A Comparative Study of Collagen Synthesis During Avian and Mammalian Secondary Palate Development: Effects of 5-Fluorouracil.

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B.D.S., University of Garyounis, 1985

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Oral Biology)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

December 1991

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Date Feb 6. 1992

DE-6 (2/88)

ABSTRACT

A study was undertaken to examine whether collagen synthesis is critical for shelf reorientation. In the initial experiments in quail, a dose of 100μg 5-fluorouracil (5-FU) administered on day 4 of incubation was determined to be the best dose-time regimen to induce cleft palate. Pregnant hamsters were given 81mg/kg 5-FU intramuscularly or 1ml saline on day 11 of gestation. Control and treated embryonic palates dissected from hamsters between days 11 and 13 of gestation, and from quail between days 5 and 10 of incubation, were incubated in a growth medium supplemented with ^{14}C-proline. The samples were used for either: 1. Collagen digestion assay to determine the rate of collagen synthesis; 2. Total protein determination; 3. High performance liquid chromatography (HPLC) to determine hydroxyproline (HYP) levels; or 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine different collagen isotypes. In addition, embryos from both hamster and quail were processed for light microscopy (LM).

The LM results showed that, in hamster 5-FU induces cleft palate by delaying the reorientation of palatal shelves, while in quail the drug widened the gap between the palatal shelves. The data on collagen synthesis showed that in control hamster a spurt in the collagen synthesis was seen in palate between days 12:00 (12 day: 0 hour) and 12:04 of gestation, which is the period of shelf reorientation. In 5-FU exposed hamster palates, the rate of collagen synthesis was lower than controls until day 12:04 of gestation followed by a spurt on day 12:12 of gestation. In 5-FU-treated embryos palatal shelf reorientation took place between days 12:16 and 13:00 of gestation. In the developing secondary palate of both the control and 5-FU-

treated quail, the rate of collagen synthesis peaked on day 8 of incubation. The collagen synthesis, however, was lower in 5-FU-treated than in the control palates. HYP levels in both control and 5-FU-treated hamster palate indicated that although an equal amount of new collagen was synthesized in both groups, the shelf reorientation was delayed in the drug-treated embryos. The HYP data from control and 5-FU-treated quail indicated that, in addition to new collagen a considerable amount of non-collagenous protein may also have been synthesized during quail palate morphogenesis. SDS-PAGE showed that only type I collagen was synthesized during palate development in both the control and 5-FU-treated hamster and quail.

It was suggested that since (1) in birds, a spurt in collagen synthesis occurs in the absence of shelf reorientation, (2) an equal amount of new collagen was synthesized in both the control and 5-FU-treated hamster embryos during the period of normal reorientation, and (3) in 5-FU-treated hamster embryos, a recovery in collagen synthesis occurs prior to, and a reduction at the time of initiation of delayed shelf reorientation, collagen synthesis may not cause shelf reorientation in mammals.

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ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. R. M. Shah for his advise, criticism and support throughout the course of this thesis. I would like to express my appreciation to Mrs. E. Feeley and Mr. J. Firth for their various technical help.

I am also grateful to Dr. K. Cheng, Director of the University of British Columbia's Quail Genetic Stock Center for providing a continuous supply of quail eggs.

I would like to express my gratitude to my brother Ashour Ben-khaial for his moral and continuous financial support. In addition. I wish to thank all the staff and students of the Department of Oral Biology and Faculty of Dentistry for providing a friendly environment.

The work conducted for this thesis was supported by a grant from the NSERC of Canada to Dr. R. M. Shah.

INTRODUCTION

Normal Development of the Secondary Palate

The vertebrate secondary palate develops intraorally as bilateral symmetrical outgrowths from the maxillary processes of the first pharyngeal arch. A review of literature indicates that much of the work on the embryology of the secondary palate was conducted on mammalian embryos (Dursy, 1869; His, 1901; Polzl, 1904; Schorr, 1907; Inouye, 1912; Pons-tortella, 1937; Lazzaro, 1940; Walker and Fraser, 1956; Asling et al., 1960; Coleman, 1965; 1967; Andersen and Matthiessen, 1967; Walker, 1969, 1971; Dostal and Jelinek, 1970; Shah and Chaudhry, 1974a; Holmstedt and Bagwell, 1977; Gulamhusein and England, 1982; Kiso et al., 1984; Young et al., 1991a). These studies indicate that in order to form the mammalian secondary palate, three sequential events during embryogenesis are essential. These events are :

1. A bilateral vertical outgrowth of palatal buds (shelves) from the maxillary processes. Eventually, as the tongue and lower jaw grow forward (anteriorly), the palatal shelves hang vertically along the sides of the tongue;
2. A change in the direction of development of the palatal shelves from a vertical to a horizontal plane (reorientation), above the dorsal surface of the tongue; and
3. Union of the opposing horizontal shelves to separate the oral and nasal cavities.

The literature further indicates that the prenatal development of the secondary palate has also been analyzed in a few other vertebrates. In fish, the

palatal shelves form vertically along side the tongue but never reorient or fuse (Shah et al, 1990). Consequently, through the ontogeny of fish, the palate remains open, i.e., physiologically clefted. Similar morphogenesis was also noted in amphibians, eg., frogs, by LeCluyse et al (1985). In reptiles, eg., alligator, the palatal shelves originate as a horizontal projections *ad initium* over the dorsal surface of the tongue and grow toward each other to eventually unite and separate the oral and nasal cavities (Ferguson, 1981; Shah and Ferguson, 1988). In other reptiles, however, the palate remains open through their ontogeny (Shah, 1984; Shah et al., 1990). In birds, as in the alligator, the palatal shelves also develop horizontally *ad initium* over the dorsal surface of the tongue. The shelves then grow toward one another over the dorsal surface of the tongue, and approximate. Subsequently, however, unlike alligator and mammals, they never fuse. Instead, a physiological cleft persists in the roof of the avian mouth through their ontogeny (Shah and Crawford, 1980; Koch and Smiley, 1981; Shah et al., 1985a, 1987, 1988; Shah and Cheng, 1988)

It is clear from the foregoing analysis that the morphogenesis of the secondary palate amongst vertebrates is different. In addition, the reorientation of palatal shelves from a vertical to a horizontal plane is a unique developmental feature of mammals.

