

**6-MERCAPTOPURINE INDUCED CLEFT PALATE IN THE HAMSTER:
MORPHOLOGICAL AND CELLULAR ASPECTS**

BY

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ABSTRACT

A study on the pathogenesis of 6-mercaptopurine induced cleft palate was undertaken using light and electron microscopic, and enzyme acid phosphatase cytochemical techniques.

Palatal development in control fetuses was observed in six stages at the gross level and five stages at the histological level. Between days 9:18 (9 days:18 hours) and 10:00 of gestation palatal primordia appeared from the roof of the oronasal cavity, and developed in the vertical direction until day 12:00 of gestation. Between days 12:00 and 13:00 of gestation the palatal shelves became horizontal and fused with one another. During closure the timely appearance of lysosomes was responsible for elimination of the intervening epithelia of the opposing palatal shelves through an intracellular process of autolysis.

Gross observations showed that 6-mercaptopurine affected the vertical development of palatal shelves. In contrast to normal development, vertically developing palatal shelves on day 10:00 of gestation showed sublethal injury of the mesenchymal cells characterized by swelling of the perinuclear space and lysosomal development. Subsequently the epithelial cells were damaged, and the basal lamina fragmented and disappeared. The epithelial and mesenchymal cells communicated with one another. Eventually, however, the epithelial and mesenchymal cells recovered and the basal lamina re-established its continuity.

It was concluded that sublethal injury of the mesenchymal and epithelial cells following 6-mercaptopurine treatment disturbed the controlled process of cytodifferentiation, and thus affected vertical development of the palatal shelves to develop a cleft palate.

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INTRODUCTION

Birth Defects

Since the turn of the century it has been increasingly recognized that chemicals present in the environment, or administered therapeutically may be hazardous to the embryo/fetus. The most overtly identifiable adverse effect of exposure to a chemical in this fashion is intrauterine death.

Alternatively the exposed fetus may develop anatomical defects or functional aberrations, including subtle behavioural or biochemical abnormalities, some of which may remain latent before being expressed at later stages of development. The recognition of such "teratogenic" effects (both anatomical and functional) gained further impetus through the tragic events of the early 1960's, when dramatic increases in the frequency of phocomelia in the newborn infants were traced to the use of thalidomide by pregnant women (Lenz, 1961; McBride, 1961). Since then both the scientist and society have been brought face to face with the devastating possibility of chemically induced birth defects in humans.

The study of birth defects has assumed more significance now than in the past because mortality and morbidity due to congenital anomalies has declined far less than those for other causes of death such as infections and nutritional irregularities (Smithells, 1966; Saxen and Rapola, 1969; Persaud, 1979). It is estimated that approximately 40% of all pregnancies terminate as miscarriages, mainly due to faulty prenatal development (Saxen and Rapola, 1969). Approximately 20-40% of all still births and infant deaths are associated with severe congenital anomalies (Elwood and Rogers 1975;

Fairweather 1982). In addition, major birth defects are found in at least 2-7% of all liveborn (Fairweather, 1982; Bower and Stanley, 1983; Fabro, 1983; Hemminki et al., 1983; Levin, 1983; Tuchmann-Duplessis, 1983). As pain and suffering caused by birth defects to both the victim and his/her family are immeasurable, birth defects have been considered among the most serious of human health problems (Pruzanski, 1961; Paul and Piazza, 1979).

Cleft Palate

Cleft palate is one of the major birth defects of humans (Elwood and Rogers, 1975; Paul and Piazza, 1979; Bower and Stanley, 1983). As it is a structural defect in the roof of the oral cavity, a cleft palate also affects the functions of mastication, deglutition, respiration and phonation. A child born with cleft palate will also present associated dental, emotional and vocational problems. Some of these functional and associated problems may be partially alleviated by surgery and/or a prosthesis, but the economic and social burden for the rehabilitation of the cleft palate individual remains very high (Paul and Piazza, 1979).

The frequency of cleft palate is reported to be, varied among different populations, from 0.05 to 1.87 per 1,000 births (Lowry and Trimble, 1977; Chung et al., 1980; Czeizel, 1980; Koguchi, 1980; Melnick et al., 1980; Shields et al., 1981; Iregbulem, 1982; Bonaiti et al., 1982; Chapman, 1983). A recent survey by Shields et al. (1981) has indicated that heritable factors can be traced only in 10-20% of cases of cleft palate. It is plausible that environmental factors, including teratogens, may account either directly or

jointly for a large proportion of cleft palate (Czeizel, 1980; Koguchi, 1980; Melnick et al., 1980; Shields, et al., 1981; Tyan, 1982).

The role of environmental factors in cleft palate formation had been implicated to some degree by experimental studies in animals. A series of experiments by Hale (1933, 1935, 1937) and by Warkany and associates (1941, 1943, 1948) showed that numerous congenital malformations, including cleft palate, occurred in the offspring of rodents who were fed nutritionally deficient diets during pregnancy. Subsequently, however, when Baxter and Fraser (1950) observed that cortisone treatment of pregnant mice produced cleft palate in the embryo, an impetus was received in the scientific community to identify other agents which might induce cleft palate in animals and in man.

During the past three decades numerous teratogenic agents which induce cleft palate in laboratory animals have been identified, and are summarized by Kalter and Warkany (1959), Dagg (1966), Schardein (1976), and Shah (1979d). While the search for environmental factors responsible for cleft palate formation continues, recent emphasis has been toward identification of abnormal mechanisms by which cleft palate may develop in an embryo.

The use of teratogenic agents as a tool for the study of cleft palate development has begun to contribute to a better understanding of normal developmental mechanisms, and their failures. More significantly, their use in laboratory animals has helped to define and improve the understanding of the factors involved in both normal and cleft palate development.

In the ensuing literature analysis both the issues involved in normal and abnormal palate development, and various experimental approaches to evaluate them, are reviewed.

Normal Development of the Secondary Palate

The first descriptive study on the normal development of secondary palate was published by Dursy in 1869. On the basis of observations in human embryos, he indicated that the secondary palate develops as two vertical projections (shelves) from the roof of the oronasal cavity lateral to the tongue. The vertical shelves then move to a horizontal position over the dorsal surface of the tongue. Subsequently the medial edges of the horizontal shelves unite with one another to separate the oral and nasal cavities.

Following publication of Dursy's work, numerous studies have been reported in the literature analyzing both in vivo and in vitro morphological, cellular and biochemical aspects of mammalian secondary palate development (Walker and Fraser, 1956; Fulton, 1957; Asling et al., 1960; Wood and Kraus, 1962; Larsson, 1962a; Coleman, 1965; Pourtois, 1966, 1972; Mato et al., 1966; Burdi and Faist, 1967; Andersen and Matthiessen, 1967; Farbman, 1968, 1969; De Angelis and Nalbandian, 1968; Walker, 1969, 1971; Brusati, 1969; Hayward, 1969; Humphrey, 1969; Dostal and Jelnick, 1970; Smiley, 1970, Bollert and Hendrickx, 1971; Andrew and Zimmerman, 1971; Smiley and Koch, 1971, 1975; Chaudhry and Shah, 1973, 1979; Shah and Chaudhry, 1974a, b; Tyler and Koch, 1975, 1977a, b; Goss, 1975; Babiarz et al., 1975; Nanda and Romeo, 1975; Luke, 1976; Shah and Travill, 1976a; Cleaton-Jones, 1976; Greene and Pratt,

1976; Holmstedt and Bagwell, 1977; Ferguson, 1977; Innes, 1978, 1981; Wee et al., 1979; Zimmerman, 1979; Shah, 1979a, b, 1980, 1984; Meller et al., 1980; Tassin and Weill, 1980; Zimmerman et al., 1980; Greene et al., 1981, 1982; Gulamhusein and England, 1982; Greene, 1983; Schupbach, 1983; Schupbach and Schroeder, 1983; Schupbach et al., 1983; Brinkley, 1984). One may infer from these studies that at least three critical events must occur for normal closure of the secondary palate:

1. a change in the direction of palatal shelf development from a vertical position on the side of the tongue, to a horizontal position dorsal to it, i.e., reorientation of the palatal shelf;
2. union between the epithelia of the opposing horizontal palatal shelves to form a seam; and
3. removal of the epithelial seam and establishment of mesenchymal continuity.

On the basis of observations in mammalian embryos several factors have been suggested in the literature to be responsible for the reorientation of palatal shelf. For the sake of simplicity and brevity the proposed factors may be grouped under the headings "extrinsic" and "intrinsic", and are summarized in Table I.

Recent reviews indicate that although extrinsic factors may play a role in fostering an intraoral environment for palatal shelves to undergo reorientation, they are probably not responsible for the actual movement of the shelves (Babiarz et al., 1975; Ferguson, 1978; Wee et al., 1979; Shah, 1979a; Zimmerman, 1979; Zimmerman et al., 1980; Greene, 1983). The

Table I. Factors Suspected to be Involved in Palatal Shelf Reorientation
(References)

Extrinsic

- A. Involving descent, depression or movement of the tongue (Asling et al., 1960; Coleman, 1965; Humphrey, 1969; Burdi and Silvey, 1969; Walker, 1969, 1971; Walker and Ross, 1972; Wragg et al., 1972; Diewert, 1974; Taylor, 1978).
- B. Involving changes in cranial base angulation (Verrusio, 1970; Brinkley and Vickerman, 1978).

Intrinsic

- A. Cell proliferation (Wood and Kraus, 1962; Mott et al., 1969; Jelnick and Dostal, 1973; Nanda and Romeo, 1975).
- B. Synthesis of extracellular matrix (Walker, 1961; Larsson, 1962a; Jacobs, 1964a, b, 1966; Anderson and Matthiessen, 1967; Andrew and Zimmerman, 1971; Ferguson, 1977, 1978; Brinkley, 1980; Jacobson, 1982).
- C. Cell movement (Babiarz et al., 1975; Wee et al., 1979; Shah, 1979a; Innes, 1981).

reorientation of the palatal shelf in mammals seems to occur via a remodelling process involving development of a bulge from the medial aspect of the vertical shelf over the dorsal surface of the tongue, with a simultaneous retraction of the vertical shelf (Walker and Fraser, 1956; Kochhar and Johnson, 1965; Greene and Kochhar, 1973; Shah and Travill, 1976a; Shah, 1979a), rather than by rotation (Coleman, 1965; Walker and Ross, 1972; Diewert, 1974; Babiarz et al., 1975). In 1956, Walker and Fraser suggested that an "intrinsic force" may be responsible for the reorientation of the palatal shelves via a remodelling process. Since then, using both in vivo and in vitro techniques, several intrinsic cellular and biochemical events have been analyzed (Table I) to determine the nature of "intrinsic force" (Larsson, 1962a; Anderson and Matthiessen, 1967; Mott et al., 1969; Babiarz et al., 1975; Nanda and Romeo, 1975; Ferguson, 1978; Shah, 1979a; Wee et al., 1979; Brinkley, 1980). The precise nature of the intrinsic force during palatal shelf reorientation, however, still remains obscure.

As the palatal shelves reorient from a vertical to a horizontal plane, and then approximate, the bilaminar epithelium of their prospective medial edges undergoes cytodifferentiation. The medial edge epithelial cells (MEE) (1) stop proliferating, as confirmed by an absence of ^3H -thymidine incorporation (Hudson and Shapiro, 1973; Pratt and Martin, 1975; Shah et al., 1985); (2) form increasing numbers of lysosomes as palatogenesis progresses (Mato et al., 1966; Brusati, 1969; Chaudhry and Shah, 1973, 1979; Shah and Chaudhry, 1974a, b; Lorente et al., 1974; Holst and Mills, 1975; Im and Mulliken, 1983), and (3) show an increasing concentration of cyclic AMP with

progressive differentiation (Pratt and Martin, 1975; Olson and Massaro, 1977; Greene and Pratt, 1979; Greene et al., 1980; Shah et al., 1985).

Simultaneously the mesenchymal cells elongate and develop intracytoplasmic filaments (Babiarz et al., 1975; Shah, 1979a; Innes, 1981). The filaments appear to be contractile in nature (Krawczyk and Gillon, 1976), and thus may allow cells to move during reorientation. Also there is a concurrent increase in the synthesis of glycosaminoglycans, especially hyaluronic acid, in the extracellular matrix of the mesenchyme (Larsson, 1962a; Jacobs, 1964a, b; 1966; Pratt et al., 1973; Ferguson, 1978; Brinkley, 1980; Jacobson and Shah, 1981; Jacobson, 1982). It has been implied that glycosaminoglycans facilitate the movement of cells during palatal shelf reorientation (Shah, 1979a, c; Jacobson, 1982; Brinkley and Vickerman, 1982).

While the debate on mechanisms of palatal shelf reorientation still continues, it is only recently that some attention has been directed towards characterization of the process by which the opposing palatal shelves unite with one another. The union between the opposing palatal shelves is a multistep process which initially involves "adhesion" between opposing epithelia, followed by their fusion to form a seam, and finally replacement of the seam by mesenchyme (Barry, 1961; Pourtois, 1972).

Originally Farbman (1968, 1969), Hayward (1969), Greene and Kochhar (1974), Hinrichsen and Stevens (1974), Souchon (1975), Pratt and Hassell (1975), De Paola et al., (1975), Meller and Barton (1978) alluded to the possibility that a sticky substance, probably of a complex carbohydrate nature, may be responsible for the formation of the epithelial seam. Recent

experimental evidence, however, suggests that carbohydrates are probably only involved in the initial adhesion (Shah, 1979b; Shah and Crawford, 1980; Heinen et al., 1982; Baeckeland et al., 1982; Pratt, 1983; Greene, 1983). Fusion of the shelves to form a seam may involve elimination of the superficial epithelial cells via an autolysosomal process or desquamation (Pourtois, 1966; Chaudhry and Shah, 1973, 1979; Shah and Chaudhry, 1974a, b; Shah, 1979b, Baeckeland et al., 1982; Heinen et al., 1982; Schupbach et al., 1983; Schupbach and Schroeder, 1983), followed by the formation of desmosomes between deeper epithelial cells of the opposing palatal shelves (De Angelis and Nalbandian, 1968; Brusati, 1969; Smiley and Koch, 1971; Chaudhry and Shah, 1973, 1979; Shah and Chaudhry, 1974b; Shah, 1979b; Pratt, 1983; Greene, 1983). Once the seam is formed, then the remaining epithelial cells must be eliminated to achieve a mesenchymal union. It is generally accepted in the literature that elimination of cells from the epithelial seam can occur through two pathways, i.e., intracellular lysosomal autophagy, and/or exfoliation of degenerating cells into the amniotic fluid or into mesenchyme (Mato et al., 1966, 1967a, b; De Angelis and Nalbandian, 1968; Brusati, 1969; Hayward, 1969; Chaudhry and Shah, 1973, 1979; Shah and Chaudhry, 1974a, b; Greene and Pratt, 1976; Greene, 1983). In the latter instance, the debris of degenerated epithelial cells are phagocytosed by macrophages.

Abnormal Development of the Secondary Palate

It is generally accepted in the literature that cleft palate following teratogenic treatment can be induced in laboratory animals by affecting any one of the three critical events of normal palate morphogenesis. For example, studies in mice, rats and hamsters have shown that cortisone, triamcinolone, radiation, high doses of vitamin A, folic acid deficiency, diazo-oxo-norleucine, 5-fluorouracil, and 5-fluoro-2-desoxyuridine induce cleft palate by delaying reorientation of the palatal shelves from a vertical to a horizontal plane (Walker and Fraser, 1957; Walker and Crain, 1960; Asling et al., 1960; Callas and Walker, 1963; Kochhar and Johnson, 1965; Coleman, 1965; Ross and Walker, 1967; Dostal and Jelinek, 1972; Ferguson, 1977; Shah, 1979e; Diewert, 1979; Diewert and Pratt, 1979; Shah and Wong, 1980). Hydrocortisone treatment of pregnant hamsters affects the fusion process in fetuses (Shah and Travill, 1976a, b). Indirect evidence has been presented to suggest that a cleft palate may develop by the rupture of a previously formed epithelial seam, or by the rupture of a mesenchymally united palate (Kitamura, 1966; Angelici, 1968).

While studies on analysing morphological aspects of palate development following teratogenic treatment continue, efforts have also been directed toward exploring cellular and biochemical alterations associated with cleft palate development.

Considerable efforts in cellular and biochemical aspects of cleft palate development have been directed toward understanding the mechanism of glucocorticoid induced cleft palate. The suggestion that glucocorticoid

treatment induced cleft palate by inhibiting the synthesis of glycosaminoglycans, mainly hyaluronic acid (Walker, 1961; Larsson, 1962b; Jacobs, 1964a, b; Pratt et al., 1973; Ferguson, 1978) has been disputed (Nanda, 1971; Andrew and Zimmerman, 1971). Recently inconclusive evidence has indicated that glucocorticoid action may be mediated via its binding to receptors in the palatal tissue (Goldman et al., 1977, 1978; Bekhor et al., 1978; Salomon and Pratt, 1979; Shah and Burton, 1980), which may subsequently lead to inhibition of intracellular protein synthesis (Zimmerman et al., 1970) and eventually to premature necrosis of palatal cells (Shah and Travill, 1976b; Shah, 1980; Kurisu et al., 1981). Mechanisms regulating the binding of glucocorticoid molecules to the receptors and the inhibition of protein synthesis, however, have not yet been clarified.

In recent years attempts have been made at analyzing the ultrastructural and biochemical effects of teratogens other than glucocorticoids on palatal tissues during development (Table II). One may deduce from Table II that depending on the chemical nature of the teratogenic agent, different aspects of growth and differentiation of either epithelial, or mesenchymal or both tissues may be affected.

6-Mercaptopurine

In 1952 Elion and associates reported the synthesis of a purine analogue, 6-mercaptopurine (6MP). The chemical is a yellowish, odourless, crystalline powder, insoluble in water, acetone, chloroform and diethyl

Table II. A Summary of Ultrastructural and Biochemical Studies of Teratogen Induced Cleft Palate Development

<u>Agent (species)</u>	<u>Mechanism</u>	<u>References</u>
<u>Ultrastructural Studies</u>		
Vincristine Sulfate & acridine orange (rat)	Inhibition of cell death	Mato & Uchiyama, 1972
Meclozine (rat)	Effect on the epithelial cell plasmamembrane permeability	Mato et al., 1975a Mato & Uchiyama, 1975
	Factors extrinsic to shelves; did not observe any cellular effects	Morgan, 1976
β -aminopropionitrile (rat)	Retarded differentiation in epithelium	Mato et al., 1975b
Hydrocortisone (hamster)	Premature necrosis of the basal epithelial cells	Shah & Travill, 1976b
Diazo-oxo-norleucine (rat)	Alteration in intracellular distribution of lysosomal enzymes	Greene & Pratt, 1978
Triamcinolone (hamster)	Alterations at the epithelial-mesenchymal interface	Shah, 1980
(mouse)	Inhibition of cell proliferation	Kurisu et al., 1981
Phenylbutazone (mouse)	Alterations at the epithelial-mesenchymal interface	Montenegro & Paz de la Vega, 1982
5-fluorouracil (hamster)	Premature, sublethal injury of the mesenchymal cells	Shah et al., 1984a

Table II. (continued)

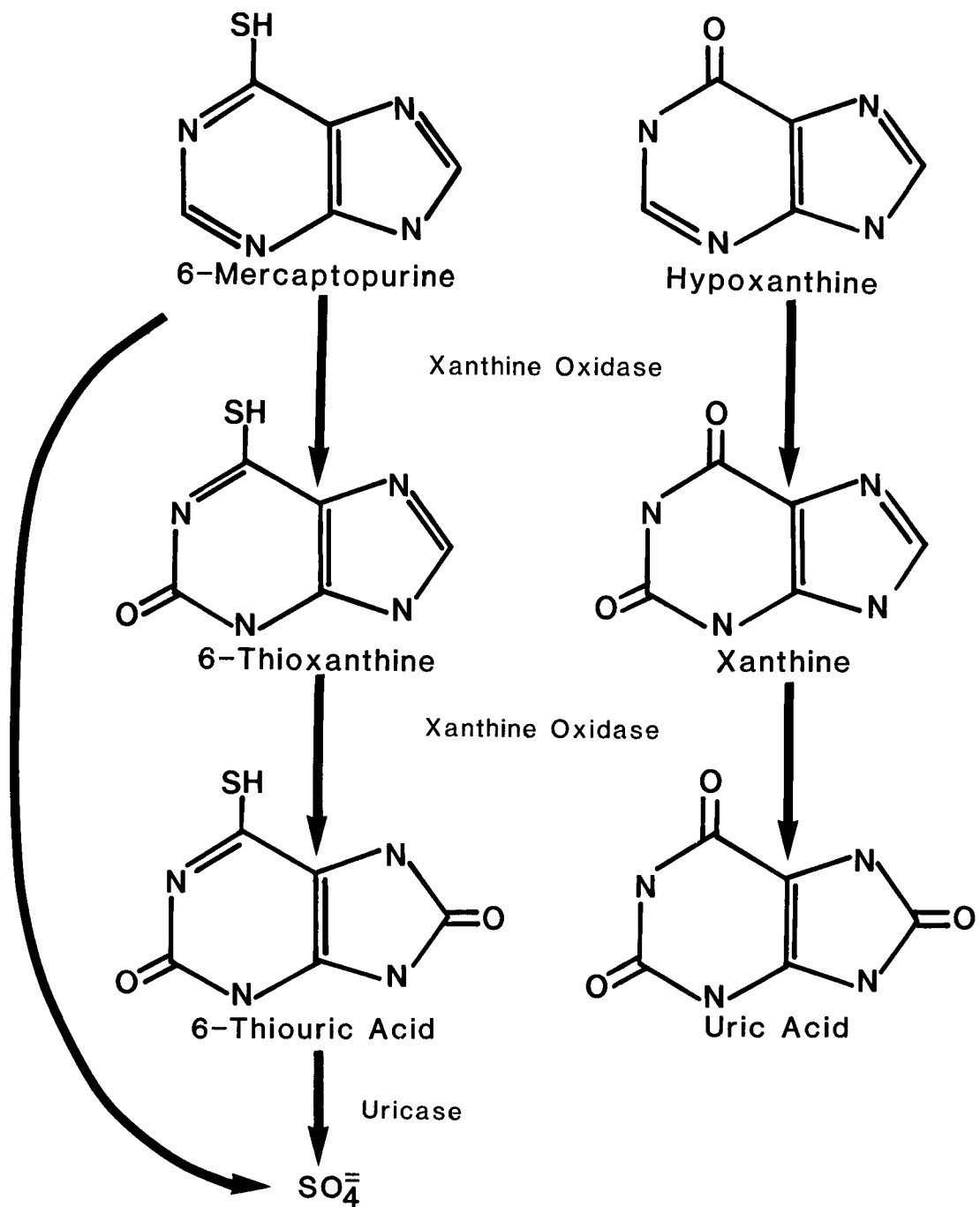
<u>Agent (species)</u>	<u>Mechanism</u>	<u>References</u>
<u>Biochemical Studies</u>		
Hypervitaminosis A (rat)	Increased synthesis of DNA and glycoprotein	Lorente & Miller, 1978
(rat)	Inhibition of DNA, collagen and glycosaminoglycan synthesis	Sauer & Evans, 1980
(rat)	Inhibition of DNA synthesis	Pick & Evans, 1981
β -aminopropionitrile (rat)	Inhibition of collagen synthesis	Pratt & King, 1972
(rat)	Stimulation of glycosaminoglycan catabolism	Del Balso & Kauffman, 1975
Diazo-oxo-norleucine (rat)	Inhibition of mucopolysaccharide synthesis	Pratt et al., 1973
(rat)	Block glucosamine formation	Pratt & Greene, 1976
(rat)	Inhibit glycoprotein synthesis	Greene & Pratt, 1977
Salicylate (mouse)	Inhibit mucopolysaccharide synthesis	Larsson & Bostrom, 1965
2-deoxyglucose (rat)	Decreased synthesis of lysosomal enzymes	Pratt & Greene, 1976
Epidermal growth factor (rat)	Reduced levels of cAMP	Hassel & Pratt, 1977
Methylmercury (mouse)	Impaired placental transfer of aminoacids	Olson & Massaro, 1977
5-fluoro-2-desoxyuridine (rat)	Reduced synthesis of mucopolysaccharide	Ferguson, 1977
Chlorcyclizine (rat)	Degradation of glycosaminoglycans	Wilk et al., 1978
Phenytoin (mouse)	Reduced protein and RNA synthesis	Sonawane & Goldman, 1981

ether, but soluble in boiling water, alkaline solutions, and warm ethanol (Windholz, 1976). It has a molecular weight of 152.19 and a melting point of 313°C.

