by rapid resynthesis of cell-surface components (Collins, Holland & Sanchez, 1973) or the resistance of essential surface components to proteolysis.

The presence of a carbohydrate surface coat may explain the phenomenon of an ‘acquired potential’ to fuse exhibited by palatal shelves of rats (Pourtois, 1966) and mice (Vargas, 1967) in vitro. It must be pointed out that the term ‘fusion’ refers only to the merging of mesenchyme of adjacent palatal shelves after midline epithelial seam breakdown. Epithelial cells from adjacent shelves adhere to one another, but there is no plasma membrane fusion during formation of the midline seam. Pourtois (1966) and Vargas (1967) have shown that palatal explants from early mouse and rat fetuses do not fuse in vitro, although explants from older fetuses show an increasing ability to fuse. Recently, however, Tyler & Koch (1975), using different culture conditions, have shown that fetal mouse palates, explanted 2½ days prior to contact in vivo, will fuse after 72 h in culture. This suggests that the activating changes necessary for shelf-fusion can occur in vitro. The acquisition of particular cell-surface properties is not an uncommon occurrence as cell-surface alterations during development have been demonstrated in a variety of embryonic systems (Moscona, 1971; Gottlieb, Merrell & Glaser, 1974; Krach, Green, Nicholson & Oppenheimer, 1974). An increasing number of observations indicate a functional role for palatal epithelial cell-surface carbohydrates, and it is likely that their programmed appearance is necessary for adhesion between adjacent palatal shelves.

B. Epithelial Cell Autolysis

Elimination of certain cells occurs during the development of a variety of tissues. This process is frequently referred to as programmed cell death, since the changes occur at precise stages of development (Lockshin & Williams, 1965; Saunders & Fallon, 1966; Dorgan & Schultz, 1971). Such changes are seen in palatal medial edge epithelial cells (Mato, Aikawa & Katahira, 1966; Smiley, 1970; Smiley & Koch, 1971; Matthiessen & Anderson, 1972; Chaudhry & Shah, 1973). Preautolytic alterations include mitochondrial swelling (Sweeny & Shapiro, 1970; Mato, Smiley & Dixon, 1972) and the appearance of lysosomal bodies in medial-edge epithelial cells (Mato, Aikawa & Katahira, 1966; Hayward, 1969).

Studies in the rat in vivo (Hudson & Shapiro, 1973) and in vitro (Pratt & Martin, 1975) have shown by measuring the incorporation of [³H]-thymidine that medial-edge epithelial cells cease DNA synthesis as early as 36 h prior to contact. These cells are not at a local nutritional disadvantage since the adjacent oral and nasal epithelial cells, as well as the mesenchymal cells, continue to divide (Fig. 5). Although DNA synthesis ceases, medial-edge cells incorporate [³H]-uridine into RNA (Pratt & Greene, 1975) and labeled glucosamine and fucose into macromolecules, some of which appear to be
Fig. 5. Autoradiograms of palatal shelves cultured for 4 h submerged in growth medium containing [3H]-thymidine at 40 μCi/ml. (a) Day 14(+12 h) (× 350) and (b) Day 15(+12 h) (× 350). The various areas of the epithelium are: M, medial edge; O, oral; N, nasal epithelium; mes, mesenchyme.

transported to the cell surface (Pratt & Hassell, 1974; DePaola, Drummond, Lorent & Miller, 1974).

Smiley & Koch (1972) demonstrated that selective death occurs in the medial-edge epithelial cells of palatal shelves explanted onto Millipore filters. Recently, Tyler & Koch (1974) demonstrated that isolated palatal epithelium explanted onto Millipore filters also displays selective cell death in the presumptive medial-edge region. Selective death of medial-edge cells was also observed in vivo in fetuses of the A/Jax mouse that displayed spontaneous cleft lip and palate (Smiley & Koch, 1972) and in rat fetuses after amniotic sac puncture (Goss, Bodner & Avery, 1970; Morgan, 1975). In both these cases inhibition of elevation due to the obstructive position of the tongue prevented epithelial contact between shelves.

Palatal epithelial–mesenchymal recombination experiments (Pourtois, 1969; Tyler & Koch, 1975) support the hypothesis that autolysis in medial-edge epithelial cells is dependent on a prior interaction between these tissues. The question of the actual time when the medial-edge cells become irreversibly committed to cell death remains unanswered, although several studies have
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Fig. 6. Photomicrograph of a plastic-embedded secondary palate illustrating early seam formation between adjacent palatal shelves. S, midline epithelial seam; NS, nasal septum. × 250.

shown that certain compounds may inhibit fusion in vitro by preventing cell death in the medial-edge epithelium (β-thienylalanine, Baird & Verrusio, 1973; hadacidin, Fairbanks & Kollar, 1974; epidermal growth factor, Hassell, King & Cohen, 1974; DON, Pratt & Greene, 1975).