The manner by which mammalian palatal shelves reorient from a vertical to a horizontal position has been an area of controversy. The historical literature on this subject was reviewed by Lazzaro (1940), Stark and Ehrmann (1958), Walker and Fraser (1957) and Shah (1979a, b). From their observations of fixed human tissues, His (1901) and Inouye (1912) suggested that the palatal shelves, due to their

inherent elasticity, "rotate" from the side of the tongue to a position above the tongue. This view, however, was disputed by Polzl (1904) who indicated that a transformation in the "form" of the vertical shelves brings them to a horizontal position. He further suggested that the transformation in the "form" was brought about by a differential growth of the tongue and mandible.

Pons-tortella (1937) forwarded another explanation for the reorientation of palatal shelves. He suggested that the vertical shelf "regresses" and then a new horizontal shelf grows from its medial surface at the approximate level of the dorsum of the tongue.

In 1940, Lazzaro reviewed the earlier literature on the development of secondary palate and proposed three possible mechanisms by which the reorientation of shelves might be achieved. These mechanisms were: (1) "external force", i.e., pressure exerted by the tongue, (2) growth changes involving regression of the ventral portion of the shelves and their outgrowth in a horizontal plane, as suggested by Pons-tortella (1937), and (3) a "rotation" of shelves due to some "intrinsic force". Lazzaro himself favoured the last mechanism and attributed the movement of shelves to an increase in the extracellular matrix (ECM) within the developing palatal shelf.

On the basis of their observations on different strains of mice, Walker and Fraser (1956) argued that the transition of shelves from a vertical to a horizontal position is too rapid to be due solely to mandibular and tongue growth as suggested by Polzl (1904). They also discarded the theory that the vertical shelf regresses and later a new shelf grow in the horizontal plane, as proposed by Pons-tortella (1937). In their opinion, the reorientation of shelf from a vertical to a horizontal plane

was achieved by a process of remodelling. The remodelling of shelves involved formation of a "bulge"on the medial wall of the vertical shelf, over the tongue, with a simultaneous "retraction" of the ventral part of the shelf. They did not find any evidence suggesting the dropping of the tongue or mandible (Lazzaro, 1940), or the pressure by the tongue (Peter, 1924), to allow the vertical shelves to become horizontal. Instead, Walker and Fraser (1956) supported Lazzaro's (1940) proposition that the palatal shelves change their position because of an "intrinsic force".

Since the publication of Walker and Fraser's (1956) work, continued efforts have been made to investigate both the extrinsic and intrinsic factors that may be involved in reorienting the palatal shelves.

A proposal that external forces such as muscular pressure by the tongue on the palatal shelves might cause shelves to reorient themselves (Lazzaro, 1940) has received attention of numerous workers. Lazzaro suggested that the withdrawal of the tongue before the shelf movements and subsequent pressure on the under surface of the palatal shelves by the tongue might push the vertical shelves into a horizontal position. He proposed five mechanisms for downward tongue withdrawal from its position between the vertical shelves: (1) a lowering of the mandible and the tongue; (2) a forward displacement of the tongue; (3) a lifting of the roof of the oral cavity; (4) changes in form of the tongue due to muscular development, and (5) muscular movement of the tongue.

Subsequent studies have supported many of the above proposals to some extent. For example, from their studies on rats, Asling et al (1960) and Coleman (1965) suggested that a lowering of the mandible and the tongue occur primarily

due to "differential growth" of both the tongue and mandible, a possibility indicated earlier by Polzl (1904). A marked growth spurt of the mandible, relative to the maxilla, at the time of shelf elevation would allow the tongue to descend towards the floor of the mouth and clear the way for the vertical shelves to become horizontal (Asling et al., 1960; Coleman, 1965; Diewert, 1976).

This proposition, however, was disputed by Humphrey (1971). She observed that in human embryos an increased mandibular growth follows rather than precedes the downward tongue withdrawal from its position between the vertical shelves. Rather the tongue withdrawal occurs as a part of fetal mouth opening reflex. She indicated that the tongue provides an "active force" by its movement to bring shelves in the horizontal plane, a view earlier proposed by Walker (1969, 1971) in his studies on the palate development in mice and rabbit embryos. These authors supported the idea that the mandible could be lowered due to lifting of head from against the chest. The tongue is simultaneously withdrawn from between the shelves, thus creating a space and making it possible for reorientation of the vertical shelves to a horizontal position. Taylor and Harris (1973) and Diewert (1976) suggested that differential growth of various areas of the craniofacial region would allow realignment of the relationship between the tongue, mandible and palatal shelves for the latter to reorient from a vertical to a horizontal plane.

While the studies continued toward defining the role of extrinsic factors during the reorientation of palatal shelves, attention was also directed toward evaluation of factors intrinsic to the shelves. These factors included both the proliferative and migratory behavior of palatal mesenchymal cells and the synthesis of extracellular matrix.