When administered intraperitoneally to mammals a large proportion (21.4%) of 6MP is excreted unchanged in the urine during the following 24 hours, and the remainder as catabolites (Fig. 1), mainly 6-thiouric acid (18.9%) and inorganic sulphate (29.5%) (Elion et al., 1963). After intravenous administration, the drug concentration rapidly becomes high within many organs. In liver the concentration reaches four times that of plasma within minutes, but falls rapidly thereafter in both the organs and plasma (Tterlikkis et al., 1977). The half life of 6MP appears to be very short. After intravenous administration of 6MP, the plasma half life of the drug was 14 minutes in mice, 9 minutes in rats (Donelli et al., 1972), and 1.5 hours in humans (Hamilton and Elion, 1954).

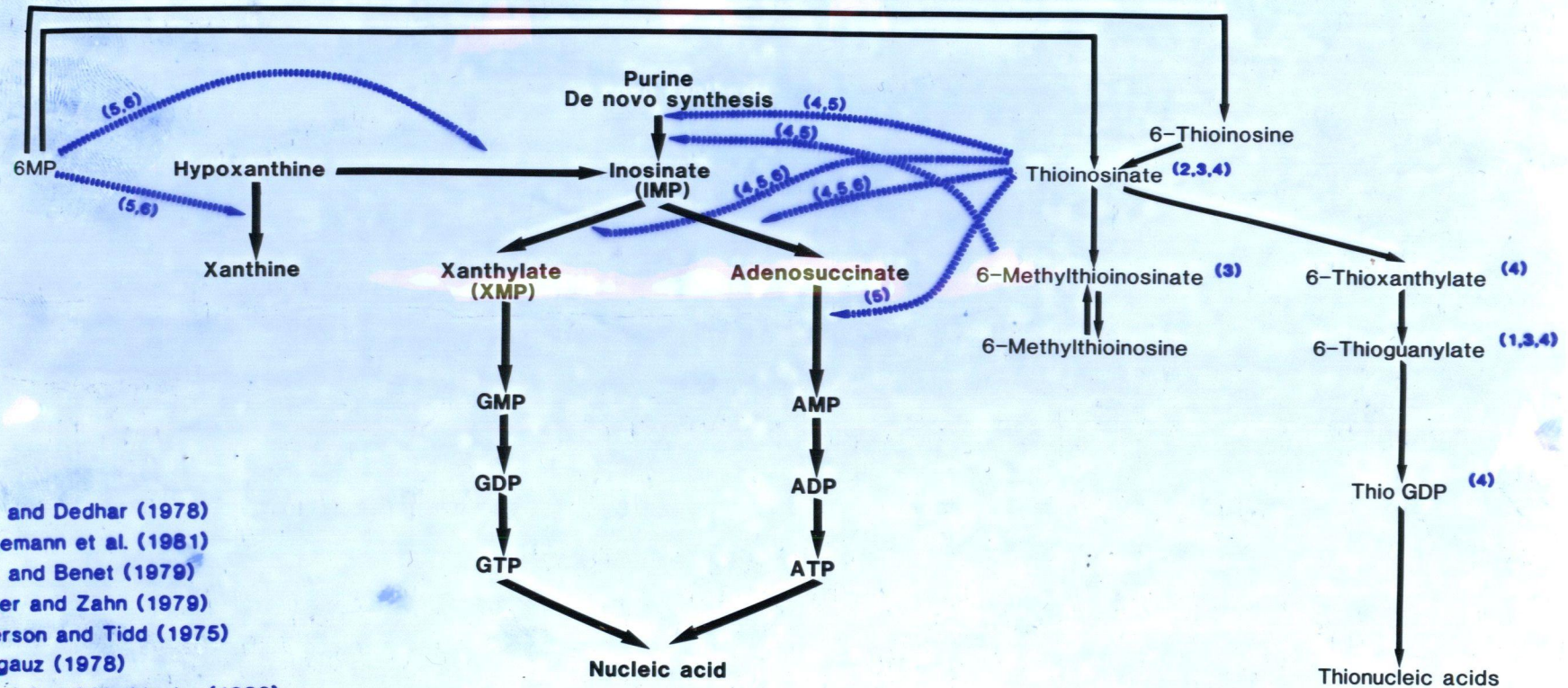
Although the precise mechanism by which 6MP exerts its effect is uncertain, it is generally agreed that the drug eventually inhibits the synthesis of purine. Several possible biochemical sites of action for inhibition of purine synthesis by 6MP have been suggested (Elion, 1967; Paterson and Tidd, 1975; Tidd and Dedhar, 1978; Gringauz, 1978; Ding and Benet, 1979; Breter and Zahn, 1979; Plagemann et al., 1981; Lennard and Maddocks, 1983), but the exact mechanism of its action is not yet clear. Acting in vitro as a free base, the chemical competitively inhibits xanthine oxidase and hypoxanthine-guanine phosphoribosyl transferase which convert hypoxanthine to xanthine and inosinate respectively (Fig. 2). However, in vivo the drug is anabolised to 6-methylthioinosinate and thioinosinate, which

Fig. 1 Catabolism of 6-Mercaptopurine and Hypoxanthine



Adapted from Gringauz (1978)

Fig. 2 Proposed Sites of Action of 6-Mercaptopurine (6MP) and its Anabolites on
6MP and Purine Metabolism



- 1) Tidd and Dedhar (1978)
- 2) Plagemann et al. (1981)
- 3) Ding and Benet (1979)
- 4) Breter and Zahn (1979)
- 5) Paterson and Tidd (1975)
- 6) Gringauz (1978)
- 7) Lennard and Maddocks (1982)

may act to inhibit de novo synthesis of purines (Paterson and Tidd, 1975; Breter and Zahn, 1979). Paterson and Tidd (1975), Gringauz (1978), and Breter and Zahn (1979) have indicated that thioinosinate may also act to inhibit purine ribonucleotide metabolism and its transconversions (Fig. 2). Others (Tidd and Dedhar, 1978; Ding and Benet, 1979; Breter and Zahn, 1979; Lennard and Maddocks, 1983), however, stress that the drug acts via the conversion of 6-thioinosinate into 6-thioxanthylate, leading to the formation of thionucleic acids (Fig. 2). Several researchers have also indicated that the effect of 6MP may be mediated via directly affecting mRNA synthesis (Roy-Burman, 1970; Neubert et al., 1970; Mewes et al., 1971). Recently Kawahata et al. (1980) suggested that 6MP may inhibit DNA-dependant RNA polymerase activity which in turn reduces RNA synthesis. It appears that eventually the interference with one or more of these aforementioned pathways is perhaps responsible for the growth suppressive effect which has made 6MP useful in the treatment of various forms of leukemia (Calabresi and Parks, 1975). The chemical also has immunosuppressive properties, and is considered useful in the treatment of ulcerative colitis (IARC, 1981).

The embryotoxic and teratological effects of 6MP are well documented in echinoderms and vertebrates (tadpole, chick, rat, mouse, rabbit, hare and hamster) (Bieber et al., 1952; Thiersch, 1954; Zunin and Borrone, 1955; Didcock et al., 1956; Tuchmann-Duplessis and Mercier-Parot, 1958, 1966, 1968; Karnofsky, 1960; Murphy, 1960, 1962; Blattner et al., 1960; Adams et al., 1961; Mercier-Parot and Tuchmann-Duplessis, 1967; Vishniakov, 1968, 1969; Bragonier and Carver, 1968; Kury et al., 1968; Adhami and Noack, 1975; Asisi and Merker, 1975; Merker et al., 1975; Puget et al., 1975; Grubb and

Montiegel, 1975; Neubert et al., 1977; Adhami, 1979; Shah and Burdett, 1979). In mammals the embryotoxic and teratological effects are manifested only after the 6MP has crossed the placenta to the fetus, where it is then anabolized by the fetus into an active form (Neubert et al., 1980). The teratogenic effects were characterized by malformations of limbs, beak, eye, brain, palate, teeth, salivary glands, tongue, mandible, respiratory tract, alimentary tract, liver, kidney and tail. In addition, sterility has been observed during postnatal life in both male and female animals which were exposed to 'sub-teratological' doses of 6MP during gestation (Reimers and Sluss, 1978; Reimers et al., 1980).

Review of the literature indicates that in at least 21 human pregnancies 6MP was administered during the first trimester. Of the twenty-one, three mothers died undelivered (Raichs, 1962; Nicholson, 1968a), eight aborted fetuses (Thiersch, 1956; Rothberg et al., 1959; Boggs et al., 1962; Raichs, 1962; Bilski-Pasquier et al., 1962; Hoover and Schumacher, 1966), one delivered a 'dysmature' infant who died shortly after birth (Merskey and Rigal, 1956), one delivered an anaemic infant (McConnell and Bhoola, 1973), seven delivered normal infants (Diamond et al., 1960; Frenkel and Meyers, 1960; Mangiameli, 1961; Sinykin and Kaplan, 1962; Bilski-Pasquier et al., 1962; Ravenna and Stein, 1963; Nicholson, 1968a), and one delivered a malformed infant who died at 10 weeks of age (Diamond et al., 1960). The malformed infant had multiple defects including a low birth weight, cloudy cornea, poorly developed genitalia, and a cleft of the soft and posterior hard palate. The mother of the malformed infant had chronic granulocytic leukaemia. For the treatment of her disease she received 100 mg 6MP per day

orally during the first six weeks of gestation, and a single treatment of 200 r of radiation during the first month of gestation. After six weeks 6MP administration was stopped in favour of 4 to 6 mg Busulfan per day for an additional thirty weeks of gestation, followed by 125 mg 6MP per day till term.

In addition, twenty-eight mothers have been reported to have received 6MP treatment during the second and third trimesters of pregnancy. Of these twenty-eight cases, three mothers died undelivered (Nicholson, 1968a,b), one aborted (O'Leary and Bepko, 1963), one had a still birth (Parekh et al., 1959), and twenty-three delivered normal infants (Schumacher, 1957; Hill, 1958; Rothberg et al., 1959; Frenkel and Meyers, 1960; Hill and Loeb, 1960; Sandberg, 1960; Loyd, 1961; Lee et al., 1962; Elizarova and Stupnitskaya, 1962; Olmer and Carcassonne, 1962; Raichs, 1962; Neu, 1962; Bilski-Pasquier et al., 1962; Stewart, 1964; Rigby et al., 1964; Rezende et al., 1965; Nicholson, 1968b).

A high incidence of fetal loss and abnormalities (11/21) following 6MP administration during the first trimester suggests that perhaps the drug may also be fetotoxic and teratogenic in humans.

PURPOSE OF THE STUDY

The foregoing analysis of the literature indicates that the sequence of events during morphogenesis and histogenesis of the secondary palate have been repeatedly studied in different mammals. These studies were mainly concerned with events and issues involved during the reorientation of palatal shelves from a vertical to a horizontal position, and their subsequent fusion. No attention, however, was paid to the events leading to the development of palatal shelves in the vertical direction in any species. One of the purposes of this investigation, therefore, was to describe the vertical development of hamster palatal shelves using both light and electron microscopy.

Cleft palate is one of the major malformations induced by GMP in mammals. When 70 mg/kg body weight of GMP was administered intraperitoneally into hamsters on day 9:00 of gestation, all the fetuses at term showed cleft palate without an appreciable embryotoxic effect (Shah and Burdett, 1979). Another purpose of the present study was, therefore, to analyze hitherto unreported aspects of the pathogenesis of GMP induced cleft palate using light and electron microscopy, and enzyme cytochemistry.

MATERIALS AND METHODS

Breeding of Animals

Seven-week-old male and female Golden Syrian hamsters were obtained from Simonsen Laboratories, California, U.S.A. The animals were caged singly and maintained in an environment of temperature ($24 \pm 1^{\circ}\text{C}$), humidity ($50 \pm 5\%$), and an alternate cycle of light (6 a.m. - 6 p.m.) and dark. Food and water were available ad libitum. In the present study 200 female hamsters were utilized.

Following at least one week of environmental acclimatization, each female (80 ± 5 grams) was placed with a male in the breeding cage, and copulation was observed. The mating was allowed to continue from 7 p.m. to 9 p.m. The midpoint of the mating period, 8 p.m., was considered as day 0 (0 days : 0 hours) of gestation.

Drug Treatment and Procurement of Tissues

On day 9:00 of gestation, each female was given an intraperitoneal injection of either 70 mg/kg 6MP (Sigma Chemicals, St. Louis, MO, Catalog #M7000, Lot #82C-1130) suspended in 1 ml distilled water, or 1 ml distilled water. The latter animals served as controls. A group of drug treated and control animals were killed at six hour intervals between days 9:00 and 14:00, and then on day 15:00 of gestation. The fetuses were delivered by caesarian section and immediately immersed in the appropriate fixative as described below.

For Gross and Light Microscopic Studies

For macroscopic and light microscopic studies the fetuses were immersed in Bouin's solution for 48 hours. They were then dehydrated through an ascending concentration of ethanol, starting at 30%. Upon reaching a 70% concentration of ethanol, each fetus was weighed, and measured for crown-rump length (CRL). The status of palate development was ascertained and recorded. The fetuses were then further processed through higher concentrations of ethanol (80%-100%), and then through three changes of chloroform, and embedded in paraffin to procure frontal sections. Six micron serial sections were stained with Hematoxylin and Eosin.

For Electron Microscopic Studies

For electron microscopic studies the fetal heads were immersed in 0.1 M phosphate buffered 2.5% gluteraldehyde (pH 7.3) at 0-4°C (Sabatini et al., 1963) for 8-12 hours. They were then given four 10 minute rinses of 0.1 M phosphate buffer (pH 7.3) at 0-4°C. The tongue and mandible were dissected from the fetal heads and discarded. The fetal heads were then immersed in 1% phosphate buffered osmium tetroxide for 90-120 minutes in the dark at 0-4°C. The heads were then quickly washed with 0.1 M phosphate buffer (pH 7.3; 0-4°C) followed by a rinse with distilled water (0-4°C). Subsequently the tissues were dehydrated through ascending concentrations of ethanol starting from 30%. Upon reaching 100% ethanol, the tissues were processed through two changes of propylene oxide. The heads were then immersed in a 3:1, and then a 1:1 mixture of propylene oxide:Epon-Araldite for at least 30 minutes each, and left overnight in 1:3 propylene oxide:Epon-Araldite mixture for

infiltration. The next morning the tissues were transferred into freshly prepared Epon-Araldite embedding medium and placed under 25 lb. vacuum for thirty minutes. Prior to embedding dimethylaminomethyl phenol (DMP-30) was added as an accelerator to the Epon-Araldite embedding medium to achieve a 2% concentration and then thoroughly mixed (Mollenbauer, 1964). The palates were trimmed by removing extraneous tissues and then cut into anterior and posterior halves. The cut palates were placed in the Beem capsule to procure frontal sections, embedded in Epon-Araldite containing 2% accelerator, and placed in a dry heat oven at 37°C for 2 hours to eliminate gases and to permit initial polymerization. Subsequently the capsules were transferred to a 60°C dry heat oven for 3-4 days. From the polymerized blocks one micron sections were stained with 1% Toluidine Blue, and examined for identification and orientation purposes. Thin sections (60 nm) were obtained from appropriate blocks and transferred to 300 mesh copper grids. They were then stained with methanolic uranyl acetate (Stempak and Ward, 1964) for fifteen minutes, followed by lead citrate (Reynolds, 1963) for five minutes. The grids were examined in a Philips 300 Electron Microscope operating at an accelerating potential of 60 KV.

For Acid Phosphatase Enzyme Cytochemistry

For acid phosphatase enzyme cytochemistry, fetal heads were immersed in formol-calcium (0-4°C) for 24-36 hours. The heads were placed on a cryostat tissue holder (International Harris Cryostat model CTD, International Equipment Corp., Needham Heights, Mass.) in a pool of Tissue Tek (Lab-Tek Products, Naperville, Ill.) and quickly frozen in a -30°C cryostat. Eight

micron serial sections were cut and transferred to cold pre-cleaned glass slides. The sections were air dried for one hour, and then incubated in a mixture (Appendix I) containing naphthol AS-TR phosphoric acid, pararosaniline hydrochloride, sodium nitrite, and veronal acetate buffer at 37°C for 90 minutes in a dry heat oven (Barka and Anderson, 1962). After incubation the slides were quickly washed in distilled water, stained for 30 seconds in methyl green (pH 4.0), and washed again in distilled water. They were then quickly dehydrated by immersion in 50%, 70%, 95%, and absolute ethanol, cleared in xylene, and mounted in DPX (BDH chemicals, Vancouver, Product Code 3303, Lot # 505309). The enzyme activity was indicated by a vivid red azo dye.

Two controls were used for the incubation reaction: for a negative control sections from each fetal head were incubated in a substrate-free medium, which was otherwise identical; for a positive control sections of normal rat liver were included in each incubation jar with the fetal head sections.

Statistical Analysis

For statistical analysis the data were subjected to the following methods. The correlations between gestational age, CRL and fetal weight were evaluated by a linear regression and exponential regression (Sokal and Rohlf, 1969). The regression lines for treated and control data were compared to assess significance. The palatal staging was tabulated against gestational age, CRL and fetal weight and evaluated by contingency table analysis (Zar,

1974). Standard curves illustrating the probability of each palatal stage in relation to gestational age, CRL and fetal weight were obtained and evaluated for significance by the chi-square method (Berkson, 1957).

RESULTS

Morphogenesis of the Secondary Palate in Control Hamster Fetuses

Based on gross observations, which were later confirmed histologically, the morphogenesis of control secondary palate can be categorized within one or another of the following stages:

Stage 1. The appearance of the palatal primordia from the roof of the oronasal cavity toward its floor (Figs. 3,4). The tongue is absent (Fig. 4).

Stage 2. The palatal shelves are vertical on the sides of the tongue, which originates from the floor of the oronasal cavity (Figs. 5, 6).

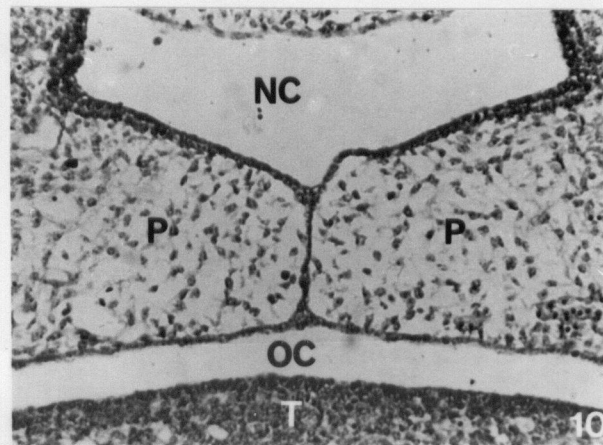
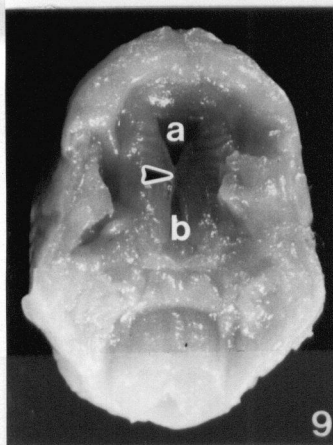
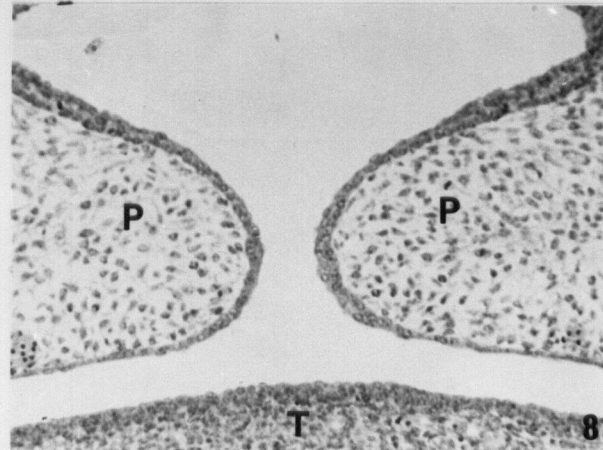
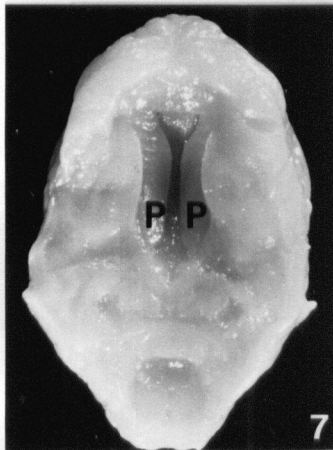
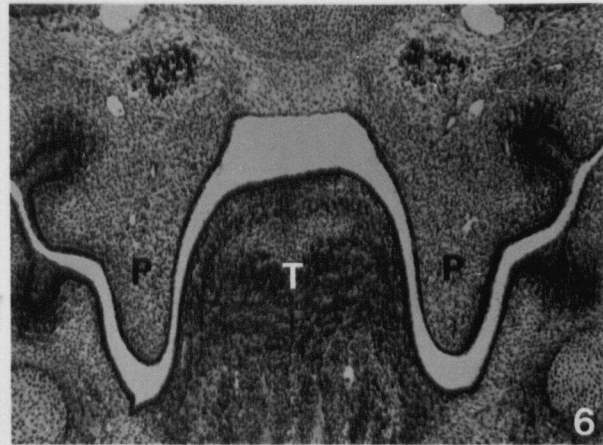
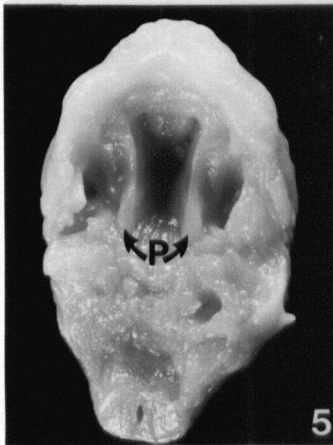
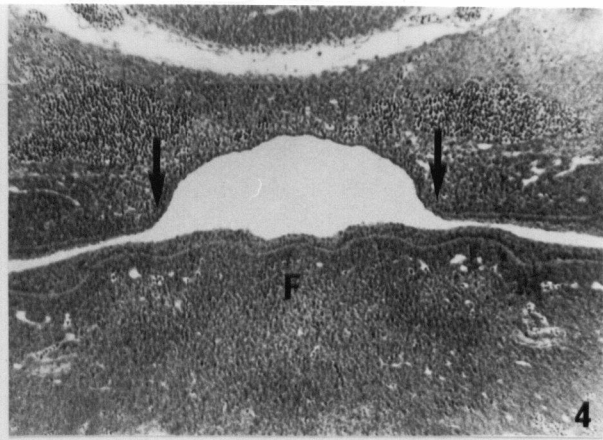
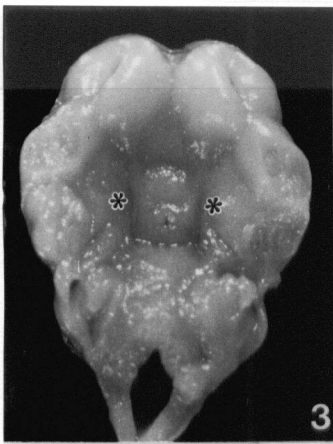
Stage 3. The palatal shelves are horizontal above the tongue (Figs. 7, 8).

Stage 4. The palatal shelves unite in the middle third, but remain open in the anterior and posterior thirds of the secondary palate (Figs. 9, 10).