The first signs of programmed cell death in the medial-edge cells is a cessation of DNA synthesis. Numerous studies with various cultured cell lines have shown that elevated levels of cyclic adenosine 3'-5' monophosphate (CAMP) are associated with reduced proliferative activity (Otten, Johnston & Pasten, 1972; Bombik & Burger, 1973). In the rat palatal shelf, levels of cyclic AMP have been shown to increase dramatically approximately 18 to 24 h prior to epithelial contact (Pratt & Martin, 1975). Whether or not this increase occurs in the epithelium or is related to cessation of DNA synthesis in the medial-edge cells is not known. The addition of dibutyryl cyclic AMP to the immature day-14 rat shelf in vitro causes a precocious decrease in medial-edge epithelial DNA synthesis and an increase in glycoprotein synthesis. These induced changes appear to mimic those that normally occur on days 15 to 16. These results suggest that activation of medial-edge cells to mature in vivo, involving both decreased proliferation and increased adhesiveness, may in part be mediated through cyclic nucleotides. This is further supported by the ability of exogenous dibutyryl cyclic AMP to prevent EGF-induced inhibition of epithelial cell death.
Fig. 7. (Inset) Photomicrograph of a secondary palate illustrating a late midline epithelial seam (S). × 250.

Fig. 8. Electron micrograph of a palatal midline epithelial seam (S) corresponding to the area indicated by the arrow in Fig. 7, demonstrating an intact basement lamina (BL) separating the cells of the seam (S) from the connective tissue cells of the palatal mesenchyme (MES). × 14200.
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(Pratt, Green, Hassel & Greenberg 1975). There are undoubtably other factors involved in this activation and maturation process, although they remain unknown at the present time.

The developing palatal shelves in the chick embryo may provide an interesting system in which to investigate these programmed changes. Chick palatal shelves do not appear to maintain epithelial contact and therefore formation of a fused secondary palate never occurs here. A comparison between the programmed changes involved in palatal adhesion and fusion of most vertebrates and the events which bring about the cleft palate of the chick should prove interesting. The developing eyelids (Anderson, Ehlers & Matthiessen, 1965; Anderson et al. 1967) may provide another system to investigate epithelial adhesion, and control of these developmental events.

After midline epithelial adhesion and the onset of autolysis, the epithelium of one shelf soon becomes indistinguishable from that derived from the adjacent shelf, as an epithelial seam is formed in the midline of the future secondary palate. The midline epithelial seam changes rapidly from the original 4–5 cell-layered seam (Fig. 6) to a 1–2 cell-layered strip of cells (Fig. 7) (Smiley & Dixon, 1968; Hayward, 1969). Superiorly, at the nasal surface of the secondary palate, the seam expands into a triangular area (Smiley & Dixon, 1968), and inferiorly, at the oral surface, the seam also expands into a triangular area. Both of these areas are characterized by large intercellular spaces with cells containing numerous electron-dense granular structures, presumably lysosomal in nature since they have been shown to be positive for acid phosphatase (Mato, Aikawa & Katahira, 1967).

Lysosomal bodies appear throughout the palatal epithelial midline seam and are rare in the epithelium covering the oral and nasal palatal surfaces (Mato, Aikawa & Katahira, 1967). In a study of the role of acid phosphatase in fusion of the secondary palate, Angelici & Pourtois (1968) localized all enzyme reactions in the epithelial cytoplasm. Autolysis of the midline seam is accomplished by lysosomal enzymes produced by the midline epithelial cells, indicating a primary lysosomal involvement in this type of programmed cell death. This is in contrast to programmed cell death in the posterior necrotic and interdigital zone of the developing chick limb, where cells are first fragmented by unknown mechanisms and these fragments taken up and degraded by macrophages containing lysosomal enzymes (secondary lysosomal involvement) (Saunders & Fallon, 1966).

As autolytic breakdown of the midline seam and the superior and inferior triangular areas proceeds, the epithelial cells become irregular in outline and are separated by increasingly larger extracellular spaces. The epithelial cells, however, remain connected throughout this process by desmosomes, and initial epithelial degeneration is not directly associated with apparent breakdown of the basal lamina (Hayward, 1969) (Fig. 8). The final phases of breakdown of the midline epithelial seam in both hard and soft palate involve disruption of the
basal lamina interposed between epithelial and mesenchymal cells, and migration of macrophages into this region with subsequent phagocytosis of degenerating epithelial cells (Kozioł & Steffek, 1969; Matthiessen & Anderson, 1972; Shah & Chaudhry, 1974). Epithelial seam breakdown has been assumed to allow extensive intermixing of the mesenchymal cells of adjacent palatal shelves. However, Koch & Smiley (1973) have combined in vitro colchicine-treated shelves labeled with [³H]thymidine and unlabeled shelves, and have thus shown that little intermixing takes place between mesenchymal cells derived from originally separate shelves.

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