In 1907, Schorr indicated that an increase in the rate of cellular proliferation within the palatal shelf tissue may account for reorientation. This possibility has received some attention in the literature. Mott et al (1969), Jelinek and Dostal (1974), Nanda and Romeo (1975) and Luke (1989) observed increased rates of cell proliferation, in the developing palate of mice and rats, several hours prior to palatal shelf reorientation. On the other hand, Walker and Fraser (1956) and Hughes et al (1967) observed only a few mitotic figures in the shelf tissue prior to, or during, reorientation and consequently they did not attach any significance to Schorr's suggestion. Also, neither an increased rate of mitosis (Cleaton-Jones, 1976) nor an increase in the synthesis of DNA, indicative of the cell proliferation rate (Shah et al., 1989a, b) have been observed immediately prior to, or during, the reorientation of shelves.

Walker and Fraser (1956), on the basis of suggestion made earlier by His (1901) and Inouye (1912), speculated that the intrinsic shelf force may reside in the elastic fiber of ECM. This speculation was, however, discarded by Frommer and Monroe (1969) who failed to demonstrate the presence of elastic fibers in the developing palatal shelves.

Following Lazzaro's (1940) and Walker and Fraser's (1956) speculation, that the intrinsic shelf force may reside in the ECM of the developing palatal shelves, a considerable amount of attention was focused on involvement of both the glycosaminoglycans (GAG) and collagen during palatogenesis. From their animal studies, and using histochemical and/or biochemical techniques, numerous researcher (Larsson, 1962; Jacobs, 1964; Nanda, 1971; Pratt et al., 1973; Ferguson, 1978; Brinkley, 1980; Jacobson and Shah, 1981; Brinkley and Morris-Wiman, 1984;

Turley et al., 1985) observed a spurt in the synthesis of GAG prior to, and during the reorientation of shelves. Although, initially, it was thought that the predominant GAG associated with reorientation of shelves was sulfated i.e., chondroitin sulfate (Larsson, 1962; Jacobs, 1964), a consensus emerged in the subsequent literature that the major GAG involved during the palatal shelf reorientation was hyaluronic acid (Nanda, 1971; Pratt et al., 1973; Ferguson, 1978; Brinkley, 1980; Jacobson and Shah, 1981; Turley et al., 1985). Cell culture studies have also shown that the palate mesenchymal cells can be stimulated to produce hyaluronic acid (Greene et al., 1982; Sasaki and Kurisu, 1983; Yoshikawa et al., 1986, Pisano and Greene, 1987). The hydrophilic properties of hyaluronic acid results in alterations in osmotic concentrations with consequent swelling of the ECM and corresponding decrease in mesenchymal cell density (Brinklely, 1980). It has also been proposed in the literature that GAG may facilitate the movement of mesenchymal cells (discussed below) during palatal shelf reorientation (Shah, 1979a, b; Brinkley, 1980; Venkatasubramanian and Zimmerman, 1983). In spite of these observations, the precise nature of the role played by GAG molecules during the reorientation of the palatal shelves is unclear.

Since an *in vivo* increase in the synthesis of collagen was observed during palatogenesis, collagen was also implicated as a candidate to account for the internal shelf force responsible for palatal shelf reorientation (Shapira, 1969; Pratt and King, 1971; Shapira and Shoshan, 1972; Hassell and Orkin, 1976; Silver et al., 1981). Collagen synthesized *in vivo* in the reorienting shelf was type I (Hassell and Orkin, 1976). During the vertical growth, however, some type III was also observed in the shelf (Silver et al., 1981). In organ culture of palatal explants (Uitto and

Thesleff, 1979), and in cell culture of palate mesenchyme (Sasaki and Kurisu, 1983) both types I and III collagen were observed. It appears that the observation of isotypes I and III depends on *in vivo* or *in vitro* methods, or on the stage of palatal development. Although the studies to date have noted a continuous increase in the synthesis of collagen during palatogenesis, the role which the increasing collagen synthesis may play in the reorientation of palatal shelves remains unknown.

The most recent hypothesis to explain the intrinsic shelf force was the migration of mesenchymal cells from the tip portion of the vertical palatal shelves into the medial bulge to form the horizontal shelves. Lassard and associates (1974) and Krawczyk and Gillon (1976) indicated that the palatal mesenchymal cells synthesize the contractile proteins, actin and myosin, which may be responsible for cell migration during the reorientation of palatal shelves. Babiarz et al (1975) observed a calcium-dependent adenosine triphosphatase activity in the mesenchymal cells around the time of shelf reorientation thus supporting the possibility of presence of a contractile system. Subsequently, Shah (1979b) observed that in a reorienting shelf, the shape of the mesenchymal cells alter from a spherical to elongated, and the cells developed cytofilaments in its sub-plasmamembrane region. The cytofilaments were oriented along the long axis of the cells. Also, the elongated cells formed junctions and appeared to be flowing into the medial bulge. An increased synthesis of hyaluronic acid during reorientation of palatal shelves (noted above) could facilitate migration of palate mesenchymal cells (Shah, 1979b; Brinkley, 1980; Venkatasubramanian and Zimmerman, 1983; Pisano and Greene, 1987). Since alterations in both cellular

morphology and contents along with an increased synthesis of ECM were believed to be associated with cellular movement during organogenesis (Trinkaus, 1984), these changes together were suggested to contribute to the intrinsic shelf force during reorientation of palatal shelves (Shah, 1979b). A spurt in the synthesis of various ECM molecules in the developing palate, it was proposed, could be one of the critical aspects in the cascade of cellular and molecular events, that may allow marshalling of the internal shelf force for reorientation of the mammalian palatal shelves (Shah, 1979b; Brinkley and Morris-Wiman, 1984).