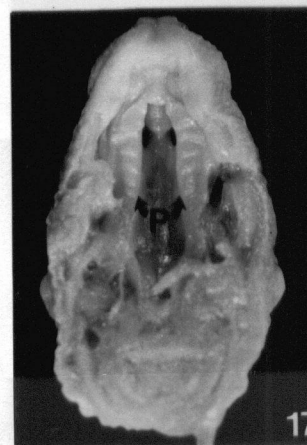
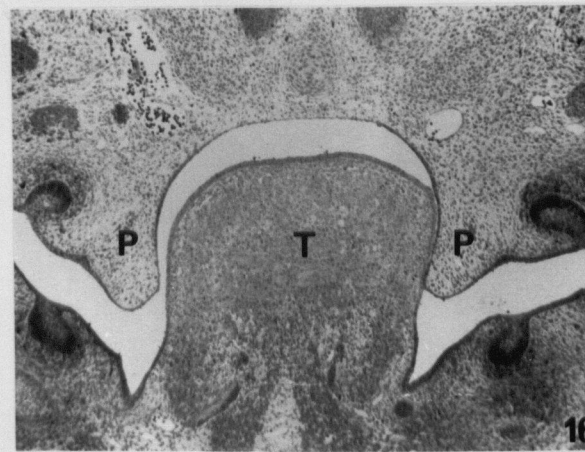
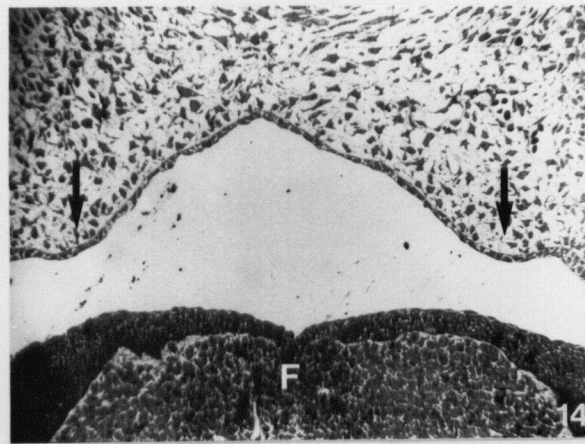
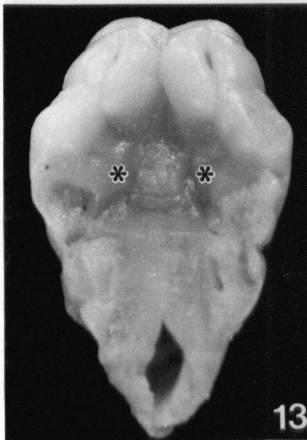
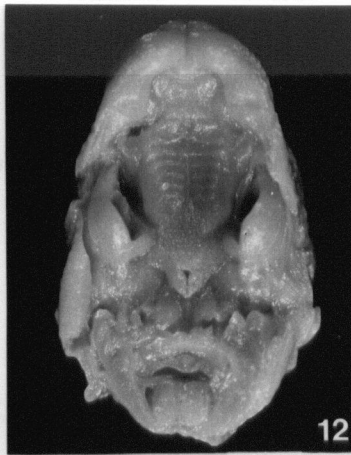
Stage 5. The palatal shelves unite in the posterior third of the secondary palate. The anterior third remains open (Fig. 11).

Stage 6. The palatal shelves unite in the anterior third of the secondary palate, and with this event the closure of the palate is complete (Fig. 12).

The data on gross observations of normal palate development in relation to gestational age of the fetus are presented in Table III. One may deduce from the table that:



- Fig. 3** Ventral view of the developing palate of a control hamster on day 10:00 of gestation. The mandible has been removed. Stage 1. The palatal primordia (asterisk) appear from the roof of the oronasal cavity. 16.5X.
- Fig. 4** Frontal section through the secondary palate region in a control hamster fetus on day 10:00 of gestation. Stage 1. The palatal primordia (arrows) develop from the roof of the oronasal cavity towards the floor (F). The tongue is absent. Paraffin section. Hematoxylin and Eosin stain. 58X.
- Fig. 5** Ventral view of the developing palate of a control hamster on day 11:18 of gestation. The mandible and tongue have been removed. Stage 2. The palatal shelves (P) are in vertical position. 8.5X.
- Fig. 6** Frontal section through the secondary palate region in a control hamster fetus on day 11:18 of gestation. Stage 2. The palatal shelves (P) are vertical on the sides of the tongue (T). Paraffin section. Hematoxylin and Eosin stain. 75X.
- Fig. 7** Ventral view of the developing palate of a control hamster on day 12:00 of gestation. The mandible and tongue have been removed. Stage 3. The palatal shelves (P) are horizontal. 8.5X.
- Fig. 8** Frontal section through the secondary palate region in a control hamster fetus on day 12:00 of gestation. Stage 3. The palatal shelves (P) are horizontal above the tongue (T). Paraffin section. Hematoxylin and Eosin stain. 258X.
- Fig. 9** Ventral view of the developing palate of a control hamster on day 12:00 of gestation. The mandible and tongue have been removed. Stage 4. The palatal shelves unite in the middle third (arrow head), but remain open in the anterior (a) and posterior (b) thirds of the secondary palate. 8.5X.
- Fig. 10** Frontal section through the secondary palate region in a control hamster fetus on day 12:00 of gestation. Stage 4. The opposing palatal shelves (P) are fused with one another. NC - nasal cavity. OC - oral cavity. Paraffin section. Hematoxylin and Eosin stain. 255X.



- Fig. 11** Ventral view of the developing palate of a control hamster on day 12:18 of gestation. The mandible and tongue have been removed. Stage 5. The palatal shelves unite in the posterior third (b) of the secondary palate. The anterior third (arrowhead) remains open. 6X.
- Fig. 12** Ventral view of the developing palate of a control hamster on day 13:00 of gestation. The mandible and tongue have been removed. Stage 6. The palatal shelves are united in the anterior third, thus marking a complete closure of the secondary palate. 6X.
- Fig. 13** Ventral view of the developing palate of a 6MP treated hamster on day 10:00 of gestation. The mandible has been removed. Stage 1. The palatal primordia (asterisk) appear from the roof of the oronasal cavity. 16.5X.
- Fig. 14** Frontal section through the secondary palate region in a 6MP treated hamster fetus on day 10:00 of gestation. Stage 1. The palatal primordia (arrows) develop from the roof of the oronasal cavity towards the floor (F). Epon-araldite section. Methylene blue stain. 92X.
- Fig. 15** Ventral view of the developing palate of a 6MP treated hamster on day 11:18 of gestation. The mandible and tongue have been removed. Stage 2. The palatal shelves (P) are vertical. 9X.
- Fig. 16** Frontal section through the secondary palate in a 6MP treated hamster fetus on day 11:18 of gestation. Stage 2. The palatal shelves (P) are vertical on the sides of the tongue (T). Epon-araldite section. Methylene blue stain. 75X.
- Fig. 17** Ventral view of the developing palate of a 6MP treated hamster on day 15:00 of gestation. The mandible and tongue have been removed. Stage 2. The palatal shelves (P) are vertical. 5X.
- Fig. 18** Frontal section through the secondary palate in a 6MP treated hamster fetus on day 15:00 of gestation. Stage 2. The palatal shelves (P) are rudimentary. T - tongue. Bone formation (B) is present within the shelves. Paraffin section. Hematoxylin and Eosin stain. 40X.

Table III. Staged Development of the Secondary Palate in Control Hamster Fetuses

Gestational Age (days:hours)	Number of Litters	Number of Fetuses at Each Stage of Palatal Development					
		1	2	3	4	5	6
9:06	5	51*					
9:12	5	61*					
9:18	5	47*					
10:00	6	65					
10:06	5		44				
10:12	6		68				
10:18	5		55				
11:00	5		55				
11:06	5		41				
11:12	5		57				
11:18	5		58	1			
12:00	5		23	13	15		
12:06	5		2	10	33	1	
12:12	5				5	50	8
12:18	4					32	11
13:00	4					8	38
14:00	3						41
15:00	4						45

*Palatal primordia are absent

1. The initiation of the palatal primordia occur between days 9:18 and 10:00 of gestation (Stage 1).
2. The vertical development of the palatal shelves (Stage 2) is accomplished in all fetuses between days 10:06 and 12:00 of gestation.
3. In almost 95% of the fetuses Stages 3 (horizontal shelves), and Stage 4 (closure of the shelves in the middle third of the secondary palate) are achieved between days 12:00 and 12:06 of gestation.
4. In 92% of the fetuses Stage 5 (closure of the shelves in the posterior third of the secondary palate) is completed by day 12:12 of gestation.
5. In over 83% of fetuses Stage 6 (complete closure of the secondary palate) is accomplished by day 13:00 of gestation.

Morphogenesis of the Secondary Palate in GMP Treated Hamster Fetuses

The observations of GMP treated fetal palatal development are summarized in Table IV. From the table, one may suggest that, like the controls, the initiation of the drug treated palatal primordia (Stage 1) occurs between days 9:18 and 10:00 of gestation (Figs. 13, 14). The palatal shelves are oriented vertically on the sides of the tongue (Stage 2) between days 10:06 and 12:00 of gestation (Figs. 15, 16). Subsequently, however, approximately 98% of the drug treated fetuses remained in Stage 2 of palatal development. In these fetuses Stages 3-6 were never accomplished. At term, the palatal

Table IV. Staged Development of the Secondary Palate in 6-Mercaptopurine Treated Hamster Fetuses

Gestational Age (days:hours)	Number of Litters	Number of Fetuses at Each Stage of Palatal Development					
		1	2	3	4	5	6
9:06	3	29*					
9:12	3	24*					
9:18	3	27*					
10:00	5	49					
10:06	3		30				
10:12	3		32				
10:18	3		30				
11:00	4		36				
11:06	5		56				
11:12	3		31				
11:18	3		26				
12:00	4		34				
12:06	4		39	4			
12:12	5		41				
12:18	5		45	6			
13:00	6		48	3			
14:00	4		36				
15:00	4		35				

*Palatal primordia are absent

shelves are rudimentary and oriented vertically on the sides of the tongue (Figs. 17, 18).

Morphogenesis of the Secondary Palate in Control Hamster Fetuses in Relation to Fetal Weight

The mean weight of control fetuses increases gradually from 11.7 mg to 345.7 mg between days 9:18 and 13:00 of gestation (Table V). Thereafter the fetal weight increases rapidly and at day 15:00 of gestation the mean fetal weight was 1901.6 mg.

Since at different times during gestation the fetuses varied in their stage of palatal development (Table III), and in weight (Table V), an attempt was made to see if there was a relationship between the fetal weight and the stage of palatal development. Fetuses were grouped by weight as shown in Table VI. The palates were examined for their morphologic stage of development. The observations in the control group revealed:

1. Morphologic Stage 2 is achieved in all fetuses weighing 51 mg.
2. Stages 3-4 are completed in all fetuses weighing 251-300 mg.
3. Stage 5 is reached in 90% fetuses weighing 301 mg.
4. In fetuses weighing 551 mg, morphologic Stage 6 of palatal development is completed.

Table V. The Mean Weights of Control and 6-Mercaptopurine (6MP) Treated Hamster Fetuses at Different Times During Gestation

Gestational Age days:hours	Mean Fetal Weight in Milligrams \pm Standard Deviation			
	Control		6MP Treated	
9:06	7.8	\pm 2.6	8.9	\pm 4.1
9:12	12.6	\pm 2.8	11.6	\pm 3.7
9:18	12.0	\pm 2.7	15.7	\pm 5.5
10:00	21.2	\pm 6.5	22.7	\pm 6.5
10:06	31.4	\pm 9.0	26.0	\pm 7.6
10:12	35.4	\pm 4.1	32.7	\pm 5.6
10:18	52.1	\pm 10.8	31.2	\pm 5.1
11:00	75.1	\pm 14.4	60.6	\pm 14.0
11:06	80.5	\pm 20.1	67.8	\pm 26.2
11:12	96.2	\pm 14.2	54.3	\pm 13.6
11:18	138.5	\pm 21.0	75.0	\pm 26.5
12:00	142.3	\pm 21.9	90.2	\pm 32.0
12:06	261.3	\pm 39.5	128.3	\pm 35.6
12:12	271.1	\pm 29.5	135.1	\pm 41.9
12:18	300.0	\pm 44.4	182.3	\pm 67.6
13:00	345.7	\pm 108.5	181.0	\pm 115.4
14:00	1061.4	\pm 320.3	583.8	\pm 210.7
15:00	1901.6	\pm 470.6	732.1	\pm 309.9

Table VI. Staged Development of the Secondary Palate in Relation to Control
Hamster Fetal Weight

Fetal Weight (mg)	<u>Number of Fetuses at Each Stage of Palatal Development</u>					
	1	2	3	4	5	6
1 - 50	224*	101				
51 - 100		161				
101 - 150		85	1			
151 - 200		46	19	3	2	
201 - 250		10	4	30	17	1
251 - 300				12	33	2
301 - 350				3	21	6
351 - 400					9	18
401 - 450					5	14
451 - 500					3	13
501 - 550					1	4
551 -2750						85

*For 159 fetuses the palatal primordia were absent

Morphogenesis of the Secondary Palate in 6MP Treated Hamster Fetuses in Relation to Fetal Weight

Following 6MP treatment, the mean fetal weight increased from 18.5 mg at day 9:18 to 181 mg at day 13:00 of gestation (Table V). On day 15:00 of gestation, the average fetal weight was 732.1 mg.

Statistical evaluation of the data from Table V indicate that there was no difference in the mean fetal weight between control and 6MP treated fetuses from day 9:06 to 10:00 of gestation, i.e., during the period of initial shelf development. In the ensuing 12 hours the mean weight of 6MP treated fetuses was lower than that of the controls. Subsequently on day 10:18 of gestation, however, a pronounced difference in the mean fetal weight was observed between the control and treated groups, and it persisted until day 15:00 ($P < 0.001$).

In Table VII the data on staged palatal development following 6MP treatment are arranged in relation to the fetal weight. One may infer that, as in the controls, the morphologic Stage 2 of palate development is reached in 6MP treated fetuses weighing 51 mg. Thereafter, even though the fetal weight continues to increase, the palatal shelves remained vertical.

Morphogenesis of the Secondary Palate in Control Hamster Fetuses in Relation to Fetal Crown-Rump Length (CRL)

During normal development, the mean fetal CRL increases progressively from 5.1 mm to 14.1 mm between days 9:18 and 13:00 of gestation (Table VIII). Subsequent increase in the fetal CRL is rapid and at day 15:00 of gestation it was 26.8 mm.

Table VII. Staged Development of the Secondary Palate in Relation to
6-Mercaptopurine Treated Hamster Fetal Weight

Fetal Weight (mg)	<u>Number of Fetuses at Each Stage of Palatal Development</u>					
	1	2	3	4	5	6
1 - 50	129*	142				
51 - 100		146				
101 - 150		86				
151 - 200		43	3			
201 - 250		25	1			
251 - 300		10	4			
301 - 350		5	2			
351 - 400		3	1			
401 - 450		9	1			
451 - 500		5	1			
501 - 550		3				
551 - 2750		42				

*For 80 fetuses the palatal primordia were absent

Table VIII. The Mean Crown-Rump Length (CRL) of Control and 6-Mercaptopurine (6MP) Treated Fetuses at Different Times During Gestation

Gestational Age days:hours	Mean CRL in Millimeters \pm Standard Deviation	
	Control	6MP Treated
9:06	4.1 \pm 0.6	4.0 \pm 0.6
9:12	5.2 \pm 0.4	4.9 \pm 0.6
9:18	5.1 \pm 0.4	5.5 \pm 0.7
10:00	5.7 \pm 0.6	6.0 \pm 0.6
10:06	7.3 \pm 0.4	6.7 \pm 0.5
10:12	7.1 \pm 0.8	7.2 \pm 0.3
10:18	7.9 \pm 0.5	6.8 \pm 0.3
11:00	8.5 \pm 0.6	8.1 \pm 0.7
11:06	9.6 \pm 0.7	8.1 \pm 0.9
11:12	9.2 \pm 0.4	8.0 \pm 0.6
11:18	10.6 \pm 0.6	8.8 \pm 1.0
12:00	10.7 \pm 0.7	9.5 \pm 0.8
12:06	12.4 \pm 0.6	10.6 \pm 1.1
12:12	12.7 \pm 0.6	10.7 \pm 0.9
12:18	13.6 \pm 0.8	11.5 \pm 1.4
13:00	14.1 \pm 1.7	11.5 \pm 2.5
14:00	21.5 \pm 1.8	16.5 \pm 2.5
15:00	26.8 \pm 2.2	19.3 \pm 3.0

Table IX shows grouping of fetuses by CRL. The palates were examined for morphologic stage of development. One may deduce from the table that:

1. Stage 2 of palate development is reached in all fetuses measuring 7 mm CRL, and is completed in all fetuses measuring 12 mm CRL.
2. Stages 3-4 are accomplished in all fetuses measuring 13 mm CRL.
3. Stage 5 is achieved in all fetuses measuring 15 mm, and stage 6 by 16 mm CRL.

Morphogenesis of the Secondary Palate in 6MP Treated Hamster Fetuses in Relation to Fetal Crown-Rump Length

The mean fetal CRL increased from 5.8 mm to 11.5 mm between days 9:18 and 13:00 of gestation (Table VIII). Subsequently on day 15:00 the average fetal CRL was 19.3 mm.

Statistical analyses of the data from Table VIII indicate that the mean fetal CRL of both the treated and control groups were similar between days 9:06 and 11:00 of gestation. During next 48 hours, i.e., until day 13:00 of gestation, the mean CRL in the treated group remained lower than that in the controls. Thereafter, a pronounced difference in the mean fetal CRL was observed between the control and treated groups which persisted on day 15:00 of gestation ($P < 0.001$).

In Table X the data on staged palatal development are arranged in relation to the 6MP treated mean fetal CRL. The observations suggest that, as in the controls, Stage 2 of palate development is reached in all fetuses measuring 7 mm CRL. Thereafter, even though the fetal CRL continued to increase, the palatal shelves remained vertical.

Table IX. Staged Development of the Secondary Palate in Relation to Control Fetal Hamster Crown-Rump Length (CRL)

CRL (mm)	Number of Fetuses at Each Stage of Palatal Development					
	1	2	3	4	5	6
0 - 1.9						
2.0 - 2.9	2*					
3.0 - 3.9	6*					
4.0 - 4.9	65*					
5.0 - 5.9	105**					
6.0 - 6.9	46	31				
7.0 - 7.9		89				
8.0 - 8.9		83				
9.0 - 9.9		69				
10.0 - 10.9		71	1			
11.0 - 11.9		48	20	16	4	
12.0 - 12.9		12	3	23	26	3
13.0 - 13.9				9	31	3
14.0 - 14.9				5	23	17
15.0 - 15.9					7	19
16.0 - 16.9						15
17.0 - 30.0						86

* Palatal primordia are absent

**For 86 fetuses the palatal primordia are absent

Table X. Staged Development of the Secondary Palate in Relation to 6-Mercaptopurine Treated Fetal Hamster Crown-Rump Length (CRL)

CRL (mm)	Number of Fetuses at Each Stage of Palatal Development					
	1	2	3	4	5	6
0 - 1.9						
2.0 - 2.9						
3.0 - 3.9	8*					
4.0 - 4.9	37*					
5.0 - 5.9	41**					
6.0 - 6.9	43	49				
7.0 - 7.9		102				
8.0 - 8.9		81				
9.0 - 9.9		62				
10.0 - 10.9		73				
11.0 - 11.9		46	2			
12.0 - 12.9		24	2			
13.0 - 13.9		11	3			
14.0 - 14.9		7	4			
15.0 - 15.9		16				
16.0 - 16.9		6	2			
17.0 - 30.0		42				

* Palatal primordia are absent

**For 35 fetuses the palatal primordia are absent

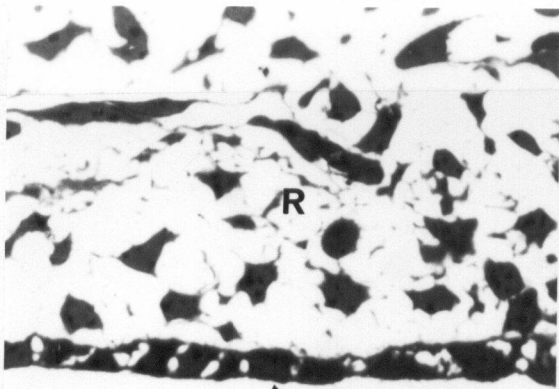
Light Microscopic Observations of the Developing Secondary Palate in Control Hamster Fetuses

On day 9:06 of gestation the palatal primordia are not formed. The roof of the oronasal cavity is flat and made of loose mesenchyme covered by a single layer of epithelial cells (Fig. 19). The epithelial cells are cuboidal, and contain a large spherical nucleus surrounded by a thin rim of cytoplasm. The mesenchymal cells are stellate and contain a large nucleus. Mitosis is occasionally observed in cells of both the epithelium and mesenchyme.

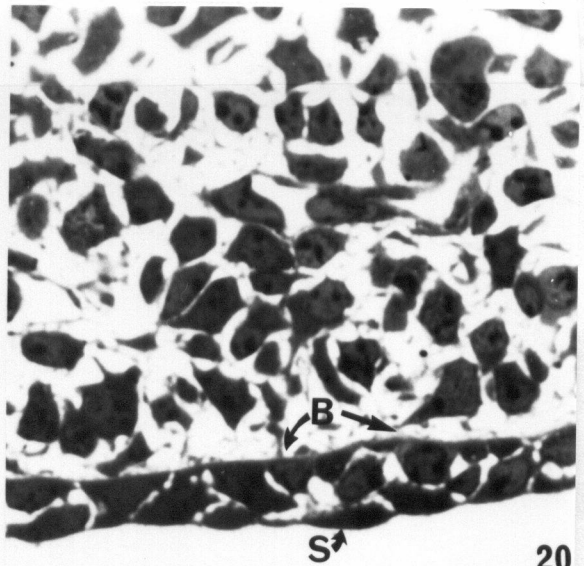
During next 18 hours, i.e., until day 10:00 of gestation, the morphology of both the epithelial and mesenchymal cells remains unchanged. Histologically, from day 10:00 onward, however, the normal palatal development can be observed in five stages.

Stage I. The palatal primordia appear from the roof of the oronasal cavity. A palatal primordium is composed of mesenchymal tissue covered by a bilaminar epithelium (Fig. 20). The mesenchymal cells are stellate and closely packed. The superficial epithelial cells are flat and contain an elongated nucleus. The basal epithelial cells are cuboidal and contain a large spherical nucleus.

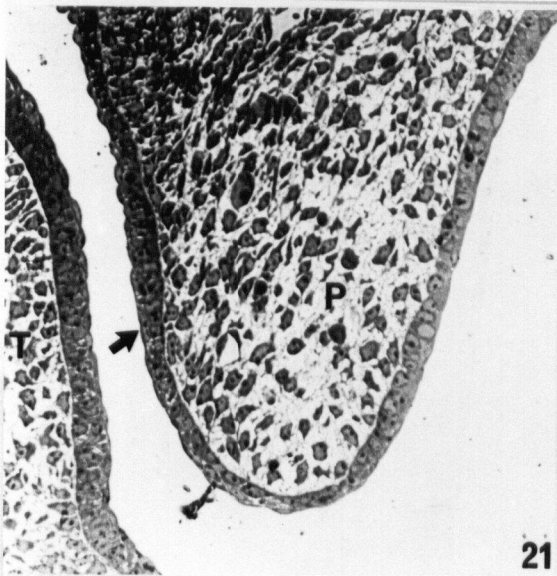
Stage II. The epithelium on the medial aspect of the vertical palatal shelf, the prospective fusion epithelium (Shah, 1979a), is two to three cell layers thick (Fig. 21). The appearance of both the epithelial and mesenchymal cells, however, remained unaltered. Few cells undergoing mitosis are observed in the epithelium and mesenchyme. In the epithelial cells, mitosis is seen only in the basal layer.