During the past decade, studies were also focused on defining the involvement of hormones and growth factors in regulation of behaviour of mesenchymal cells of the developing palate in mammals. It was generally recognized in the literature that epidermal growth factors (EGF), transforming growth factors (TGF), cyclic adenosine monophosphate (cAMP), prostaglandines, catecholamines and neurotransmitters may be involved in regulation of growth and differentiation of mammalian palatal cells during development (Greene et al., 1982, 1989; Zimmerman and Wee, 1984; Pratt et al., 1984; Shah et al., 1985b; Pisano and Greene, 1986, 1987; Pratt, 1987; Greene, 1989; Gehris et al., 1991). For example, levels of cAMP are altered during palatal development (Greene and Pratt, 1979; Olson and Massaro, 1980; Shah et al., 1985b). Adenylate cyclase activity, which catalyzes the synthesis of cAMP from adenosine triphosphate (ATP), has been demonstrated in the developing palate by both cytochemical and biochemical techniques (Waterman et al., 1976; Greene and Pratt, 1979; Palmer et al., 1980). Adenylate cyclase activity is maximum prior to and during fusion of the palatal processes and temporally corresponds to the increase in levels of cAMP. The

activities of adenylate cyclase, and cAMP, in palatal cells can be modulated by several agents. For example, treatment of embryonic palate mesenchymal cells *in vitro* with prostaglandin PGE_2 and prostacycline results in dose-dependent accumulation of intracellular cAMP (Greene et al., 1981). Palmer et al (1980) observed that PGE_1, E_2 and $F_{2\alpha}$ stimulated adenylate cyclase activity in the intact hamster palate. Phenylbutazone, an agent which inhibits prostaglandin biosynthesis, produces a high incidence of cleft palate (Montenegro et al., 1976), by preventing fusion of palatal processes *in vitro* (Montenegro et al., 1982). George and Chepenik (1985) have observed the presence of several phospholipases which hydrolyse membrane phospolipids yielding free fatty acids utilized in prostaglandin synthesis by the embryonic palate mesenchymal cells. These observations on the activity of prostaglandins in the developing palate indicates a role for these compounds in cAMP mediated growth and differentiation of the secondary palate (Chepenik et al., 1984; Pisano and Greene, 1986).

Catecholamines have also been implicated as modulators of cAMP during palatogenesis. For example, quantitative alterations in catecholamines, i.e., dopamine, norepinephrine, and epinephrine within the embryonic palatal tissue have been observed during development (Zimmerman and Wee, 1984; Pisano and Greene, 1987). Catecholamines can stimulate adenylate cyclase in the developing palate (Waterman et al., 1976; Palmer et al., 1980). Murine embryonic palate mesenchymal cells *in vitro* have been shown to respond to various catecholamines with dose-dependent elevations of intracellular cAMP. In these cells, β-adrenergic receptors have also been characterized (Greene, 1989). Also, it has been shown that

the neurotransmitters, serotonin and acetylcholine, were capable of stimulating palatal shelf reorientation while γ-amino-n-butyric acid (GABA) exerted an inhibitory effect (Zimmerman and Wee, 1984). Further, the levels of both serotonin and GABA in the embryonic palate changes during palatogenesis (Zimmerman and Wee, 1984). It was suggested that serotonin would stimulate palate mesenchymal cell motility, by altering the intracellular levels of cAMP and cGMP and by increasing cellular protein carboxymethylation to bring about the shelf reorientation (Zimmerman and Wee, 1984).

Growth factors are also involved in regulation of palate development. Human and murine embryonic palate mesenchymal cells contain EGF receptors and are responsive to growth stimulation by EGF (Nexø et al., 1980; Yoneda and Pratt, 1981). Hassell (1975) and Hassell and Pratt (1977) observed that the programmed cell death in the medial edge epithelium (MEE) can be prevented by the addition of EGF to organ culture of developing palate. Subsequently, Yoneda and Pratt (1981) indicated that EGF influences mouse palatal MEE differentiation via action on the underlying mesenchyme. The effect of EGF on the synthesis of ECM in the developing palate in organ culture has also been reported (Silver et al., 1984; Turley et al., 1985). Silver et al (1984) observed that mouse palatal shelves grown in the presence of EGF were substantially larger with increases occurring in DNA content as well as in protein synthesis. They also observed that the net collagen synthesis in palatal organ culture appears to be stimulated by EGF.

Recently, transforming growth factor (TGF) α and β's have been shown to be involved in palate development (Gehris et al., 1991). TGF-β has been

immunolocalized in embryonic mesenchyme of neural crest origin destined to develop into a number of craniofacial structures including the secondary palate (Heine et al., 1987; Fitzpatrick et al., 1990; Williams et al., 1991). Greene et al (1989) observed that TGF-β and basic fibroblast growth factor (bFGF), stimulates proliferation of human embryonic palate mesenchymal cells *in vitro*. In contrast, however, murine embryonic palate mesenchymal cells growth is inhibited by TGF-β. Pelton et al (1990) observed a differential expression of the three TGF-β genes (TGF-β_1, β_2, β_3) in both the mesenchymal and epithelial cells of the palatal shelves and suggested that they may be involved in the morphogenesis of the secondary palate. Gehris et al (1991) suggested that TGF-β_s may have both autocrine and paracrine mode of action during palate development. They further indicated that TGF-β_1, synthesized in epithelial tissue, may exerts influence on the underlying mesenchyme. Also, a similar mode of action for TGF-β_2 has been suggested by Gehris et al (1991) whereby this gene product, synthesized in the mesenchyme (Pelton et al., 1990; Fitzpatrick et al., 1990), may exert its effects on the overlying epithelium.

While the studies on growth and differentiation of developing palate were mainly conducted in mammals, those in the other vertebrates are only a few. It has been observed that the developing palatal tissues of vertebrates differ in their biological behaviour. For example, unlike mammals where the DNA synthesis in the MEE ceases approximately 24 hours prior to reorientation and fusion of the palatal shelves (Hudson and Shapiro, 1973; Pratt and Martin, 1975; Shah et al.,

1985b), DNA synthesis in avian MEE continues at a steady pace through the morphogenesis of the secondary palate (Shah et al., 1985b, 1987; Shah and Cheng, 1988). In mammals, cAMP levels change during palatogenesis (Greene and Pratt, 1979; Olson and Massaro, 1980; Shah et al., 1985b), but in birds the cAMP activities in the developing palate remains unaltered (Shah et al., 1985b, 1987; Shah and Cheng, 1988). The programmed cell death observed during mammalian palatogenesis (Mato et al., 1966; Chaudhry and Shah, 1973, 1979; Shah and Chaudhry, 1974a, b) is absent during palate development in birds (Shah and Crawford, 1980; Koch and Smiley, 1981; Shah et al., 1985, 1987, 1988; Shah and Cheng, 1988) as well as fish (Shah et al., 1990) and reptiles (Ferguson, 1981). Thus, these observations underscore differing life histories of palatal tissues during embryonic development.

Abnormal Development of the Secondary Palate:

In human, clefting of the palate is a major birth defect. It allows a communication between both the oral and nasal cavities, thus, affecting the functions of mastication, deglutition, respiration and phonation.

Researchers have been using teratogens, that induce cleft palate in animals, as a tool to understand both the normal and abnormal aspects of palatogenesis. Almost all research work on teratogen induced cleft palate have been carried out in mammals. From these studies, it is suggested that a teratogen may affect any one of the three events of normal palatogenesis in mammals to induce a cleft palate in the offspring (Table 1). The mechanism(s) by which these teratogens induce cleft palate, however, are largely unknown. In addition, it has also been suggested that

a cleft palate may result from rupture of previously fused palate (Veau, 1931; Kitamura, 1966, 1991; Goss, 1977).

During the last thirty years much of the efforts were focused on understanding the mechanism of glucocorticoid-induced cleft palate. Glucocorticoids, when administered to various mammalian species during midgestation induce cleft palate in the offspring (Baxter and Fraser, 1950; Shah and Kilistoff, 1976). The mechanism by which glucocorticoids induce cleft palate is, however, unclear. It has been shown that, in the developing palate, glucocorticoids can affect cell proliferation (Jelinek and Dostal, 1974; Nanda and Romeo, 1978), ECM synthesis (Larsson, 1962; Jacobs, 1964; Shapira, 1969; Pratt et al., 1973; Jacobson and Shah, 1981; Sasaki and Kurisu, 1983), lysosomal enzyme synthesis (Shah and Chaudhry, 1974a; Herold and Futran, 1980; Goldman et al., 1981; Ads et al., 1983; Shah et al., 1991a, b), cyclic nucleotide levels (Erickson et al., 1979; Greene et al., 1981) and mobilization of arachidonic acid, the precursor for prostaglandin synthesis (Piddington et al., 1983; George and Chepenik, 1985) which affects the growth of the cells. Glucocorticoid action may be mediated via its binding to receptors in the palatal tissues (Goldman et al., 1978; Bekhor et al., 1978; Salomon and Pratt, 1979; Shah and Burton, 1980) which may affect aforementioned cellular functions.

Several phospholipase inhibitory proteins (PLIP) have recently been shown to mimic the teratogenic effects of glucocorticoids on the developing palate *in vivo*. PLIP inhibited terminal differentiation of the MEE of palatal shelves *in vitro*, an effect which could be reversed by addition of arachidonic acid to the culture medium (Gupta et al., 1984). On the basis of these results, it was postulated that

effect which could be reversed by addition of arachidonic acid to the culture medium (Gupta et al., 1984). On the basis of these results, it was postulated that

Table 1. Summary of the Morphological Aspects of Teratogen-induced Cleft Palate Development.

Affected Stage of Palate Development	Agents	Reference
1. Vertical	6-mercaptopurine, bromo-deoxyuridine, hadacidin.	Burdett and Shah, 1988; Burdett et al., 1988; Shah et al., 1991a, b.
2. Reorientation	cortisone, triamcinolone, vitamin A, folic acid deficiency, diazo-oxo-norleucine, 5-fluorouracil, cyclophosphamide, radiation, 6-amino-nicotinamide.	Walker and Fraser, 1957; Walker and Crain, 1960; Asling et al., 1960; Callas and Walker, 1963; Kochhar and Johnson, 1965; Dostal and Jelinek, 1972; Ferguson, 1977; Shah, 1979c; Diewert, 1979 Diewert and Pratt, 1979; Shah and Wong, 1980; Shah et al., 1989.
3. Fusion	hydrocortisone, phenylbutazone	Shah and Travill, 1976a; Montenegro et al., 1982.

glucocorticoids may inhibit both the arachidonic acid release and prostaglandin biosynthesis, and consequently affect various cellular functions including ECM synthesis, during the critical period of palate morphogenesis (Gupta et al., 1984) to induce cleft palate.

5-Fluorouracil:

In 1957, Duschinsky and Pleven reported the synthesis of a pyrimidine analogue, 5-fluorouracil (5-FU). The drug is a white, odourless, crystalline powder (Windholz, 1976), sparingly soluble in water and ethanol, and insoluble in chloroform, benzene, and diethyl ether (Rudy and Senkowski, 1973). Solubility of 5-FU in aqueous solution can be increased at alkaline pH and by increasing the temperature. It has a molecular weight of 130.08. The drug is stable when exposed to air, hydrolyses under strongly basic conditions, and decomposes at about 280°-282°C.

One of the most widely used anticancer drugs, 5-FU is used in the treatment of a number of different malignancies, either alone or in combination with other drugs. For example, 5-FU has been shown to have a palliative activity in the management of cancer of the breast, gastrointestinal tract, colon and ovary, and curative effect in the treatment of non-invasive basal cell carcinomas (Heidelberger et al., 1957; 1962; DeVita et al., 1985).