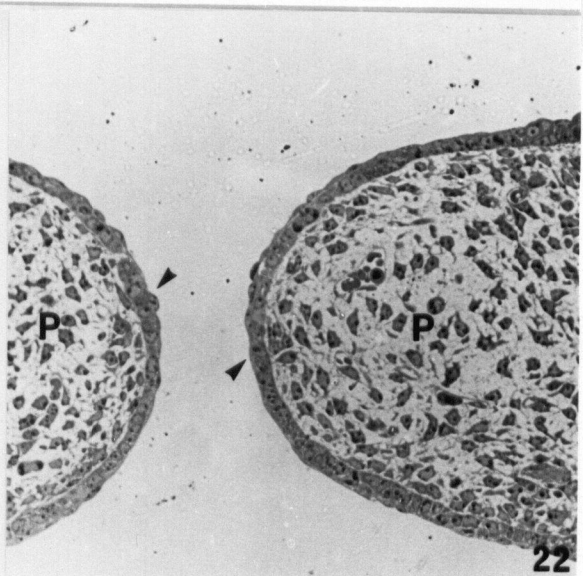
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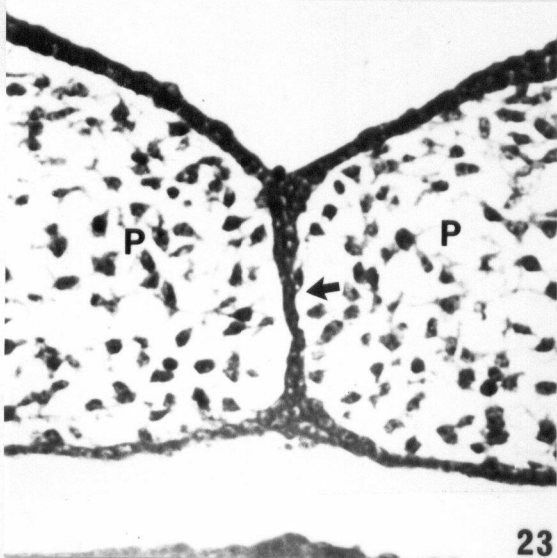
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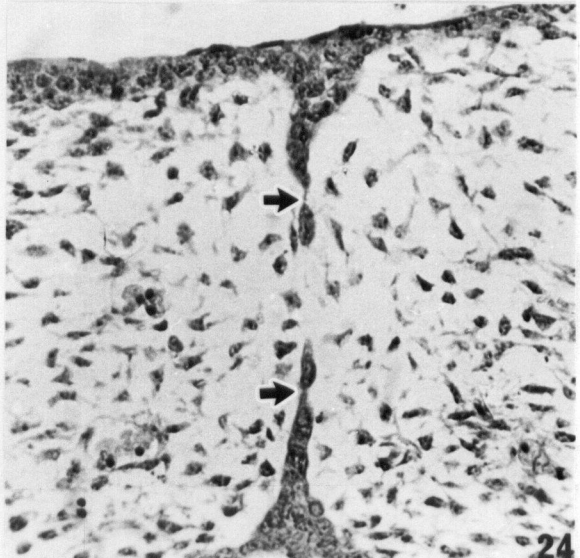
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- Fig. 19** Frontal section through the secondary palate region of a control hamster on day 9:06 of gestation. The roof (R) of the oronasal cavity is covered by a single layer of cuboidal epithelial (E) cells. The mesenchymal cells are stellate. Epon-araldite section. Methylene blue stain. 920X.
- Fig. 20** Frontal section through the secondary palate region of a control hamster fetus on day 10:00 of gestation showing the bilaminar epithelium of the palatal primordia (Stage I). The bilaminar epithelium is made of flat superficial (S) and cuboidal basal (B) cells. The mesenchymal cells are stellate. Epon-araldite section. Methylene blue stain. 980X.
- Fig. 21** Frontal section through the secondary palate of a control hamster fetus on day 11:18 of gestation showing the two to three cell layered epithelium (arrow) of the medial aspect of the vertical palatal (P) shelf (Stage II). I - tongue. Epon-araldite section. Methylene blue stain. 360X.
- Fig. 22** Frontal section through the secondary palate of a control hamster fetus on day 12:00 of gestation. The medial edge epithelium (arrow heads) of the horizontal palatal shelves (P) is bilaminar (Stage III). The mesenchymal cells are stellate or elongated. Epon-araldite section. Methylene blue stain. 410X.
- Fig. 23** Frontal section through the secondary palate of a control hamster fetus on day 12:00 of gestation. The medial edge epithelia of the opposing horizontal palatal shelves (P) have contacted to form a seam (arrow) (Stage IV). Paraffin section. Hematoxylin and Eosin stain. 410X.
- Fig. 24** Frontal section through the secondary palate of a control hamster fetus on day 12:06 of gestation showing a fragmenting (arrows) (Stage V) epithelial seam. Epon-araldite section. Methylene blue stain. 504X.

Stage III. The palatal shelves are horizontal above the tongue (Fig. 22). The prospective fusion epithelium on the medial edge (MEE) of the horizontal shelf shows no histological change from the previous stage. The mesenchymal cells are stellate or elongated in shape (Fig. 22). Mitosis is absent in the MEE, but is occasionally seen in the mesenchymal cells.

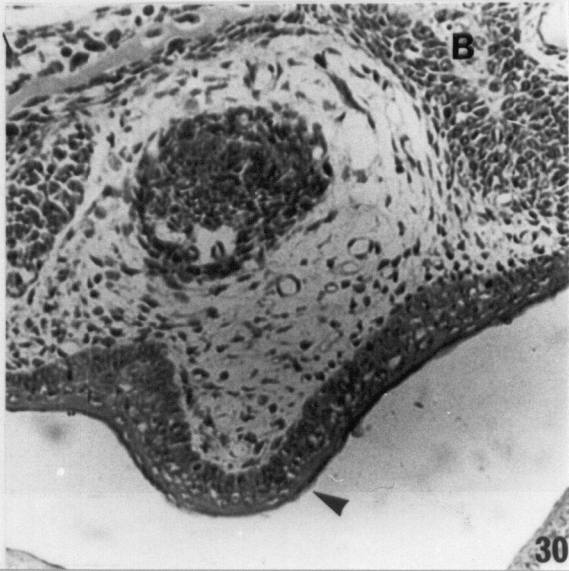
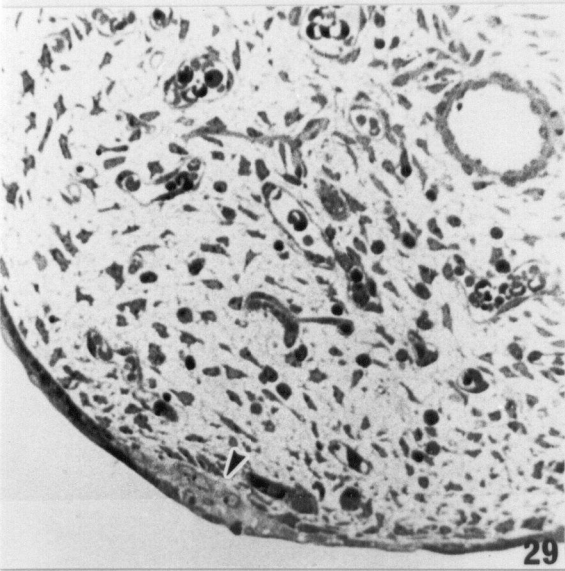
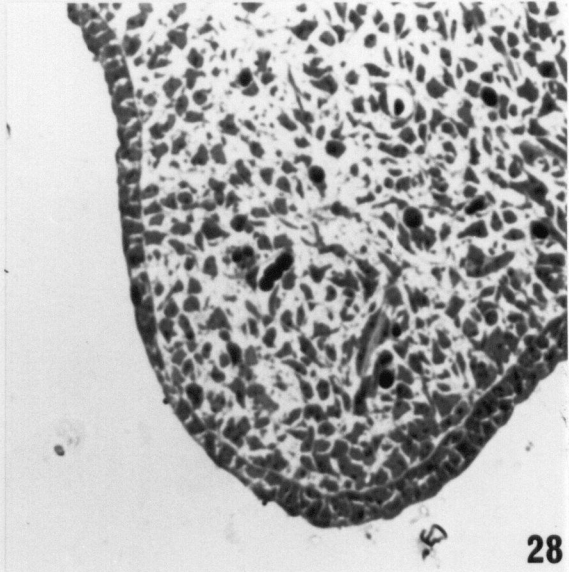
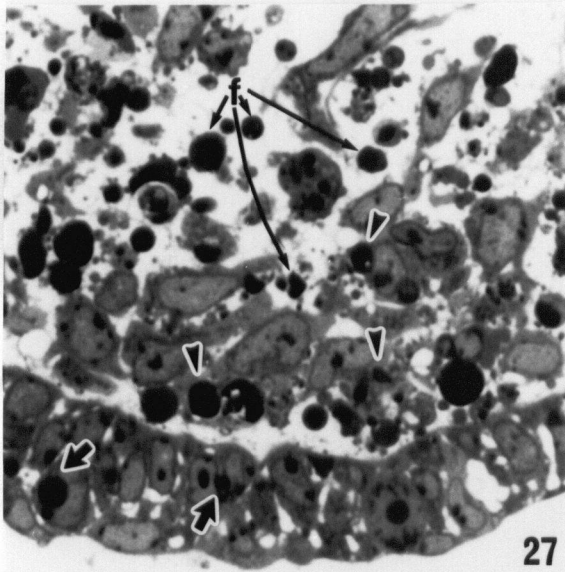
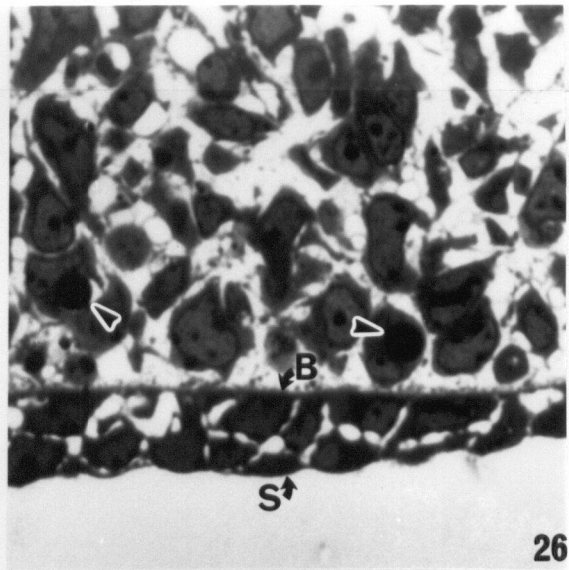
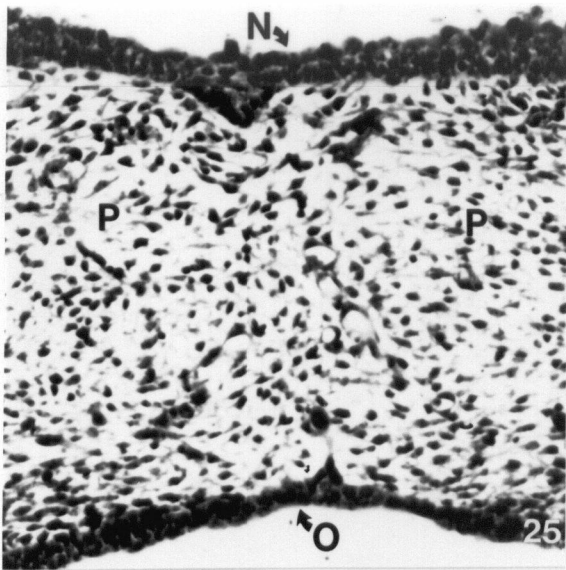
Stage IV. The MEE of the opposing horizontal shelves are in contact with one another. The epithelial contact results in the formation of a seam (Fig. 23). The seam is two to three cell layers thick. The cells are irregular in shape and contain an oval nucleus. The mesenchymal cells did not show any alteration in their morphology.

Stage V. The epithelial seam is broken into fragments (Fig. 24). The space between the fragments is occupied by mesenchymal cells. The epithelial fragments eventually disappear, and mesenchyme of the two palatal shelves becomes continuous (Fig. 25).

Light Microscopic Observations of the Developing Secondary Palate in Hamster Fetuses Following GMP Treatment

During first 18 hours, i.e., between days 9:06 and 10:00 of gestation, the histological appearance of both the epithelial and mesenchymal cells in the roof of the oronasal cavity of GMP treated fetuses is similar to that of the controls.

Stage I. The palatal primordia develop from the roof of the oronasal cavity on day 10:00 of gestation. During the first six hours of primordial development the histological appearance of the bilaminar epithelial cells resembles that of the controls. An occasional mesenchymal cell shows a small



- Fig. 25** Frontal section through the secondary palate of a control hamster fetus on day 12:12 of gestation showing mesenchymal continuity between opposing palatal shelves (P). O - oral epithelium. N - nasal epithelium. Paraffin section. Hematoxylin and Eosin stain. 410X.
- Fig. 26** Frontal section through the secondary palate of a 6MP treated hamster fetus on day 10:00 of gestation showing the primordial epithelium (Stage I). The epithelium is bilaminar with flat superficial (S) cells and cuboidal basal (B) cells. An occasional mesenchymal cell shows a small darkly stained dense body (arrow heads). Epon-araldite section. Methylene blue stain. 980X.
- Fig. 27** Frontal section through the secondary palate of a 6MP treated hamster fetus on day 10:06 of gestation. The histological appearance of the epithelial and mesenchymal cells of the vertically developing shelf (Stage II). Dense bodies are present in the mesenchymal cells (arrow heads), epithelial cells (arrows) and in the cell fragments (f). Epon-araldite section. Methylene blue stain. 980X.
- Fig. 28** Frontal section through the secondary palate of a 6MP treated hamster fetus on day 11:00 of gestation. The dense bodies are absent in the mesenchymal and epithelial cells of the vertical palatal shelf (Stage II). Epon-araldite section. Methylene blue stain. 510X.
- Fig. 29** Frontal section through the secondary palate of a 6MP treated hamster fetus on day 12:18 of gestation. The basal epithelial cells are light in appearance (arrow head). Epon-araldite section. Methylene blue stain. 280X.
- Fig. 30** Frontal section through the secondary palate of a 6MP treated hamster fetus on day 15:00 of gestation. The epithelium of the stunted palatal shelf is stratified squamous (arrow head). Bone formation (B) is observed in the mesenchyme. Paraffin section. Hematoxylin and Eosin stain. 208X.

darkly stained dense body in its cytoplasm (Fig. 26). Such cells with dense bodies are not present at the corresponding stage during normal development.

Stage II. In the next 12 hours, i.e., between days 10:00 and 10:12 of gestation, the histological appearance of both the mesenchyme and epithelial cells changes, and on day 10:12 of gestation they are strikingly different from that of the controls. The dense bodies are present in many mesenchymal cells (Fig. 27) of the treated palatal shelf. Also small fragments of cells containing dense bodies are present in the extracellular matrix of the mesenchyme. A few epithelial cells, in addition, show dense bodies in their cytoplasm (Fig. 27).

Subsequently, during next the 12 hours, only a few epithelial and mesenchymal cells show dense bodies. On day 11:00 of gestation none of the epithelial and mesenchymal cells show dense bodies (Fig. 28). Histologically both the epithelial and mesenchymal cells of the treated vertical palatal shelf resemble those of the controls.

Between days 11:00 and 12:18 of gestation the epithelial and mesenchymal cells of QMP treated palatal shelves appear unaltered. Unlike those of the controls, some basal cells in the epithelium, on day 12:18 of gestation, are relatively light in appearance (Fig. 29). The mesenchymal cells, however, appear unchanged. The treated vertical palatal shelves appear smaller than those of the controls (cf. Figs. 6, 16).

The light cells persist in the epithelium during the ensuing 24 hours, i.e., until day 13:18 of gestation. On day 14:00 of gestation, and thereafter, the light cells are absent. The epithelium on the stunted

vertical shelf undergoes stratification. On day 15:00 of gestation, the epithelium is stratified squamous (Fig. 30). The mesenchyme shows evidence of bone formation (Figs. 18, 30).

Stages III-IV. Histologic Stages III (horizontal shelves), IV (formation of an epithelial seam), and V (fragmentation of epithelial seam) of the normal palatal development are not seen in the 6MP treated fetuses at any time during gestation. The palatal shelves instead remain vertical and appear stunted at term (Fig. 18).

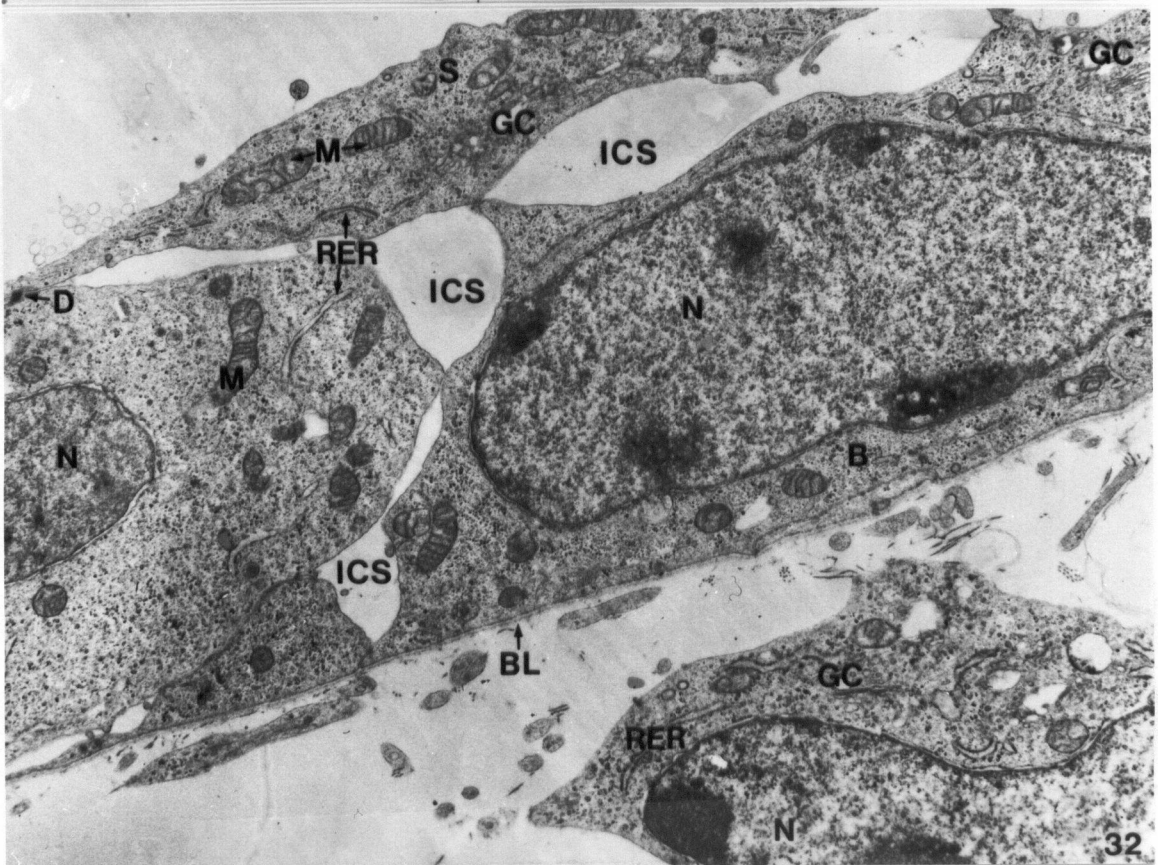
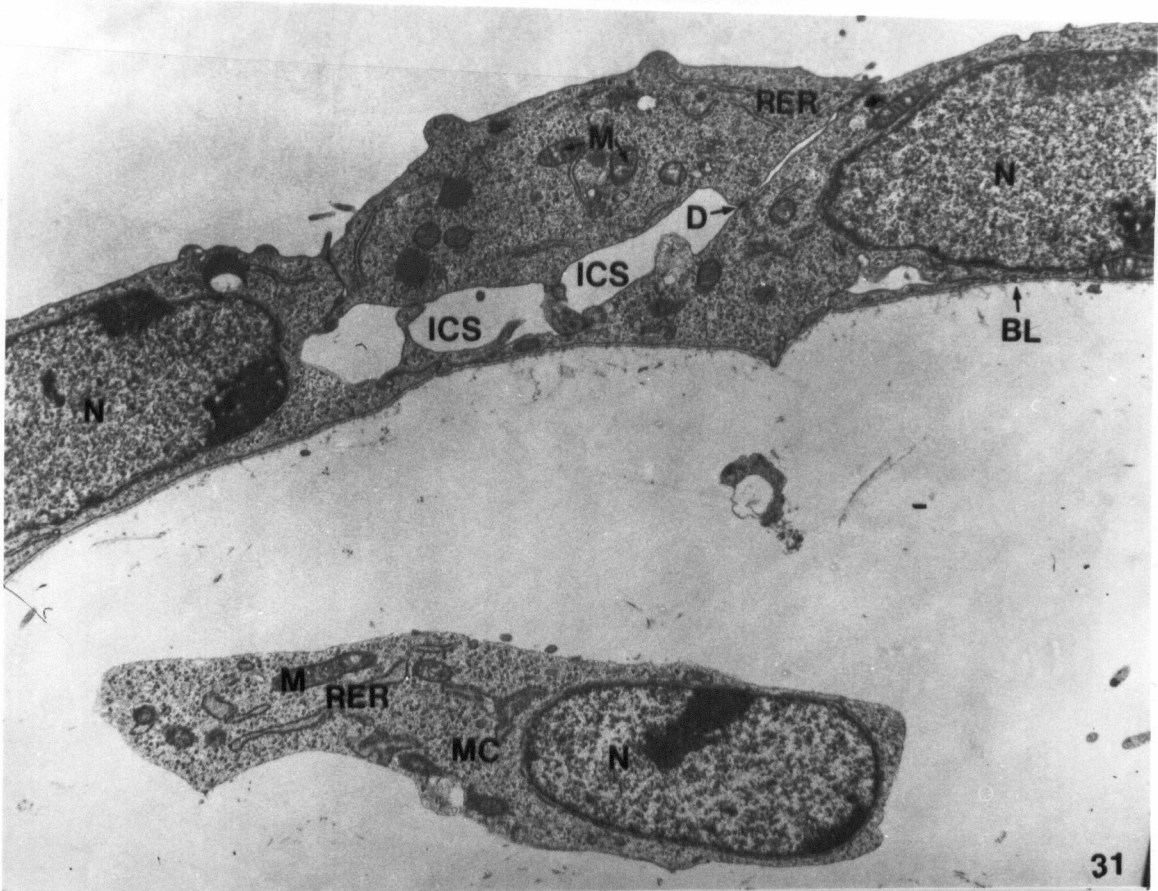
Electron Microscopic Observations of the Developing Secondary Palate in Control Hamster Fetuses

Prior to the appearance of palatal primordia, the roof of the oronasal cavity is lined by simple cuboidal epithelium. The epithelium is separated from the underlying mesenchyme by a continuous basal lamina (Fig. 31). Spaces are present between the epithelial cells which are otherwise attached to one another by desmosomes.

The epithelial cells (Fig. 31) contain a large nucleus surrounded by polyribosomes, a few mitochondria, and occasional cisternae of rough endoplasmic reticulum.

The mesenchymal cells (Fig. 31) contain a round to oval nucleus surrounded by organelles similar to the epithelial cells.

With the appearance of palatal primordia histological Stages I through V of palatogenesis were studied at the ultrastructural level.