When administered parenterally to mice or rats, 5-FU rapidly enters all body compartments with large amounts reported in bone marrow, small intestine, kidney, liver and spleen (Liss and Chadwick, 1974; Chabner, 1982). As much as 80% of the administered drug is eliminated through metabolic degradation, while

approximately 5-20% by urinary excretion. In human, rapid intravenous administration of 5-FU produces plasma concentrations of 0.1mM with a plasma half-life of 10-20 minutes (Chabner, 1982; Diasio and Harris, 1989). In hamster, the drug crosses the placental barrier rapidly and reaches the fetuses within 30 minutes (Tuchmann-Duplessis, 1975). Oral absorption of 5-FU is variable, unpredictable and incomplete due to the significant variation in the bioavailability (0-80%) of the drug (Diasio and Harris, 1989).

Following parenteral administration, 5-FU is inactive , and requires metabolic activation to a nucleotide forms to be effective (Figure 1). The catabolism of 5-FU proceeds largely in the liver to α-fluoro-β-alanine, urea, carbon dioxide and ammonia (Chaudhuri et al., 1958; Mukherjee and Heidelberger, 1960; Miller, 1971; Chabner et al., 1975).

Depending on the relative significance of deoxyuridine or uridine phosphorylase during metabolic activation, 5-FU may exert its effect on cells via two major mechanisms: (1) inhibition of DNA synthesis, and (2) alteration in the processing and function of RNA (Heidelberger et al., 1983; Carrico and Glazer, 1979). The metabolic activation of the drug (Figure 1) can be achieved by three possible pathways: (1) the reaction with ribose-1-phosphate catalysed by uridine phosphorylase to form 5-fluorouridine, followed by phosphorylation by uridine kinase; (2) the reaction with phosphoribosyl pyrophosphate catalysed by pyrimidine phosphoribosyl transferase directly to form 5-fluorouridine-5'-monophosphate; and (3) the reaction with deoxyribose-1-phosphate catalysed by thymidine phosphorylase to convert 5-FU to 5-fluoro-2'-deoxyuridine-5'-

monophosphate (FdUMP) by thymidine kinase (Kessel et al., 1966; Heidelberger et al., 1983). The deoxyuridine pathway leads to the formation of 5-fluorodeoxyuridine monophosphate, which is a potent inhibitor of thymidylate synthetase (TS), an enzyme essential in the *de novo* synthesis of DNA. A covalent complex is formed between 5-fluorodeoxyuridine monophosphate, methylene-tetrahydrofolate and TS which, although reversible, has a sufficiently long half-life to prevent thymidylic acid synthesis (Chaudhuri et al., 1958; Mukherjee and Heidelberger, 1960; Miller, 1971; Chabner et al., 1975).

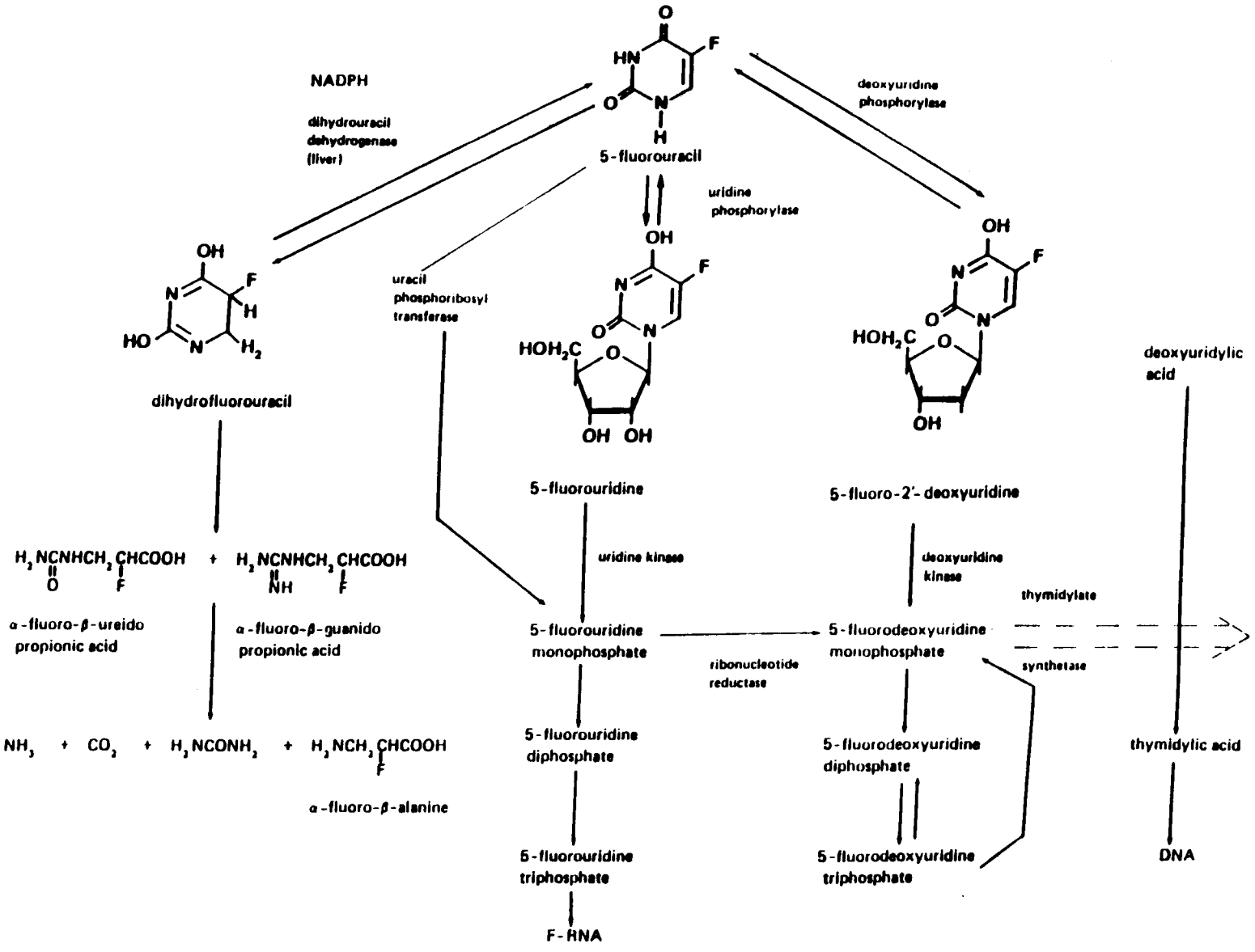
Most mammalian cells use TS to make the thymidine-5'-monophosphate (dTMP) needed for DNA synthesis from 2'-deoxyuridine-5'-monophosphate (dUMP), obtaining the methyl group for the "5" position of the pyrimidine ring from 5,10 methylene tetrahydrofolate (Danenberg and Danenberg, 1978). Because FdUMP has a greater affinity for TS than the natural substrate dUMP, it can prevent the formation of dTMP (Danenberg and Danenberg, 1978) Consequent inhibition of DNA synthesis, combined with continued RNA and protein synthesis, it is suggested, may produce an imbalance in the cell that is incompatible with its survival (Chaudhuri et al., 1958; Mukherjee and Heidelberger, 1960; Miller, 1971; Chabner et al., 1975; Stevens et al., 1984; Uchida et al., 1989; Prior et al., 1990).

While some of the actions of 5-FU are explained by the inhibition of DNA synthesis through the thymidylate pathway, the drug can also alter RNA metabolism. For example, Chaudhuri et al (1958) identified 5-fluorouridine-5'-monophosphate (FUMP) in a hydrolysate of RNA from Ehrlich ascites cells and sarcoma-180 cells treated with ^{14}C-FU. Mandel (1969) demonstrated that another

metabolite, 5-fluorouridine-5'-triphosphate (FUTP) could be incorporated into RNA in place of uracil. Additional experimental evidence from *in vivo* studies also support the contention that the action of 5-FU is, at least, partially independent of its effect on TS. For example, co-administration of 5-FU and thymidine prevents the early inhibition of DNA synthesis, but increases 5-FU incorporation into the RNA of normal and malignant cells (Carrico and Glazer, 1979). Maybaum and colleagues (1980) observed that mouse lymphoma cells experienced two phases of drug effect when exposed to 5-FU. During an early phase (1-24 hours) the inhibition of cell growth was reversed by addition of thymidine to the culture medium, but during the later phase (after 24 hours) the inhibition was not reversible. The authors suggested that the second phase of inhibition was probably caused by progressive incorporation of 5-FU into RNA. Evans and co-workers (1980) noted that a three-hour incubation with 5-FU at low concentrations (5-20μM) produced a thymidine reversible toxicity, whereas high concentrations (50-200μM) produced toxicity not reversible by thymidine. On the basis of these data it was suggested that the RNA incorporation mechanism would probably be favored by high concentrations, and longer durations of exposure to 5-FU, and would be enhanced by the presence of thymidine, whereas the opposite conditions would favour TS depletion (Chabner, 1982).

5-FU also affects other cellular activities. The drug decreases the surface charge and transmembrane potential in tumor cells (Ingraham et al., 1980), decreases fucose incorporation into membrane proteins (Kessel, 1980), and decreases protein synthesis (Kessel, 1980).

Figure 1 Metabolic pathways of 5-fluorouracil. *(Adapted from IARC monographs, pp. 217-235, 1981)*


NADPH
dihydrouracil dehydrogenase (liver)
5-fluorouracil
deoxyuridine phosphorylase
uridine phosphorylase
uracil phosphoribosyl transferase
dihydrofluorouracil
5-fluorouridine
5-fluoro-2'-deoxyuridine
deoxyuridylic acid
α-fluoro-β-ureido propionic acid
α-fluoro-β-guanido propionic acid
uridine kinase
deoxyuridine kinase
thymidylate synthetase
5-fluorouridine monophosphate
5-fluorodeoxyuridine monophosphate
ribonucleotide reductase
5-fluorouridine diphosphate
5-fluorodeoxyuridine diphosphate
thymidylic acid
5-fluorouridine triphosphate
5-fluorodeoxyuridine triphosphate
DNA
α-fluoro-β-alanine
F-RNA

The embryotoxic and teratological effects of 5-FU has been documented in mammals such as rats, mice, monkey, guinea pigs and hamsters (Dagg, 1960; Morris et al., 1967; Wilson, 1971; Kromka and Hoar, 1973; Forsthoefel et al., 1978; Shah and Mackay, 1978; Skalko and Jacobs, 1978; Imagawa et al., 1979) and birds (Karnofsky et al., 1958; Ruddick and Runner, 1974). In these species, the teratogenic effects of 5-FU were characterized by malformations of limb, eye, beak, tail, palate, lower jaw, gut and brain.

Stadler and Knowles (1971) reported a case of human pregnancy in which the mother received a total dose of 7.5g 5-FU during the second and third trimesters. The newborn showed transient effects, characterized by mild respiratory distress and petechiae. No gross malformations were described. In 1980, Stephens and associates reported multiple congenital anomalies in a human fetus from a mother exposed to 5-FU during the first trimester. The anomalies included bilateral radial aplasia and absent thumbs and fingers, single umbilical artery, hypoplastic aorta, pulmonary hypoplasia and renal dysplasia.

In hamster, a single intramuscular injection of 81mg/kg 5-FU induces cleft palate by delaying reorientation of the shelves (Shah and Wong, 1980; Shah et al., 1984, 1989c). Arvystas and Cohen (1971) also observed 5-FU-induced cleft palate in mice due to a delay in the reorientation of palatal shelves.