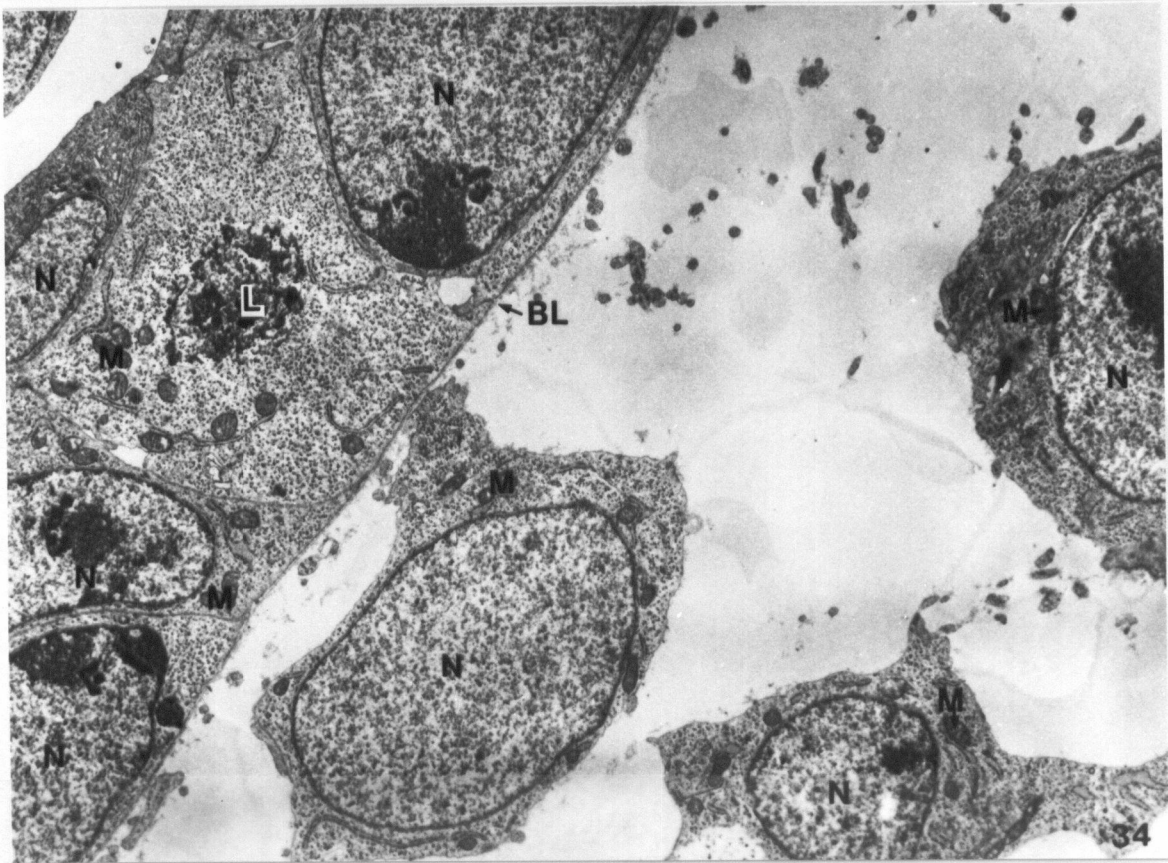
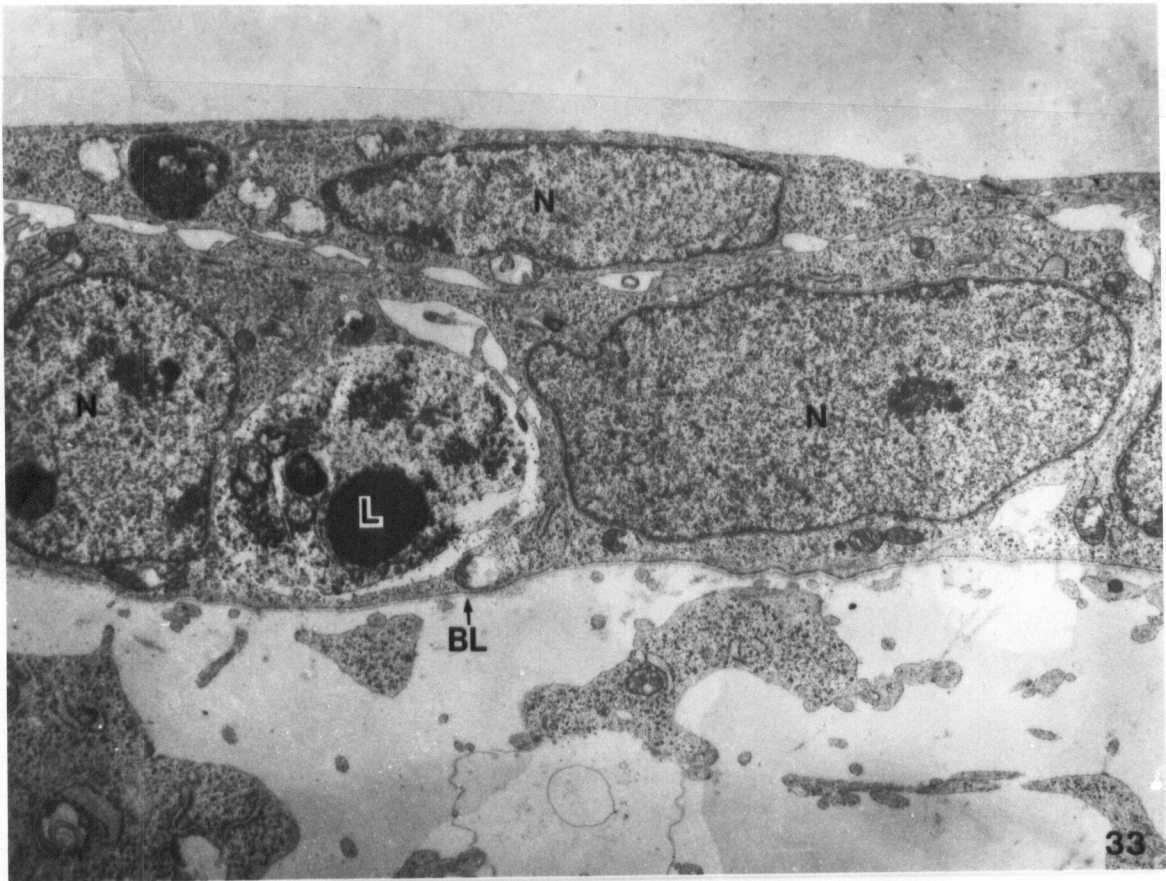
- Fig. 31** Electron micrograph of the roof of the oronasal cavity in a control hamster fetus on day 9:12 of gestation. The epithelium is separated from the mesenchyme by a continuous basal lamina (BL). The mesenchymal cell (MC) contains a round to oval nucleus surrounded by organelles similar to the epithelial cells. ICS - intercellular space. N - nucleus. M - mitochondrion. D - desmosome. RER - rough endoplasmic reticulum. 8664X.
- Fig. 32** Electron micrograph of the secondary palate primordia in a control hamster fetus on day 10:00 of gestation showing a continuous basal lamina (BL) separating the bilaminar epithelium from the mesenchyme. A small golgi complex (GC) appears in the mesenchymal cell. B - basal cell. S - superficial cell. M - mitochondrion. ICS - intercellular space. N - nucleus. RER - rough endoplasmic reticulum. D - desmosome. 10180X.

Stage I. The palatal primordium is covered by a bilaminar epithelium (Fig. 32). The basal cells are cuboidal and the superficial cells flat. Both cell types contain a large nucleus surrounded by polyribosomes, mitochondria, cisternae of rough endoplasmic reticulum and a small Golgi complex. Numerous large spaces are present between cells of the epithelium. Both the superficial and basal cells are attached to one another by desmosomes. The basal lamina separating the epithelium from the mesenchyme is continuous.

The mesenchymal cells of the vertically developing palatal primordia (Fig. 32) contain more organelles than those observed in the roof of the oronasal cavity. In addition, a small Golgi complex appears in the mesenchymal cells of the vertically developing palatal primordia (Fig. 32).

Stage II. The morphology and contents of both the epithelial and mesenchymal cells seen during Stage I remain unchanged in the vertical palatal shelf.

Stage III. The epithelium of the horizontal palatal shelf is separated from the mesenchyme by a continuous basal lamina (Figs. 33, 34). In comparison to large intercellular spaces seen at Stages I and II, the intercellular spaces in the epithelium of the horizontal palatal shelf are smaller. The cytoplasm of the epithelial cells show membrane-bound and membrane-free areas containing aggregations of electron dense granules, and dense bodies (Figs. 33, 34). On a morphological basis, these areas are interpreted as lysosomes (DeDuve, 1963; Erickson, 1969). Other cytoplasmic contents of the epithelial cells remain unchanged from Stage II.



- Fig. 33** Electron micrograph of the secondary palate in a control hamster fetus on day 12:06 of gestation showing the epithelium of the horizontal palatal shelf. Lysosomes (L) are present in both the superficial and basal epithelial cells. BL - basal lamina. N - nucleus. 6412X.
- Fig. 34** Electron micrograph of the secondary palate in a control hamster fetus on day 12:06 of gestation showing the mesenchymal cells of the horizontal palatal shelf. N - nucleus. M - mitochondrion. BL - basal lamina, L - lysosome. 8000X.

With the exception of lysosomes, the mesenchymal cells contain organelles (Fig. 34) similar to those of the epithelial cells.

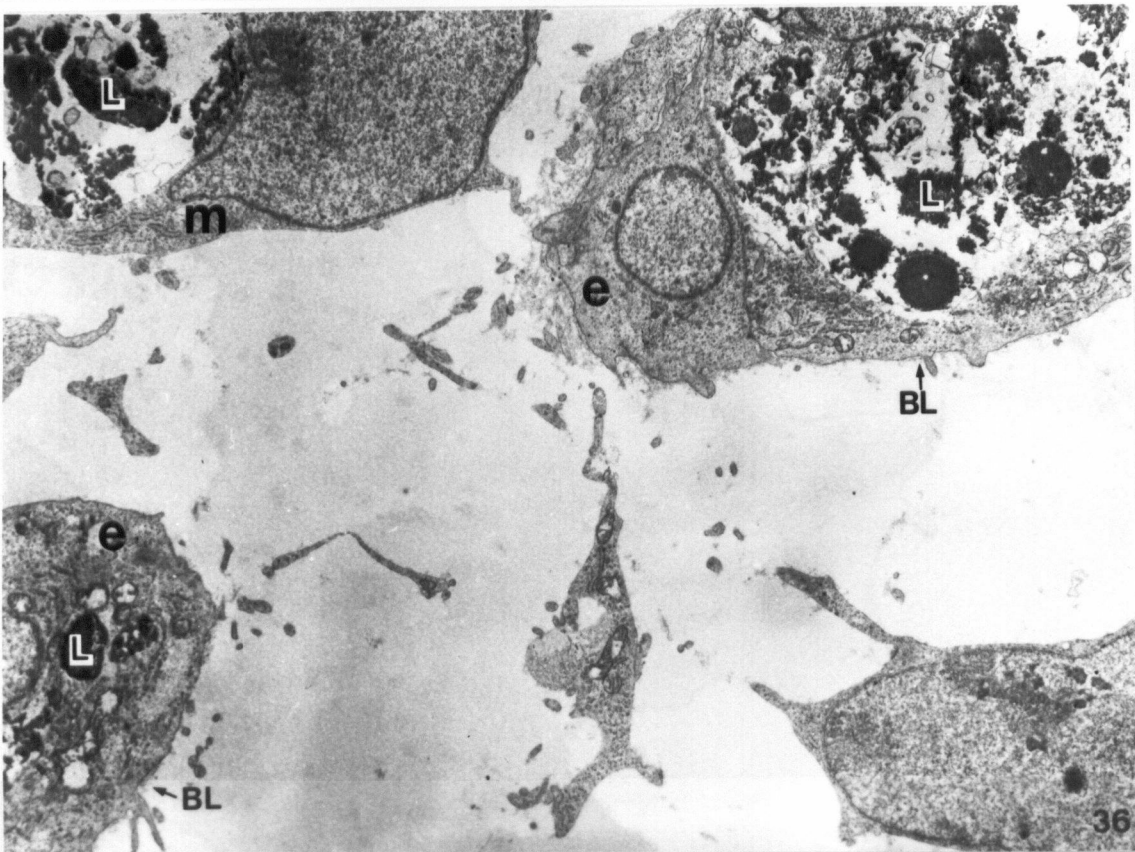
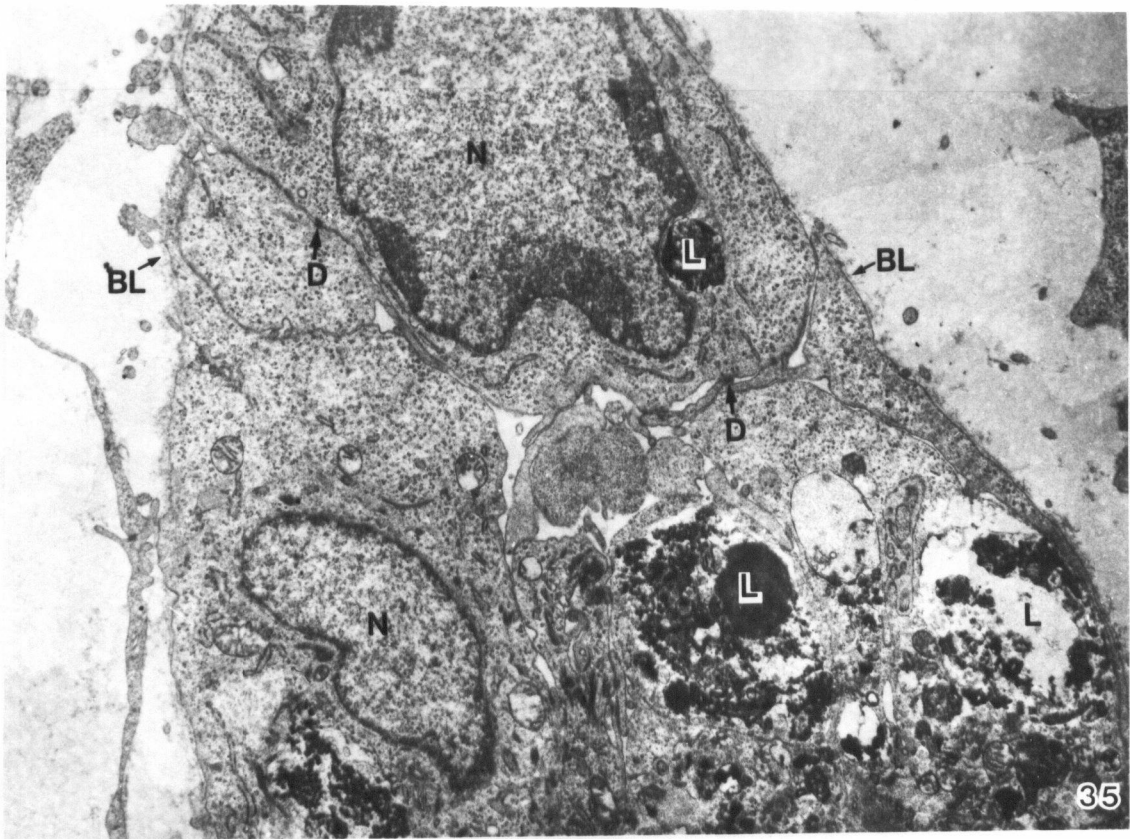
Stage IV. During this stage the epithelia of the opposing shelves come into contact with one another thus forming a seam (Figs. 23, 24). The epithelial seam is two to four layers in width (Fig. 35). The cells in the seam are attached to one another by desmosomes. A continuous basal lamina separates the epithelial seam from the surrounding mesenchyme.

The cells of the epithelial seam show considerable variation in size, shape and contents (Fig. 35). They are cuboidal to irregular in shape and contain an oval or irregular nucleus surrounded by numerous polyribosomes, Golgi complex, and a few cisternae of rough endoplasmic reticulum. The mitochondria show disruption in their cristae. Lysosomes are more numerous than those observed at Stage III.

The mesenchymal cells are stellate and resemble those of Stage III.

Stage V. In contrast to previous stages, the basal lamina separating the fragments of epithelial seam from mesenchyme is not continuous (Fig. 36). The cells of fragmented seam contain large lysosomes which appear to dissolve the cytoplasmic content.

The mesenchymal cells, near the epithelial fragments, contain a few vacuoles and lysosomes (Fig. 36). These cells are interpreted as macrophages. They appear to carry away the debris of autolysed epithelial cells, thus clearing the area for mesenchymal union.



- Fig. 35** Electron micrograph of the secondary palate in a control hamster fetus on day 12:06 of gestation showing the epithelial seam. The cells of the seam are attached to one another by desmosomes (D). A basal lamina (BL) separates the epithelial cells from the surrounding mesenchyme on both sides of the seam. N - nucleus. L - lysosomes. 8330X.
- Fig. 36** Electron micrograph of the secondary palate in a control hamster fetus on day 12:06 of gestation showing a fragment of an epithelial seam (e) and a macrophage (m). A discontinuous basal lamina (BL) surrounds the cells of the epithelial fragment. Lysosomes (L) are present in both the epithelial cells and the macrophage. 6125X.

Electron Microscopic Observations of the Developing Secondary Palate Following 6MP Treatment

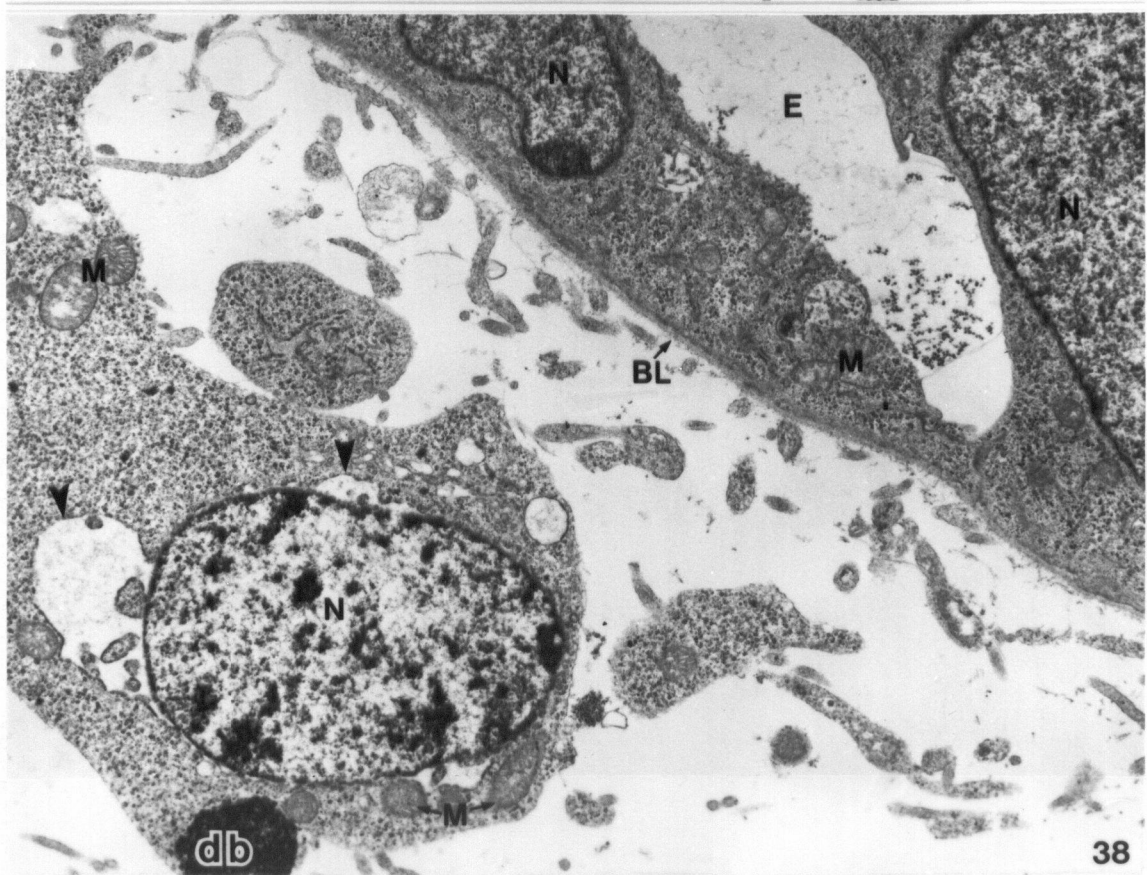
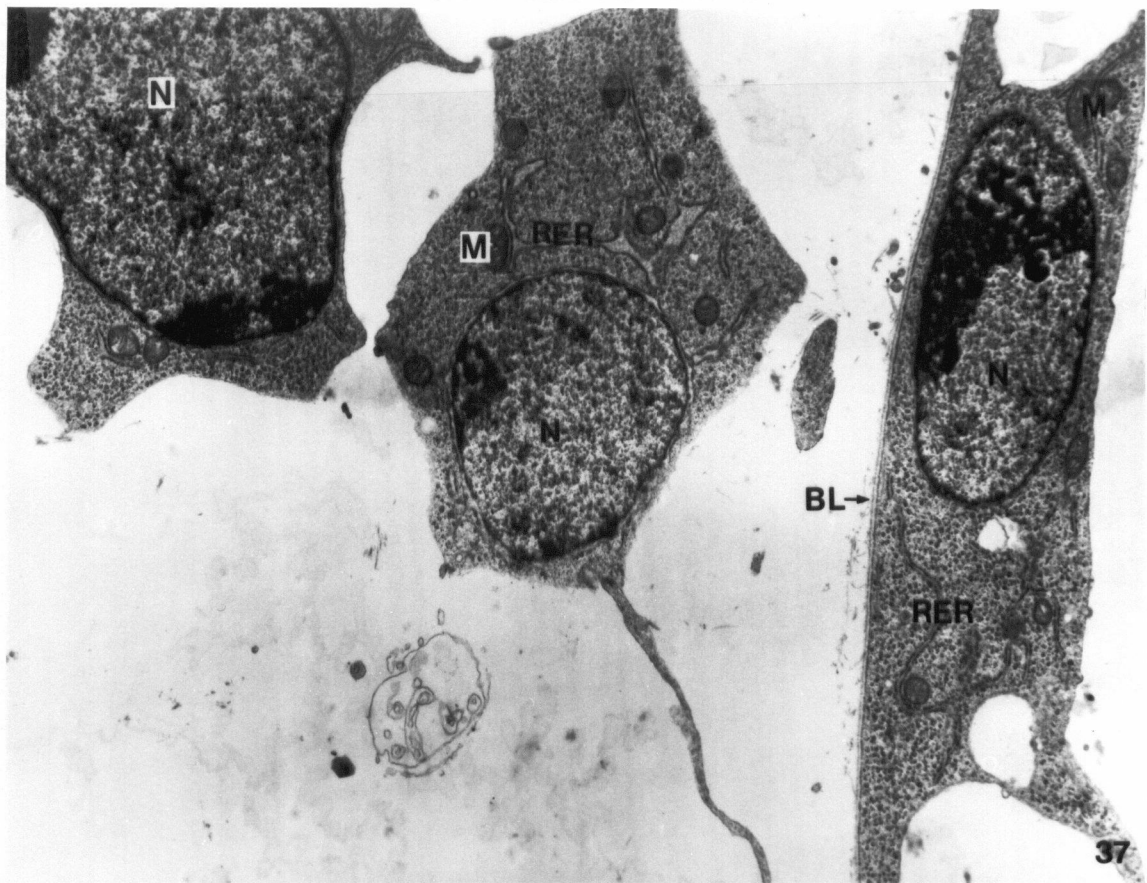
During first 18 hours following drug administration, i.e., until day 9:18 of gestation, the morphology and contents of both the epithelial and mesenchymal cells of the roof of the oronasal cavity (Fig. 37) resemble those of the controls. The epithelium is simple and made of cuboidal cells which are separated from the subjacent stellate mesenchymal cells by a continuous basal lamina (Fig. 37). The cytoplasm of both the epithelial and mesenchymal cells contains a nucleus surrounded by polyribosomes, mitochondria, and rough endoplasmic reticulum.

Subsequently, histological Stages I and II of palatal development following 6MP treatment were examined at the ultrastructural level.

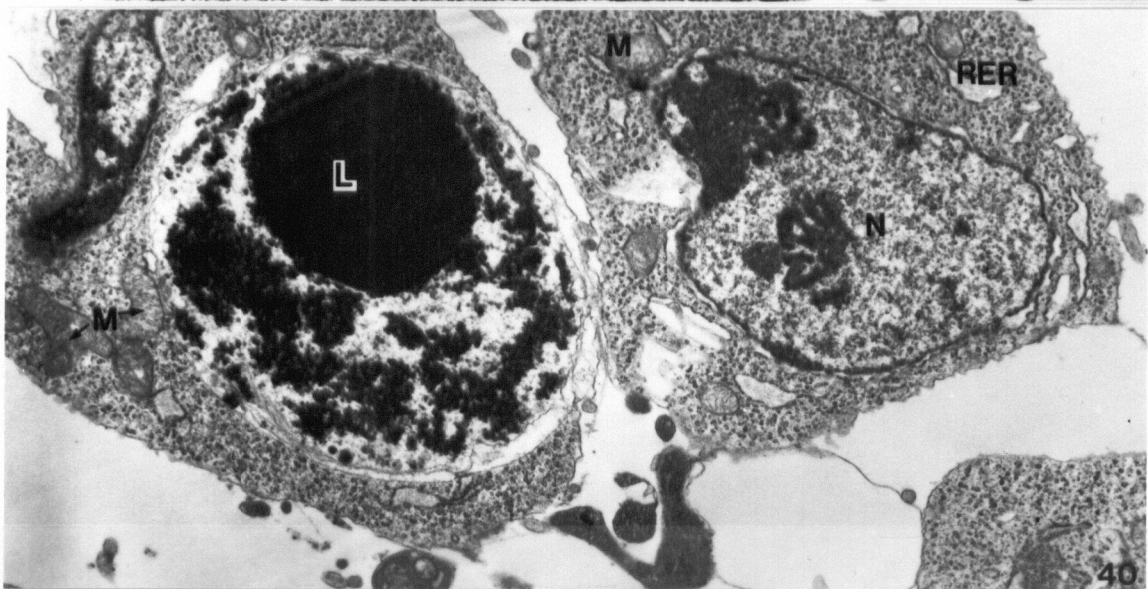
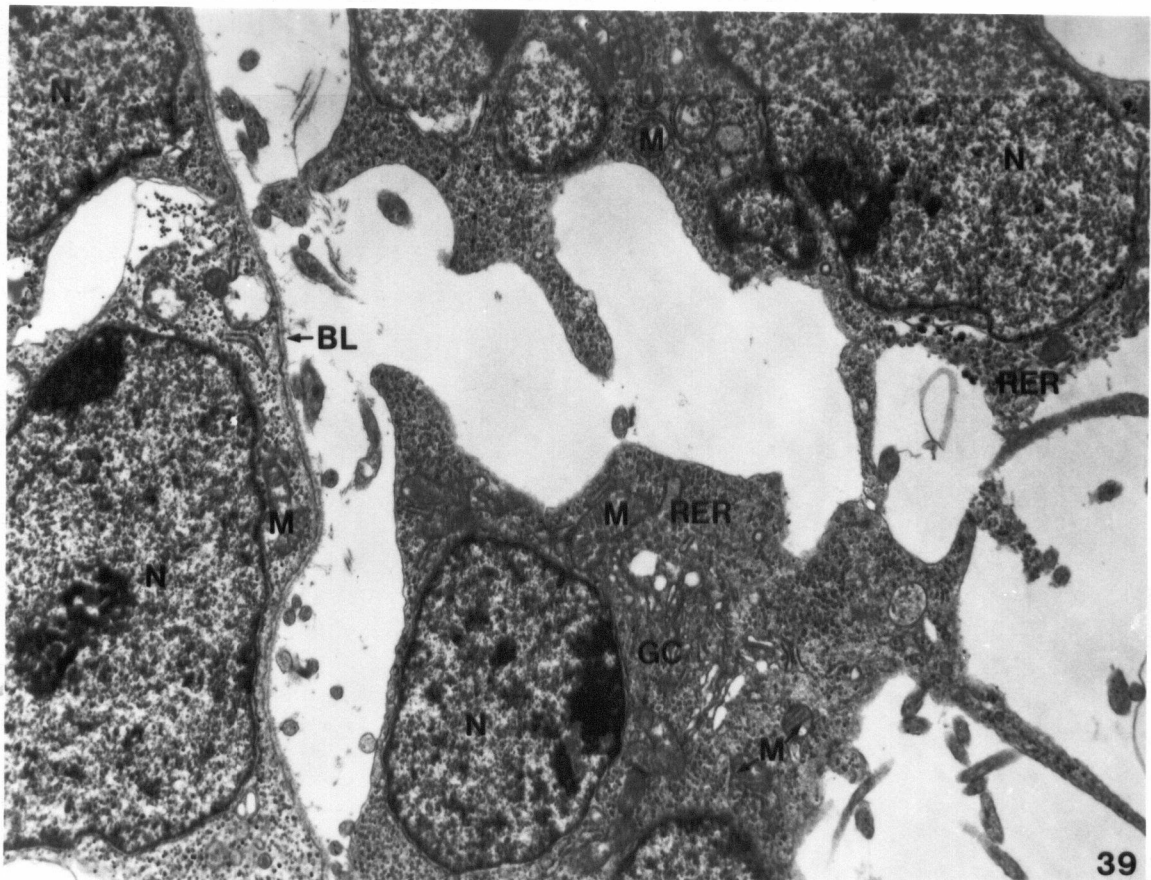
Stage I. On day 10:00 of gestation, the ultrastructural appearance of the epithelial cells of the 6MP treated palatal primordia remains identical to that of the control. The basal lamina separating the epithelium and mesenchyme is continuous (Fig. 38).

Alterations are, however, seen in the mesenchyme of the 6MP treated, palatal primordia. Some of the mesenchymal cells (Fig. 38) appear irregular, and contain an oval nucleus surrounded by polyribosomes, few mitochondria, and an occasional dense body. The perinuclear space of the nuclear envelope is swollen, and the nucleoplasm appears clear due to clumping of chromatin (Fig. 38).

Other mesenchymal cells (Fig. 39) are stellate. They contain an oval or indented nucleus surrounded by polyribosomes, mitochondria, a few cisternae of rough endoplasmic reticulum, and a well developed Golgi complex. An



- Fig. 37** Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 9:06 of gestation. The epithelium is separated from the mesenchyme by a continuous basal lamina (BL). N - nucleus. M - mitochondrion. RER - rough endoplasmic reticulum. 5897X.
- Fig. 38** Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 10:00 of gestation. The basal lamina (BL) separating the epithelium and mesenchyme is continuous. The perinuclear space of the mesenchymal cell nuclear envelope is swollen (arrow heads), and the nucleoplasm appears clear due to clumping of chromatin. N - nucleus. M - mitochondrion. db - dense body. E - epithelium. 5783X.



- Fig. 39** Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 10:00 of gestation. The mesenchymal cells show a nucleus (N) surrounded by polyribosomes, mitochondria (M), a few cisternae of rough endoplasmic reticulum (RER), and a well developed Golgi complex (GC). BL - basal lamina. 5880X.
- Fig. 40** Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 10:00 of gestation. The mesenchymal cell shows a membrane bound lysosome (L). N - nucleus. M - mitochondrion. 5927X.

occasional cell also shows, in addition, membrane-bound and membrane-free structures of varying electron densities containing granular material and cytoplasmic organelles such as mitochondria (Fig. 40). On a morphological basis, these structures were interpreted as lysosomes (DeDuve, 1963; Erickson, 1969). The lysosomes were absent at a comparable time during normal development.

Stage II. On day 10:06 of gestation, a few mesenchymal cells show massive disruption of their cytoplasmic morphology (Fig. 41). The cytoplasm appears vacuolated, and shows numerous lysosomes. The lysosomes vary in size, and contain dense granules, dense bodies and vacuoles. The nucleus is indented, and, along with other cytoplasmic organelles, pushed to the side. These mesenchymal cells are interpreted as macrophages. Small membrane-bound structures containing dense bodies and cytoplasmic organelles (Fig. 41) are also found in the intercellular space near the macrophages. Presumably these structures represent fragments of expelled material from the mesenchymal cells, and are in the process of being phagocytosed by the macrophages.

Subsequently, on day 10:12 of gestation, the mesenchymal cells continue to show disruptive changes in their cytoplasm. In addition, alterations are also seen for the first time in the epithelium. Some cells of the bilaminar epithelia are altered, and show a nucleus surrounded by polyribosomes, a few mitochondria, cisternae of rough endoplasmic reticulum, lipid droplets, and lysosomes (Fig. 42). Other epithelial cells are not altered (Fig. 43), and resemble their control counterpart. The basal lamina beneath both the

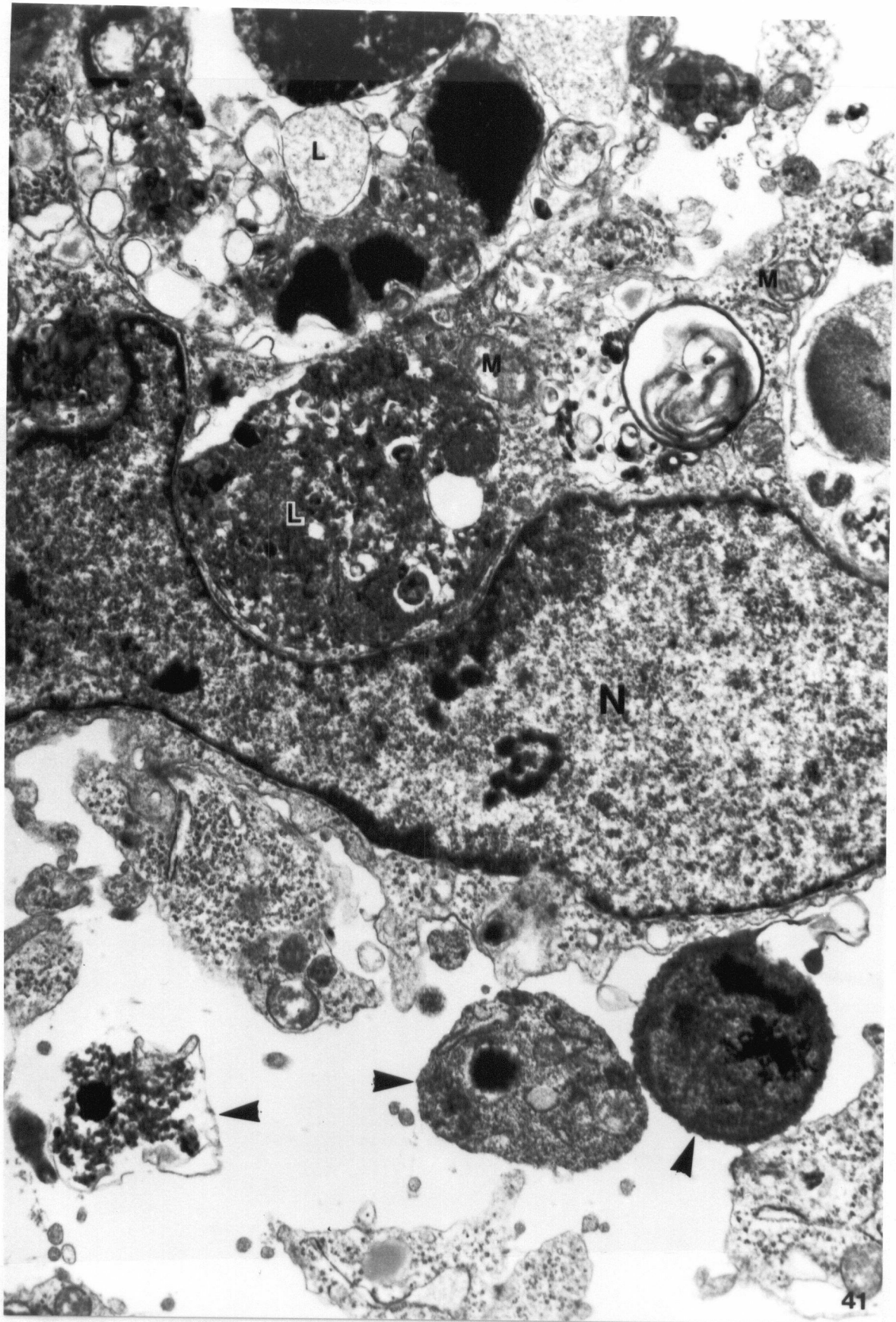


Fig. 41 Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 10:06 of gestation. A mesenchymal cell showing massive disruption of its cytoplasmic morphology, and interpreted as a macrophage. The cytoplasm appears vacuolated, and shows numerous lysosomes (L). The nucleus (N) is indented and is pushed to the side. Small membrane bound structures (arrow heads) containing dense bodies and cytoplasmic organelles are present near the macrophage. M - mitochondria. 7594X.

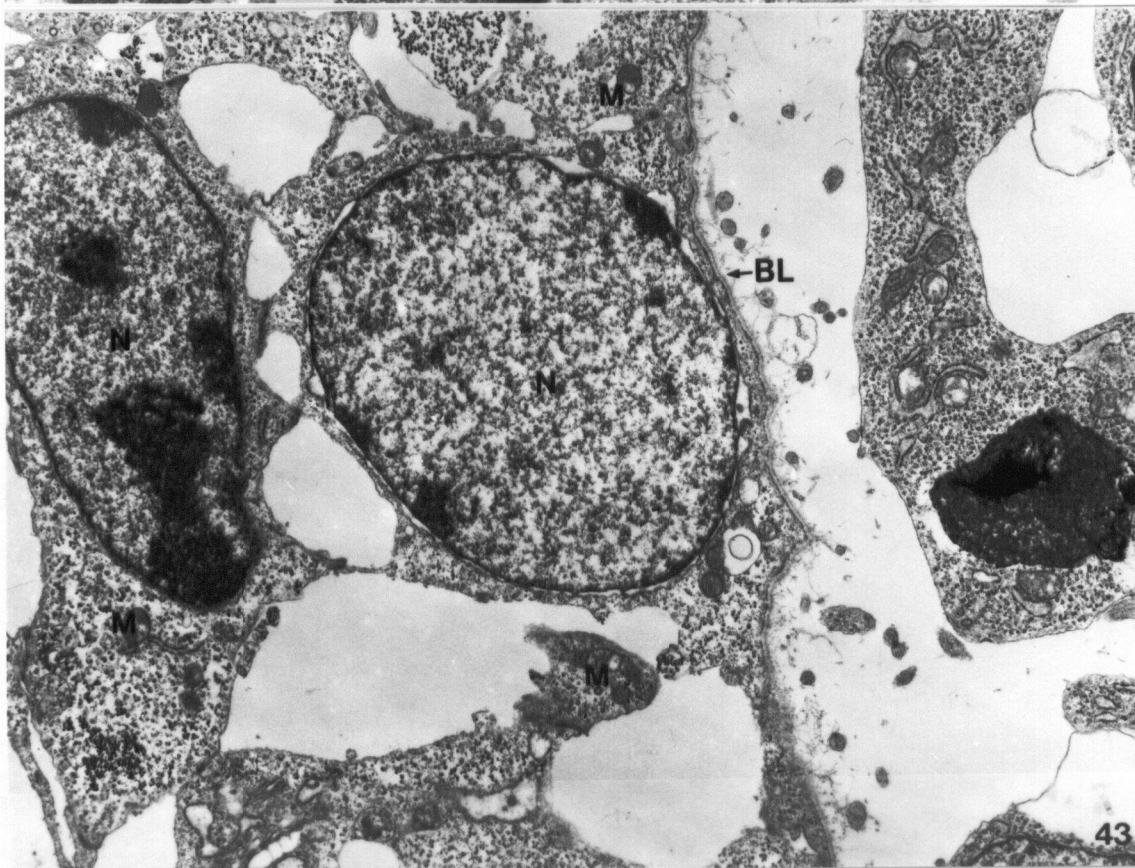
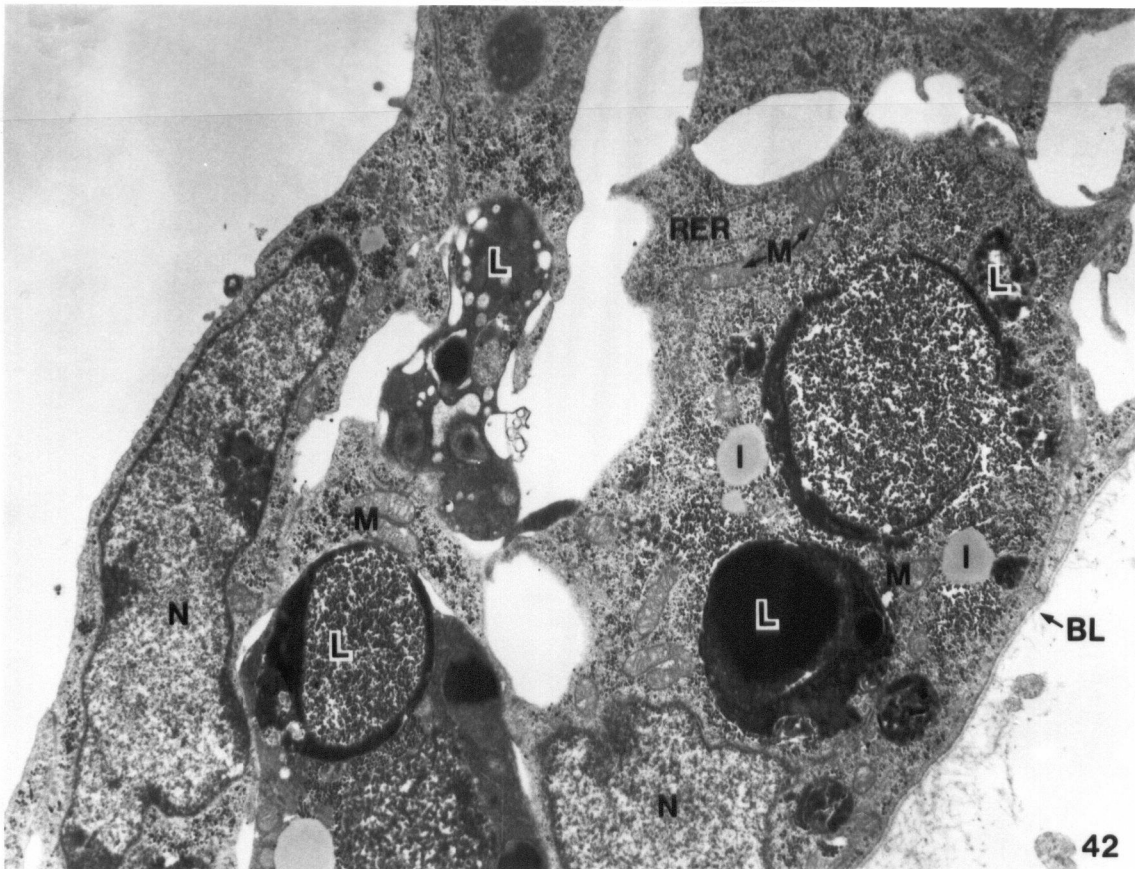


Fig. 42 Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 10:12 of gestation showing cytoplasmic features of the epithelial cells. The nucleus (N) is surrounded by polyribosomes, mitochondria (M), cisternae of rough endoplasmic reticulum (RER), lipid droplets (l), and lysosomes (L). The basal lamina (BL) is continuous. 5789X.

Fig. 43 Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 10:12 of gestation showing unaffected epithelial cells (cf. Fig. 42). The basal lamina (BL) is continuous. N - nucleus. M - mitochondrion. 5894X.

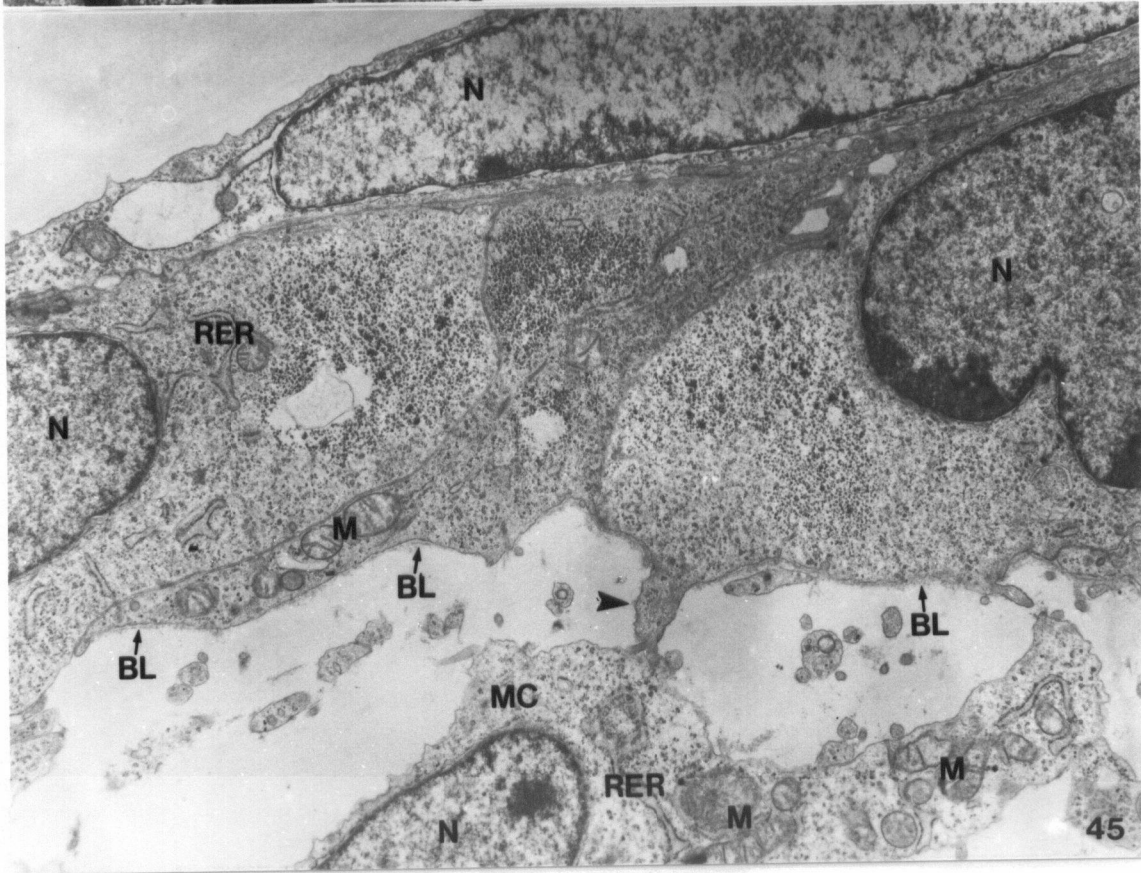
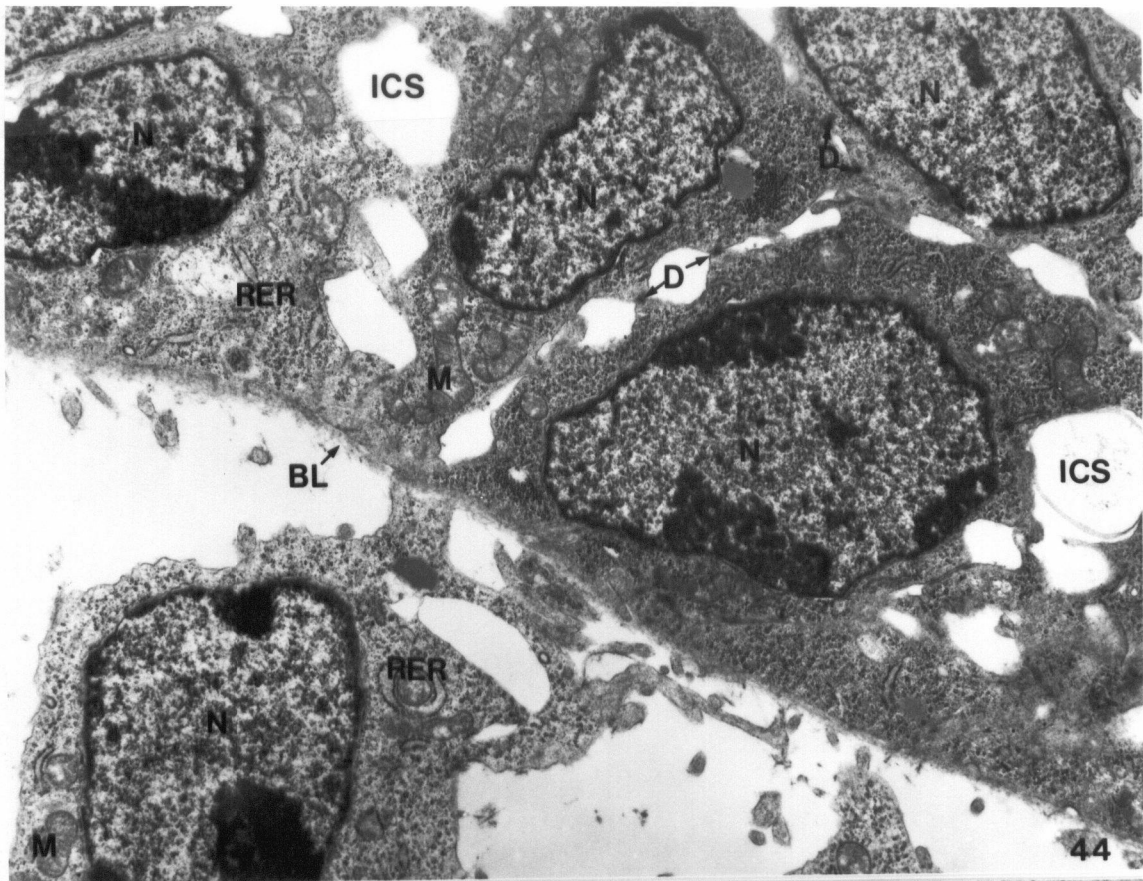
altered and unaltered epithelial cells, however, is continuous (Figs. 42, 43).

In the ensuing 24 hours, i.e., between days 10:18 and 11:18 of gestation, the epithelial and mesenchymal changes in the 6MP treated palate are gradually reduced. On day 11:18 of gestation, the epithelium on the medial aspect of the vertical palatal shelf is bilaminar (Fig. 44). Both the superficial and basal epithelial cells are irregular in appearance. They contain an irregular nucleus surrounded by polyribosomes, a few mitochondria, an occasional cisterna of rough endoplasmic reticulum and lipid droplets. The basal cells are attached with one another, and with the superficial cells by desmosomes. Numerous spaces otherwise intervene between cells of the epithelium. The basal lamina separating the epithelium and mesenchyme is continuous.

The mesenchymal cells are stellate and contain organelles similar to those described for epithelial cells (Fig. 44).

Subsequently, unlike those of the control palates, the epithelial cells of 6MP treated did not show any appreciable alterations between days 11:18 and 12:18 of gestation. On day 12:18 of gestation, the epithelium on the medial aspect of the vertical palatal shelf is bilaminar (Fig. 45). Both the superficial and basal cells contain a large nucleus surrounded by few cytoplasmic organelles. In comparison to 6MP treated palatal epithelium on day 11:18, however, the epithelial cells are closely packed and intercellular spaces are small or absent on day 12:18 of gestation (Fig. 45).

The mesenchymal cells on day 12:18 of gestation are irregular in appearance (Fig. 45). Their cytoplasmic contents remain unchanged.



- Fig. 44** Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 11:18 of gestation showing a bilaminar epithelium. Both the superficial and basal epithelial cells contain a nucleus (N) surrounded by polyribosomes, mitochondria (M), and rough endoplasmic reticulum (RER). The epithelial cells are attached to one another by desmosomes (D). A mesenchymal cell shows a nucleus (N) surrounded by polyribosomes, mitochondria (M) and rough endoplasmic reticulum (RER). ICS - intercellular space. BL - basal lamina. 6892X.
- Fig. 45** Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 12:18 of gestation showing discontinuity in the basal lamina (BL). A thin cytoplasmic extension (arrow head) of the basal epithelial cell extends through the basal lamina and contacts a mesenchymal cell (MC). Intercellular spaces are missing (cf. Fig. 44). N - nucleus. M - mitochondrion. RER - rough endoplasmic reticulum. 6105X.

At the epithelial-mesenchymal interface, striking changes are visible on day 12:18 of gestation. At various places the basal lamina is discontinuous (Fig. 45). A thin cytoplasmic extension of the basal epithelial cell extends through the basal lamina, and either contacts a mesenchymal cell (Fig. 45), or remains free (Fig. 46).

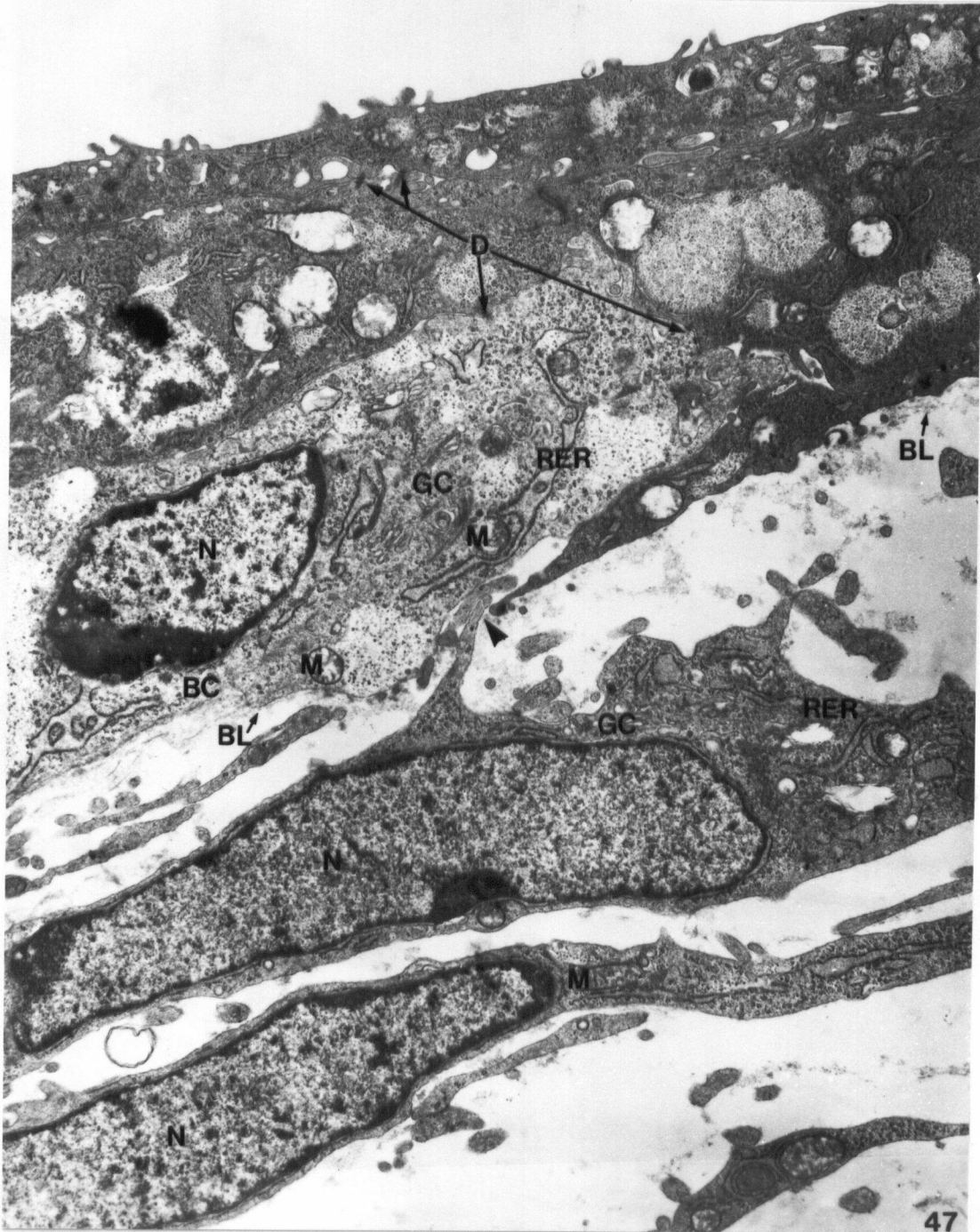
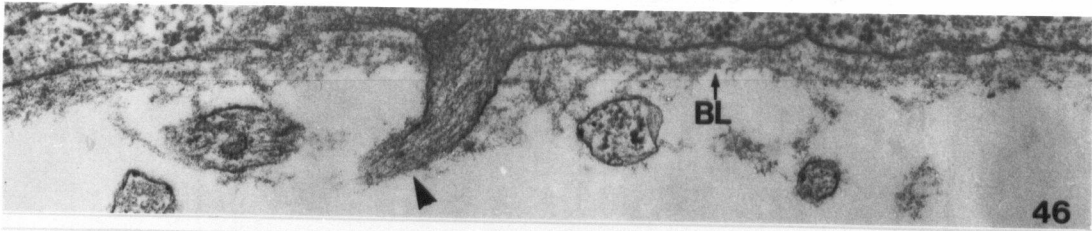
During the ensuing 24 hours, i.e., between days 12:18 and 13:18 of gestation, the basal lamina changes become progressively more severe in the 6MP treated palates. On day 13:18 of gestation, when palates of control fetuses are already closed, changes are observed in the epithelium, the mesenchyme and the basal lamina of the 6MP treated vertical palatal shelf.

In comparison to the superficial epithelial and mesenchymal cells, the basal epithelial cells appear light due to reduced polyribosome content (Fig. 47). In addition, their cytoplasm contains a few mitochondria, cisternae of rough endoplasmic reticulum, and a small Golgi Complex (Fig. 47).

In contrast to those observed at day 12:18 of gestation, the mesenchymal cells on day 13:18 of gestation vary in shape from stellate to elongated (Fig. 47). They contain a flat nucleus surrounded by polyribosomes, numerous cisternae of rough endoplasmic reticulum, few mitochondria and a well developed Golgi Complex.

The basal lamina at the interface of the epithelium and mesenchyme is almost missing (Fig. 47). In many instances a cytoplasmic extension of the mesenchymal cell appears to have penetrated the disappearing basal lamina to contact the epithelial cell (Fig. 47).

Eventually, however, the alterations in the epithelial cells become less severe. Between days 14:00 and 15:00 of gestation, the epithelium on the 6MP



- Fig. 46** Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 12:18 of gestation showing a cytoplasmic extension (arrow head) of the basal epithelial cell. BL - basal lamina. 42325X.
- Fig. 47** Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 13:18 of gestation showing a light basal cell (BC). The cytoplasm of the light basal cell contains a reduced polyribosome content, mitochondria (M), rough endoplasmic reticulum (RER), and a Golgi complex (GC). The elongated mesenchymal cells contain a nucleus (N) surrounded by polyribosomes, rough endoplasmic reticulum (RER), mitochondria (M) and a Golgi complex (GC). The basal lamina (BL) is almost missing. A cytoplasmic extension (arrow head) of the mesenchymal cell contacts the epithelial cell. N - nucleus. D - desmosome. 7565X.

treated cleft palatal shelf is made of 3-5 cell layers (Fig. 48). The basal cells are roughly cuboidal, and contain an irregular nucleus surrounded by polyribosomes, mitochondria, few cisternae of rough endoplasmic reticulum, and tonofilaments. The outer 2-4 layers of cells are flat, and contain organelles similar to those in the basal epithelial cells. Spaces are numerous between the basal, and between the basal and suprabasal epithelial cells (Fig. 48). Both the basal and outer flat cells are attached to their neighbours by desmosomes. The basal lamina separating the epithelium from the mesenchyme is continuous (Fig. 48). Numerous hemidesmosomes are seen for the first time between the plasma membrane of the basal cell and the basal lamina (Fig. 48). The mesenchymal cells of the cleft palatal shelves remain unaltered.

Light Microscopic Observations of Enzyme Acid Phosphatase in the Developing Secondary Palate of Control Hamster Fetuses

Prior to day 12:00 of gestation, i.e., histological Stages I and II of palatal development, the reaction product of enzyme acid phosphatase is absent in both the epithelial and mesenchymal cells. A mild reaction product of the enzyme is observable in cells of the medial edge epithelium of the horizontal palatal shelves (Stage III), and epithelial seam (Stage IV). At histological Stage V, the acid phosphatase reaction product is seen in the epithelial cells of the fragmented seam, and in the nearby mesenchymal cells.

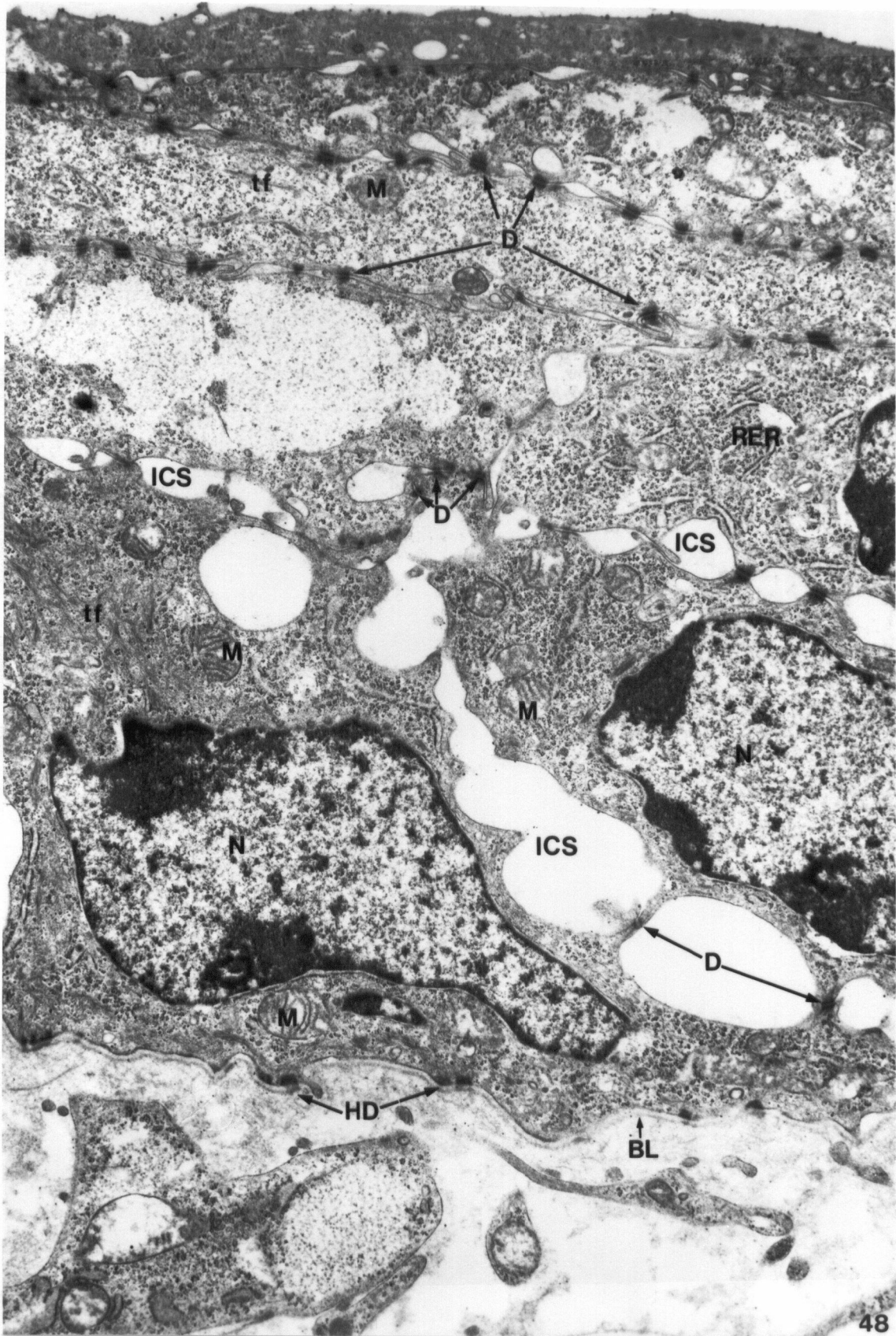


Fig. 48 Electron micrograph of the secondary palate in a 6MP treated hamster fetus on day 14:00 of gestation showing a stratified epithelium. The basal cells contain irregular nuclei (N) surrounded by polyribosomes, mitochondria (M), cisternae of rough endoplasmic reticulum (RER), and tonofilaments (tf). The outer 3 to 4 layers of cells are flatter, and contain organelles similar to those in the basal epithelial cells. Numerous spaces (ICS) intervene between epithelial cells. D - desmosome. HD - hemidesmosome. BL - basal lamina. 8373X.

Light Microscopic Observations of Enzyme Acid Phosphatase in the Developing Secondary Palate of 6MP Treated Hamster Fetuses

Unlike controls, the enzyme acid phosphatase reaction product in the 6MP treated palate is first visible in the mesenchymal cells on day 10:06, and then in both the epithelial and mesenchymal cells on day 10:12 of gestation (histological Stage II). Subsequently, the enzyme activity is not seen at any time in 6MP treated palates during development.

The appearance of intracytoplasmic areas containing dense granules and dense bodies at ultrastructural level, and corresponding localization of enzyme acid phosphatase reaction product at light microscopic levels, in both the control and 6MP treated palatal tissues, confirms the lysosomal nature of the dense granules and dense bodies (Novikoff, 1963; Erickson, 1969).

DISCUSSION

Normal Palatal Development

Results of the present study have shown that the normal palatal development in hamster is completed between days 10:00 and 13:00 of gestation. During this period the palatal shelves initiate vertically from the roof of the oronasal cavity toward its floor. The shelves then change the direction of their development from vertical to horizontal. Subsequently, opposing epithelia of the horizontal shelves fuse with one another to form a seam. The epithelial seam, thereafter, undergoes fragmentation to allow the mesenchyme of the two shelves to become continuous. These sequential events of hamster palatal development, with the exception of the initial formation of palatal shelves (Stage 1), have also been described in other mammals such as mice (Walker and Fraser, 1956; Dostal and Jelnick, 1970; Waterman et al., 1973), rats (Asling et al., 1960; Zeiler et al., 1964; Coleman, 1965; Ferguson, 1978; Schupbach et al., 1983), monkeys (Asling and Van Wagenen, 1967; Steffek et al., 1968; Bollert and Hendrickx, 1971), gerbils (Holmstedt and Bagwell, 1977), rabbits (Walker, 1971; Meller et al., 1980), ferrets (Gulamhusein and England, 1982), and human (Fulton, 1957; Wood and Kraus, 1962; Kitamura, 1966; Burdi and Faist, 1967; Anderson and Matthiessen, 1967; Luke, 1976).

Furthermore, observations between days 12:00 and 13:00 of gestation have shown that the reorientation of the palatal shelves from vertical to horizontal plane was completed in all fetuses by day 12:06 of pregnancy. Subsequently, the closure of the middle third of the secondary palate was

completed in all fetuses by day 12:12 and that of the posterior third and anterior third by days 12:18 and 13:00 of gestation, respectively. Similar chronological observations on palatal closure in hamster have been made in other studies (Shah and Chaudhry, 1974a; Shah and Travill, 1976a; Shah, 1979a, b; Shah and Wong 1980). In addition, the present study also reconfirmed the observations made in these studies that not only the age, but also the weight and CRL of the fetus are reliable indicators of stages of normal palatal development in hamster. In their studies on mouse palatogenesis Walker and Crain (1960) and Dostal and Jelnick (1972) also noted a good correlation between the stages of palatal development and fetal weight, but not between the stages of palatal development and fetal age. The reliability of chronologic age as an indicator of the stages of normal palatal development in hamster may be due to a relatively shorter period of organogenesis and a corresponding smaller period of palatogenesis in hamster as compared with mice. It is anticipated that the reproducibility of the accurate timings, fetal weights, and fetal CRL for the important events of palatogenesis in hamster should strengthen its usefulness as a biological system for further embryological and teratological studies.

In contrast to mammals, the palatal shelves in the other vertebrates such as chick (Shah and Crawford, 1980; Koch and Smiley, 1981), quail (Shah et al., 1984b), and alligator (Ferguson, 1981) originate horizontally over the dorsal surface of the tongue. Subsequently the horizontal shelves never fuse. One may, therefore, suggest that Class differences exist among vertebrates with regard to the sequence of palatal development. Further investigation of difference in palatogenesis between various vertebrate

classes may prove useful in understanding normal and abnormal biology of the palate.

There is a paucity of literature on the issue of why the mammalian palatal shelves first develop in a vertical direction when their eventual position is horizontal. It has been presumed that a large tongue occupies the oronasal cavity before the appearance of the palatal primordia. A lack of room over the tongue would consequently direct the palatal shelves to grow vertically along the sides of the tongue (Keibel and Mall, 1912; Fraser, 1947; Hayward and Avery, 1957; Patten, 1971; Tuchmann-Duplessis and Haegel, 1974; Dryden, 1977). The results of the present study, however, show that the palatal shelves appear prior to the development of the tongue. By the time a large tongue occupies the space in the oronasal cavity, the vertical palatal shelves are already well developed. One may, therefore, suggest that initial formation of palatal primordia is not related to the development of the tongue. Further credence to this suggestion comes from an analysis of human cleft palate cases where it was noted that in many instances of aglossia associated with cleft palate in embryos, the shelves were vertical (Shah, 1977b).

Recently Flint (1980) observed the initiation of mouse palatal primordia from the roof of the oronasal cavity, but he did not indicate the status of tongue development at the time of palatal initiation. However, observations on embryonic palatal development in chick (Shah and Crawford, 1980; Koch and Smiley, 1981), alligator (Ferguson, 1981) and quail (Shah et al., 1984b) have shown that, despite the presence of a large tongue, the palatal shelves appear horizontal from the beginning. Thus these observations in lower

vertebrates would also argue against an active role of tongue in guiding the initial development of the palate in a vertical direction.

Earlier Shah (1977b) and Ferguson (1981) had hypothesized that the initiation of mammalian embryonic palate in the vertical direction may be determined genetically, and is probably not dependant on the physical presence of surrounding structures such as the tongue. The observations of the present study, along with those made from lower vertebrates, lends support to this hypothesis.

It seems that although the basic sequence of palatal development in mammals is similar, there are species differences in the pattern of palatal closure. For example, in mice (Walker and Fraser, 1956), rats (Zeiler et al., 1964; Coleman, 1965; Schupbach et al., 1983), gerbils (Holmstedt and Bagwell, 1977), ferrets (Gulamhusein and England, 1982), and humans (Fulton, 1957; Kitamura, 1966; Burdi and Faist, 1967; Waterman and Meller, 1974; Luke, 1976) the closure starts in the middle third of the palate and then simultaneously extends anteriorly and posteriorly, whereas in rabbits (Walker, 1971; Meller et al., 1980) and monkeys (Asling and van Wagenen, 1967; Steffek et al., 1968; Bollert and Hendrickx, 1971) the closure begins anteriorly and proceeds posteriorly. The present investigation showed that in hamsters closure starts in the middle third of the secondary palate, extends posteriorly, and finally anteriorly in that order. Thus it seems that even though the basic sequence of mammalian palatogenesis is similar, species differences in the patterns of palatal closure may be reflected during abnormal palatogenesis and result in the morphologically different types of palatal clefts seen in humans (Veau, 1931; Lynch et al., 1966; Fara,

1971; Smiley, 1972; Mitts et al., 1981), and in laboratory animals after experimental manipulation (Giroud and Martinet, 1956; Buresh and Urban, 1964; Walker, 1967; Dostal and Jelinek, 1971; Shah and Travill, 1976a; Shah and Kilistoff, 1976; Shah, 1977a, b; Shah and Mackay, 1978; Shah and Burdett, 1979; Shah et al., 1979; Kusanagi, 1983; Schupbach, 1983).

Lysosomes were observed in the epithelial cells and met the morphologic features of secondary lysosomes and autophagic vacuoles or cytosegresomes (DeDuve, 1963; Erickson, 1969). The lysosomes were first seen in the medial edge epithelial cells of the horizontal palatal shelves. After the formation of the epithelial seam, the number and size of lysosomes had increased in the cells of the seam until they were lost. These observations are in accordance with those reported earlier by Chaudhry and Shah (1973, 1979) and Shah and Chaudhry (1974a, b). However, De Angelis and Nalbandian (1968), Farbman (1968, 1969) and Brusati (1969) did not observe any lysosomes in the prefused epithelia. Mato et al. (1966, 1967a), Hayward (1969), Morgan (1969) and Smiley (1970) observed them in the basal cells prior to fusion but did not attach any significance to the timings of lysosomal appearance. In their studies Angelici and Pourtois (1968), Koziol and Steffek (1969), Vargas et al. (1972), Shah and Chaudhry (1974a), Lorente et al. (1974), Holst and Mills (1975), and Im and Mulliken (1983) have noted that during and after the fusion of opposing epithelia there is a significant increase in the acid hydrolytic activity which reaches its maximum when the epithelial seam starts to undergo fragmentation. As a corresponding increase in the number and size of lysosomes was observed during the present investigation, the observations give further credence to the proposition that the timely appearance and

subsequent increase in number of lysosomes play an important role in the autophagic degeneration of cells (Mato et al., 1966; Smiley, 1970; Chaudhry and Shah, 1973, 1979; Shah and Chaudhry, 1974a, b; Greene, 1983; Shah, 1984).

With regard to the role of macrophages during palatal development, the observations in the present study are in harmony with those reported by Mato et al. (1967a), Chaudhry and Shah (1973, 1979) and Shah and Chaudhry (1974b). At no time was one able to observe the macrophage penetrating the basal lamina to destroy the epithelial cells, as has been previously suggested by Anderson and Matthiessen (1967). It would, therefore, appear that macrophages are mainly concerned with the removal of cellular debris, rather than the destruction of cells during palatal closure.

6-Mercaptopurine Induced Cleft Palate

In 1979, Shah and Burdett reported that 6MP is a potent teratogen when injected into pregnant hamsters, and that it produces cleft palate in the fetuses. An extensive review of the literature indicates that this is the first comprehensive investigation which shows that a teratogen, 6MP, induces cleft palate by affecting vertical development of the palatal shelves.

Flint (1980) observed that in the CBA/101 mutant mouse, cleft palate arises due to a failure of the primordia to grow from the beginning. Morphological observations of palatogenesis in mice and rats following teratogenic treatment have shown that cortisone, high doses of vitamin A, radiation, folic acid deficiency, 5-fluoro-2-desoxyuridine, and

diazo-oxo-norleucine delay reorientation of the palatal shelves from a vertical to horizontal plane (Walker and Fraser, 1957; Walker and Crain, 1960; Asling et al., 1960; Callas and Walker, 1963; Kochhar and Johnson, 1965; Coleman, 1965; Ross and Walker, 1967; Nanda, 1971; Dostal and Jelnick, 1972; Ferguson, 1977; Diewert, 1979; Diewert and Pratt, 1979). Similar observations in hamster have shown that triamcinolone and 5-fluorouracil treatment retard the palatal shelf reorientation (Shah, 1979e; Shah and Wong, 1980), whereas hydrocortisone treatment prevents the fusion between opposing horizontal shelves (Shah and Travill, 1976a, b). During the present investigation the timing of the initial appearance of palatal primordia in the treated fetuses resembled that of the controls. Thereafter, the vertical development of the palatal shelves was affected. The reorientation of the palatal shelves from vertical to horizontal, and their subsequent fusion never occurred. One must be cautious in comparing the results reported in the present study with those noted earlier in the paragraph. The caution is in part due to the species differences involved, and partly due to the chemical natures of teratogens and different schedules of treatment used. For example, triamcinolone, 5-fluorouracil and hydrocortisone were administered intramuscularly on day 11:00 of gestation into the pregnant hamster (Shah and Travill, 1974; Shah, 1979e; Shah and Wong, 1980) as compared to intraperitoneal injection of 6MP given to the hamsters on day 9:00 of gestation in the present study.

It was shown during the present study that both fetal weight and CRL provide reliable indicators for the stages of normal palatal development. Following 6MP treatment, however, only the first stage of palatal development

could be predicted on the basis of both fetal weight and CRL. The palatal closure was completed in normal fetuses weighing 551 mg and measuring 16 mm (Tables VI, IX), but in the treated ones with similar weight and CRL (Tables VII, X), the palatal shelves were vertical. At birth, however, the treated fetuses were significantly lighter than the controls. One may, therefore, suggest that following 6MP treatment (a) both fetal weight and CRL are poor indicators to determine the stages of palatal development, and (b) there is an association between the cleft palate and the reduction in fetal CRL, and in fetal weight. Similar observations were made earlier by Fraser and Fainstat (1951), Chaudhry et al. (1967), Shah and Travill (1974) and Shah and Wong (1980) following treatment of mice and hamsters with different teratogens.

At cellular and subcellular levels, four main differences were observed during palatal development between 6MP treated fetuses and controls. These were (a) nuclear alterations, (b) premature appearance of lysosomes, initially in the mesenchymal cells and then in the epithelial cells, (c) subsequent changes in the epithelial cells, and (d) alterations in the basal lamina.

Following 6MP treatment in the present study, the initial ultrastructural alteration observed was swelling of the perinuclear space in the mesenchymal cells of the developing palatal primordia. Similar alterations have been observed in the neural epithelium of developing rats and mice fetuses exposed to trypan blue (Peters et al., 1979), high doses of vitamin A (Morris, 1973; Theodosis and Fraser, 1978), and fluoro-deoxyuridine (Langman and Cardell, 1978), human epithelial cells

maintained in vitro after cadmium exposure (Ree et al., 1982), rabbit erythrocyte (Glauert, 1963) and rat dermal fibroblast (Daniel et al., 1966) after retinol exposure, leukemic monocytes (Smetana and Hermansky, 1966), cat muscle fibres degenerating after long term immobilization (Cooper, 1972), and in porcine muscle affected with congenital myofibrillar hypoplasia (Zelena et al., 1978). The swelling of the perinuclear space is, however, not reported in other ultrastructural studies of cleft palate (Table II), or following 6MP induced limb malformations in rats (Merker et al., 1975). The precise nature of the defect of the nuclear envelope is not clear. Most investigators did not attach any significance to the observation. It has been suggested that the swollen nuclear envelope may reflect its altered permeability as a sequela of primary injury to the membrane system of the cell (Morris, 1973), or as part of a sequela of cell death (Peters et al., 1979). Normally the perinuclear space contains several enzymes (Whaley et al., 1971) which regulate the interaction of chromosomes with the nuclear envelope (Franke et al., 1981), and subsequently allow nuclear-cytoplasmic interaction (Baud, 1963; Goldstein, 1974; Franke, 1974). It is plausible that following 6MP assault of the mesenchymal cells, the enzyme systems of the nuclear membrane may be affected, thereby altering its permeability (Morris, 1973), and subsequently disrupting nuclear-cytoplasmic interaction and thus the cytoplasmic homeostasis. At no time in the present study was degeneration of mesenchymal cells observed to follow swelling of the perinuclear space, as was observed by Peters et al. (1979).

In the treated embryos lysosomes were first recognized in the mesenchymal cells of the vertically developing palatal primordia at a very

early age (i.e., day 10:00 of gestation). The number of affected mesenchymal cells increased for approximately 12 hours, and then decreased. On day 10:12 of gestation, lysosomes also appeared in the superficial and basal epithelial cells of the vertically developing palatal shelves. Lysosomally affected cells in the mesenchyme, however, were always more than in the epithelium. On day 11:00 of gestation, and thereafter, lysosomes were absent in both the epithelial and mesenchymal cells of the cleft palatal shelves. The appearance of lysosomes in the epithelial cells of developing vertical palatal shelves is temporally abnormal, and thus not related to the physiological cell death observed during normal palatal development. On the other hand, during normal palatogenesis lysosomes do not appear in differentiating mesenchymal cells, except in macrophages. The appearance of lysosomes in the mesenchymal cells following 6MP treatment is, therefore, abnormal. A similar abnormal lysosomal response in the mesenchymal cells following 6MP treatment has been observed in developing limb bud (Merker et al., 1975), and in cleft palate induced by other chemicals (Ferguson, 1978; Shah et al., 1984a).

Several investigators have observed a transient appearance of lysosomes in a variety of embryonic tissues following treatment with numerous teratogens (Jurand, 1966, 1968; Crawford et al., 1972; Schweichel and Merker, 1973; Morriss, 1973; Merker et al., 1975; Sadler and Cordell, 1977; Kochhar et al., 1978; Herken et al., 1978; Langman and Cardell, 1978; Ferguson, 1978; Merchant-Larios and Coello, 1978; Peters et al., 1979; Abramovici et al., 1980; Desesso, 1981). They assumed that the appearance of lysosomes and their morphological variants was an expression of cell death.

It has been, however, repeatedly suggested that autophagocytosis will be followed by cell death, only if the processes of sequestration and subsequent digestion of the damaged portions of the cell cannot cope with the toxic stress (Swift and Hruban, 1964; Erickson, 1969; Kerr, 1971; Wyllie, 1981; Trump et al., 1983). Since, in the present study, the cytoplasmic features of the mesenchymal cells containing lysosomes appeared otherwise normal, one may suggest that such cells were able to overcome the effects of 6MP. A similar deduction was also reached by Theodosis and Fraser (1978) in their study on hypervitaminosis A induced limb defects in mice and by Shah et al. (1984a) in their study on 5-fluorouracil induced cleft palate development in hamster. It seems that the differentiating palatal mesenchymal cells are capable of either neutralizing and/or detoxifying the exogenous substrate, 6MP, or its metabolic product through the synthesis of lysosomal enzymes (Kerr, 1971; Arstila et al., 1974; Trump et al., 1983; Gedigk and Totovic, 1983). Thus the premature appearance of lysosomes in the present study may be interpreted as a sublethal, and protective response. The interpretation is further strengthened by observations that, unlike a dying cell where cytoplasmic organelles undergo profound changes (Morris, 1973; Peters et al., 1979; Trump et al., 1983; Constantinidis, 1984), the general appearance of cytoplasmic organelles in the mesenchymal cells remained healthy.

The sublethal injury to the mesenchymal cells, however, seems to have irreversibly altered their path of differentiation. Data on the rate of mesenchymal cell proliferation, and the synthesis of extracellular matrix molecules by mesenchymal cells at the time of development of palatal

primordia is lacking. These issues are, however, considered to be important during subsequent palatal development (Table II). It is not clear whether the stunted vertical palatal shelves seen at term reflect a reduction in the mesenchymal cell population, or synthesis of extracellular matrix, or both.

The premature appearance of lysosomes in the palatal epithelial cells may be the result of either a direct sublethal response to 6MP treatment or its metabolic product, or an indirect response to the preceding mesenchymal cellular injury. There is no direct evidence in the present study to support either view. As discussed for the mesenchymal cells in the earlier paragraphs, premature lysosomal formation in the epithelial cells may also indicate a sublethal response to neutralize and/or detoxify the harmful cytotoxic effects of 6MP or its metabolic product. The plausibility of this explanation is further enhanced by the fact that the morphology of cytoplasmic organelles of the epithelial cells appeared healthy. On the other hand, although an epithelial-mesenchymal interaction is considered to be important for terminal differentiation of the palatal epithelial cells (Pourtois, 1972; Tyler and Koch, 1975, 1977a, b; Shah et al., 1983; Shah, 1984), there is no evidence in the present study or in the literature, that the mesenchyme may affect differentiation of epithelial cells at the time of palatal primordial development. Furthermore, the basal lamina separating the injured epithelial and mesenchymal cells was intact (Fig. 42). An exchange of information, if any, between them may still occur at the molecular level through the basal lamina. Under such circumstances the appearance of lysosomes in the epithelial cell may reflect a response to injury in the mesenchymal cell. Thus this possibility cannot be ruled out.

The injury to epithelial cells, like the mesenchymal cells, also appears to have irreversibly altered their path of differentiation. Consequently between days 12:00 and 13:00 of gestation the 6MP treated palatal epithelia do not exhibit any signs of lysosomally mediated programmed cell death observed during normal development (Mato et al., 1966; Smiley, 1970; Chaudhry and Shah, 1973; Shah and Chaudhry, 1974a, b; Greene, 1983; Shah, 1984). Thereafter, however, the basal epithelial cells show transient cytoplasmic alterations on day 13:18 of gestation (Fig. 47). The alterations seem to be reversible because subsequently on day 14:00 of gestation the epithelial cells appear healthy (Fig. 48). A similar latent response of the epithelial cells has not been noted in other ultrastructural studies on chemically induced cleft palate (Table II). One may perhaps suggest that the epithelial cells, having changed their path of differentiation, were discarding organelles which are normally involved in programmed elimination. During the present study no loss of epithelial cells was evident at any time.

The lysosomally-mediated sublethal response in 6MP-induced cleft palate is different from the effect of other teratogens (Table II). The autolytic degeneration in the palatal epithelium of mice and rats was either reduced or prevented following treatment with β -aminopropionitrile (Mato et al., 1975b), meclozine (Morgan, 1976), epidermal growth factor (Hassell, 1975; Hassell and Pratt, 1977), diazo-oxo-norleucine (Pratt and Greene, 1976; Morgan and Pratt, 1977), triamcinolone (Kurisu et al., 1981 phenylbutazone (Montenegro and Paz de la Vega, 1982), and cortisol (Goldman et al., 1983). The response of the mesenchyme was not reported in these studies. In

hamster, following hydrocortisone and triamcinolone treatment (Shah and Travill, 1976b; Shah, 1980) a premature nonlysosomal necrosis of the epithelium was observed but the mesenchymal cells appeared unaffected. Shah et al. (1984a) observed a lysosomally-mediated sublethal response first in the epithelial, and then in the mesenchymal cells of developing hamster palate following 5-fluorouracil treatment. Ferguson (1978) reported cell death in the developing rat palatal mesenchyme following 5-fluoro-2-desoxyuridine treatment. It seems that the chemical nature of the drug, its species specific effect, and the metabolic state of the developing tissues at the time of treatment, among other factors, may determine the nature of the tissue response.

In the present study, epithelial and mesenchymal cytoplasmic extensions into each others territory, and their contacts with one another were observed following sublethal injury. Cytoplasmic extensions of the epithelial cells and epithelial-mesenchymal contacts have also been observed in other studies on teratogen induced cleft palate (Shah, 1980; Shah et al., 1984a). It was suggested that the extension of epithelial cell processes into the mesenchyme increased the surface area of the cell to receive nutrients following injury (Shah, 1980). In the present study the cellular projections and contacts were seen late during cleft palate development, i.e., on day 12:18 of gestation. After day 11:00 of gestation the injured epithelial and mesenchymal cells appeared to have been repaired, and structurally resembled their normal counterparts. Furthermore, mesenchymal cytoplasmic extensions also extended into the epithelium (Fig. 47). Thus it is unlikely that the epithelial and mesenchymal cytoplasmic projections and contact between them,

as observed in the present study, develop for nutritional purposes. Epithelial-mesenchymal communications have been considered to play an important role during normal development in determining the differentiation and fate of the palatal epithelium (Pourtois, 1972; Tyler and Koch, 1977b; Tyler and Pratt, 1980; Shah et al., 1983; Shah, 1984). It is possible that, following 6MP treatment, since the injured epithelial and mesenchymal cells may have deviated from the normal path of differentiation, they prepared themselves for a new role in the cleft palate by communicating with one another. The observations of light cells on day 13:18 of gestation (Fig. 47) may indeed be the response to an epithelial-mesenchymal interaction.

Alterations in the configuration of the basal lamina have been observed in many pathological and experimental situations (Pearson and Spargo, 1961; Caulfield and Wilgram, 1962; Johnson and Fry, 1967; Frithiof, 1969; Woods and Smith, 1969, 1970; Vracko, 1972, 1979; Tarin, 1972; Yamanishi et al., 1972; Sugimoto et al., 1973; Takarada et al., 1974; Martinez-Hernandez et al., 1976; Jao et al., 1978; Frei, 1978; Ingber et al., 1981; White and Gohari, 1981; Otsubo and Kameyama, 1982; Yasuzumi et al., 1983). The alterations include irregularities in thickness, reduplication, detachment from the basal cells, fragmentation and complete disappearance. In the present study, the alterations in the basal lamina were seen long after injury to the epithelial and mesenchymal cells. The changes were characterized initially by breaks in the continuity of the basal lamina followed by its total loss. Similar observations on the basal lamina configuration have been made following treatment with several teratogens (Mato et al., 1975a, b; Shah and Travill, 1976b; Morgan, 1976; Ferguson, 1978; Shah, 1980, 1984; Shah et al., 1984a).

In pathological circumstances, the fragmentation and loss of basal lamina has been related either to its destruction by enzymes, or to a failure of epithelial cells to form it (Woods and Smith, 1969, 1970; Tarin, 1972; Yamanishi et al., 1972; Takarada et al., 1974; Martinez-Hernandez et al., 1976; Frei, 1978; White and Gohari, 1981). Although the present study lacks evidence to support either view, the latter seems more plausible. At the ultrastructural level there was no evidence that lysosomal enzymes escaped the epithelial and/or mesenchymal cells to affect the basal lamina. Fragments of cellular material, which appeared to be expelled from the mesenchymal cells on day 10:06 of gestation (Fig. 41) were membrane bound, and it is doubtful that enzymes would escape during their phagocytosis by macrophages. Furthermore, from a chronological viewpoint, the lysosome-mediated cellular injury subsided by day 11:00 of gestation, while early basal lamina changes were visible first on day 12:18 of gestation, i.e., after 42 hours. It is highly unlikely that enzymes would persist extracellularly over such a long duration. On the other hand, it is generally agreed that the basal lamina is an epithelial product (Kurtz and Feldman, 1962; Pearce et al., 1963; Briggman et al., 1971; Timpl et al., 1979; Stanley et al., 1982). Altered differentiation of the epithelial cells due to sublethal injury, as implicated in the earlier paragraphs, could affect the synthesis of molecules required for basal lamina formation and maintenance, thus causing the defect.

Merker et al. (1975) indicated that, following GMP treatment, the mesenchymal cell damage in the developing rat embryonic limb bud occurred after 5 hours. They further indicated that the epithelial cells were not

affected. Scott et al. (1980), on the other hand, noted that the effect of 6MP treatment on the rat embryo limb bud was observed in the mesenchymal cells, and then in the epithelial cells, after 24 hours. Adhami and Noack (1975), and Adhami (1979) also noted a duration of 24 hours for 6MP to affect the rat embryonic brain tissues. Observations of the present study in hamster show that the initial adverse effect of 6MP is seen after 24 hours in the mesenchymal cells, and later on in the epithelial cells. The difference in the interval for cellular response observed by Merker et al. (1975) and others may possibly be attributed to the vehicle used to inject the drug. Merker et al. (1975) used 0.9% NaCl and 2.5% propylene glycol to administer 6MP, whereas Adhami and Noack (1975), Adhami (1979), Scott et al. (1980), and in the present study water was used as a vehicle for 6MP injection. Furthermore Scott et al. (1980) injected 6MP on day 11:00 whereas Merker et al. (1975) injected on day 12:00 of gestation to induce limb malformations. It is possible that these differences in vehicle may affect the duration of placental transfer, and that of metabolism of 6MP in the maternal, placental and fetal tissues, and hence in the eventual timings of the tissue response. It is also possible that cells at the initial stages of differentiation are highly susceptible to 6MP induced injury (Merker et al. 1975). The proposition is further strengthened by the observations that 6MP affected the primordial stage of the development of limb (Merker et al., 1975; Scott et al., 1980), and palate (Present study) to induce the malformations.

As indicated in the INTRODUCTION, several biochemical pathways have been suggested by which 6MP may exert its action. The mechanism, however, by which 6MP affects differentiating cells and tissues in a developing embryo is

not known. It is suggested that 6MP goes through the placenta and is anabolized by the fetus into active antimetabolite (Neubert et al., 1977, 1980). The activated antimetabolite then may affect the cellular functions by one of the following mechanisms: (1) directly affecting RNA synthesis, especially that of mRNA and consequently affecting protein synthesis (Roy-Burman, 1970; Neubert et al., 1970; Mewes et al., 1971; Kawahata et al., 1980), or (2) interference with DNA synthesis either via directly inhibiting enzymes such as adenylosuccinate synthetase, or feedback inhibition of amidotransferase (Neubert et al., 1977), thus affecting purine metabolism (Fig. 2) (Elion and Hitchings, 1965; Elion, 1967; Roy-Burman, 1970; Malamud et al., 1972; Paterson and Tidd, 1975; or (3) by direct incorporation into DNA (Martin et al., 1972; Tidd and Paterson, 1974; Zimmerman et al., 1974; Breter and Zahn, 1979; Ding and Benet, 1979; Lennard and Maddocks, 1983). Merker et al. (1975) proposed that the rapid necrosis, which occurs in the limb mesenchyme within 5 hours after 6MP treatment cannot be solely due to reduced nucleic acid synthesis, because other rapidly proliferating tissues such as the epithelium and endothelium were not affected. They suggested that the rapid effect of 6MP may be due to inhibition of a short lived substance such as mRNA or cAMP which play a role in carrying information for cytodifferentiation. A lack of availability of such a substance, Merker et al. (1975) indicated, would not produce information necessary for differentiation and lead to reduced protein synthesis and eventual necrosis. It is unlikely, therefore, that the initial cellular lesions observed in the present study, and those observed earlier by Adhami and Noack (1975), Adhami (1979) and Scott et al. (1980) could be due to reduced protein synthesis,

because the duration between drug administration and the initial cellular effect was long (24 hours). Furthermore, there was no electron microscopic evidence of alterations in the protein synthetic machinery or in the polyribosomal content of the epithelial and mesenchymal cells of developing palatal primordia. Hence one may deduce that in the present study perhaps 6MP did not directly affect mRNA and protein synthesis in the palatal cells, as implicated by Merker et al. (1975) for differentiating limb mesenchymal cells. Taylor et al. (1962), Webster et al. (1973), Scott (1977) and Adhami (1979) suggested that if 6MP or its metabolites rapidly inhibited DNA synthesis the first morphologically demonstrable lesion could be expected within a few hours after drug administration. In the delayed cytotoxic response following 6MP administration, however, Tidd et al. (1972) and Horakova et al. (1974) suggested that the cells must go through the DNA synthesis-phase at least once. Also in their study on the effect of 6MP on HeLa cells in vitro Horakova et al. (1974) observed that following DNA synthesis and mitosis, HeLa cells showed reduced DNA and RNA content per cell eventually leading to cell damage or necrosis. In the present study, the observation that after 6MP treatment the palatal primordia indeed developed, suggests that perhaps at least some of its mesenchymal and epithelial cells may have had the opportunity to undergo DNA synthesis and mitosis before manifesting injury caused by the drug or its metabolite. The delayed cytotoxicity thus may reflect incorporation of 6MP into DNA as proposed by Tidd et al. (1972) and Tidd and Paterson (1974).

The mechanism by which lysosomes develop in the mesenchymal and epithelial cells following 6MP administration, and how it affects

cytodifferentiation is not clear. Menkes et al. (1970), Scott (1977), Scott et al. (1980) and others have suggested that teratogen-induced lysosomal alterations may alter some pathways involved in cellular differentiation, which consequently can delete or injure one or more cellular functions essential for histo- and morphodifferentiation. Information on metabolic pathways involved in the early differentiation of palatal tissue is, however, virtually non-existent. It is perhaps reasonable to suggest that 6MP induced lysosome-mediated sublethal injury could perturb the intracellular homeostasis, or vice versa, and subsequently alter the programmed differentiation of palatal mesenchyme and epithelium by affecting DNA synthesis, either by affecting purine metabolism, or by direct incorporation into DNA (Fig. 2) (Elion and Hitchings, 1965; Elion 1967; Tidd and Paterson, 1974). Eventually the assaulted cells of the palatal primordia, following their repair, may adapt themselves for a different role in the cleft palate.

Foregoing RESULTS and DISCUSSION indicate that normal palatal development in hamster consists of an orderly sequence of events involving growth, differentiation, and death at both cellular and tissue levels. 6MP treatment injured first the mesenchymal and then the epithelial cells of the developing palatal primordia, which in turn appear to irreversibly alter the differentiation of cells and tissues to cause the cleft palate. The regulatory mechanisms of cell and tissue differentiation during normal palatal development, however, are only partially understood. Also, the biochemical mechanism(s) of the action of 6MP on developing palatal tissues and cells are still unclear. Future investigations may be directed toward understanding these mechanisms before the pathogenesis of cleft palate following 6MP treatment can be completely defined.

SUMMARY AND CONCLUSIONS

1. Formation of the secondary palate in hamster occurs in distinct but sequential stages.
2. The age, weight, and crown-rump length of the fetus are reliable indicators of stages of normal palatal development in hamster.
3. The initial formation of the palatal shelves is independent of tongue development.
4. The timely appearance of lysosomes play a crucial role in the autolytic elimination of medial edge epithelia during palatal closure.
5. Macrophages are involved in removal of epithelial cellular debris.
6. A single intraperitoneal injection of 6-mercaptopurine on day 9:00 of gestation affects the vertical development of palatal shelves, and thus produces cleft palate.
7. An association exists between the reduction in weight and crown-rump length of the fetus and the cleft palate.
8. At cellular and subcellular levels, four main differences were observed which distinguished palatal development between the 6-mercaptopurine treated and control fetuses. In order of appearance in the treated palates, these were (a) alterations in the nuclear membrane, (b) premature appearance of lysosomes first in the mesenchymal, and then in the epithelial cells, (c) subsequent changes in the epithelial cells, and (d) alterations in the basal lamina.

9. administration of 6-mercaptopurine induces sublethal injury in the mesenchymal and epithelial cells during vertical development of the palatal shelves. The injury altered the path of cytodifferentiation.
10. The alterations in the basal lamina were secondary in response to the epithelial and mesenchymal injury following 6-mercaptopurine treatment.
11. Inhibition of DNA synthesis following 6-mercaptopurine administration may have disrupted the process of differentiation in both the epithelial and mesenchymal cells and thus caused cleft palate.

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APPENDIX 1

Preparation of solutions for Acid Phosphatase Cytochemistry (Barka and Anderson, 1962).

Formol-Calcium: Fresh formol-calcium was prepared by dissolving one gram anhydrous calcium chloride (Allied Chemical Canada Ltd., catalog# 1502, Lot# X070) in 60 ml of distilled water. To this solution, 10 ml of 40% formaldehyde was added, and adjusted to pH 7.1. The volume was then brought to 100 ml with distilled water.

Pararosanilin: With gentle warming, one gram of pararosanilin hydrochloride (J.T. Baker Chemical Co., Phillipsburg, N.J., catalog # 2903, Lot # 508512) was dissolved in 20 ml of distilled water and 5 ml concentrated hydrochloric acid. After the solution cooled, it was filtered and stored at room temperature for future use.

4% Sodium Nitrite: A fresh solution of sodium nitrite (Fisher Scientific Co., Fair Lawn, N.J., catalog # S-347, Lot # 781768) was prepared by the addition of 4 g sodium nitrite to 100 ml of water.

Michaelis Veronal Acetate Buffer: 5.85 g anhydrous sodium acetate (Fisher Scientific Co., Fair Lawn, N.J., catalog # S-210, Lot# 774397) was added to 14.714 g Barbitol Sodium (BDH Chemicals Canada Ltd., Vancouver). They were then dissolved in 500 ml of distilled water.

Naphthol AS-TR Solution: 100 mg naphthol AS-TR phosphate (Nutritional Biochemicals Co., Cleveland, Ohio, catalog # 4308) was dissolved in 10 ml N, N-dimethyl formamide (Fisher Scientific Co., Fair Lawn, N.J., catalog# D-131, Lot # 786597) and then quickly chilled in the freezer to prevent the formation of a precipitate. The solution was freshly prepared for each incubation.

Methyl Green Stain: 1 g of methyl green (Fisher Scientific Co., Fair Lawn, N.J., catalog # M-295, Lot # 773099) was dissolved in 100 ml of veronal acetate buffer, and adjusted to pH 4.0.

Incubation Solution: The incubation solution was prepared in two parts. In the first part, 30 ml of Michaelis veronal acetate buffer was mixed with 72 ml of distilled water and 6 ml of the substrate naphthol AS-TR solution. In the second part 4.8 ml of pararosanilin solution was diazotized by mixing with 4.8 ml of 4% sodium nitrite solution. The two parts were mixed and adjusted to pH 5.0 with 0.5 N NaOH. The solution was then filtered and used as the incubation medium.