Collagen:

Collagen is an important extracellular matrix protein of the connective tissue. It represent one of the principle structural elements of the connective tissue and the most widely distributed protein in the animal kingdom.

In 1942, Schmitt and his colleagues observed cross striations of collagen fibrils through the electron microscope. Since then numerous reviews have appeared in the literature about the assembly, structure, synthesis and functions of collagen (Gross, 1974; Shoshan and Gross, 1974; Miller and Matukas, 1974; Weinstock and Leblond, 1974; Fessler and Fessler, 1978; Prockop et al., 1979a, 1979b; Jackson, 1979; Burgeson, 1988).

The basic type I collagen molecule is composed of three continuous helical polypeptide α-chains (two identical α_1 chains and one α_2 chain) (Gross, 1974; Miller and Matukas, 1974; Shoshan and Gross, 1974). Each chain has a molecular weight of approximately 100,000 and contains approximately 1,000 amino acid residues along its length. These chains are coiled into a left-handed triple helix with three amino acids per turn. The three helical chains are then twisted around each other into a right-handed helix. This unusual conformation gives the molecule a rigid rod like shape with dimensions of approximately 15x3000Å. The chains are composed of repeating triplet structures, X-Y-Gly in which every third amino acid residue is glycine with the Y position often occupied by hydroxyproline (HYP) or hydroxylysine and the X position often occupied by proline (Miller, 1976; Prockop et al., 1979a; Burgeson and Morris, 1987). The occurrence of HYP in collagen is unique since this amino acid has been found in only a few other proteins of vertebrates like elastin (Miller, 1976). In addition to the triple helical region there are short non-helical domains present at both the amino and carboxy terminals (Fessler and Fessler, 1978; Prockop et al., 1979a, b).

In recent years, it has become increasingly apparent that different tissues of the same organism are characterized by different collagen types. According to Burgeson and Morris (1987), collagen can be grouped into two main categories (Table 2) : (a) those that form periodically banded fibers, and (b) those that do not form banded fiber systems.

A. Collagen that form fibers with uniform periodic cross striations:

This group of collagens are capable of forming large fibers. These fibers represent the most common fiber form in connective tissue and are largely responsible for the rigidity of connective tissue matrices. These banded fibers can be formed from three major types of collagen, type I, II, and III. This group is often known as interstitial collagens.

TYPE I. Type I is the most ubiquitous of all collagen species. It has been isolated from the connective tissues of various structures and organs, including skin, bone, tendon, dentin, cornea, fascia, periodontal ligament, gingiva and hard palate (Bornstein and Sage, 1980). This type of collagen is largely responsible for the rigidity of connective tissue matrices. It has been described in cell culture, tumors and in rapidly growing tissues (Bornstein and Sage, 1980). The tropocollagen molecule of type I collagen is composed of $[\alpha 1(I)]_2\alpha 2(I)]$. Also there is a variant of type I collagen of chain organization $[\alpha 1(I)]_3$ called type I trimer.

TYPE II. The tropocollagen molecule of type II collagen is composed of three identical $\alpha 1$ chains designated as $[\alpha 1(II)]_3$. It is mainly seen in tissues such as hyaline cartilage, vitreous humor, the notochord and the sclera of the eye (Mayne and Von der Mark, 1983). It has been suggested that type II collagen may be capable

of forming an association between cartilage collagens and cartilage proteoglycans (Burgeson and Morris, 1987) through type IX collagen.

TYPE III. The second most prevalent collagen species is type III. Type III collagen is generally found in close association with type I (Burgeson, 1982). The type III tropocollagen molecule is composed of three identical α1-chains $[\alpha 1(III)]_3$ (Miller et al., 1971; Chung and Miller, 1974). It appears to form small fibrils in which the aminopropeptide of the collagen form of the molecule remains uncleaved (Fleishmajer et al., 1981). This type of collagen is prevalent in tissues which require elasticity for normal function, eg, skin, blood vessels, gut and chorioamniotic membranes. It may play a role in development since it occurs in various tissues of a developing fetus (Bhatnagar and Rapaka, 1975).

B. Collagens that do not form uniformally banded fiber system:

The most consistent feature distinguishing these collagens from the interstitial collagens is their inability to form broad banded collagen fibers *in vivo* and *in vitro*. The genetically distinct collagen species of this group are summarized in Table 2.

TYPE IV. This collagen form contains two α chains, α1(IV) and α2(IV). This type of collagen is present in all structurally defined basement membrane and, for the most part secreted, by epithelial/endothelial cells (Timpl and Martin, 1982; Kleinman et al., 1986). Type IV collagen is present at the interface between epithelia and mesenchymal structures and it may play a direct role in morphogenesis (Bornstein and Sage, 1980). The molecule is characterized by both collagenous and noncollagenous domains, and there are also interruptions in

morphogenesis (Bornstein and Sage, 1980). The molecule is characterized by both collagenous and noncollagenous domains, and there are also interruptions in

Table 2 Genetically Distinct Collagens [Adapted from Burgeson and Morris (1987)]

A. The interstitial collagens-Collagens that form broad-banded fibers		
Type I	$[\alpha1(I)]_2\alpha2(I)$	Found in most tissues except cartilage; major component of bone, tendon, skin, and dentin.
Type I "trimer"	$[\alpha1(I)]3$	Some fetal tissues; product of certain malignant and normal cell lines.
Type IIM	$[\alpha1(IIM)]_3$	Cartilaginous tissues.
Type IIm	$[\alpha1(IIm)]_3$	Cartilagenous tissues.
Type III	$[\alpha1(III)]_3$	Found together with type I; in relatively high concentrations in extensible tissues such as blood vessels, skin, and gut.
B. The minor collagens-less abundant collagens that do not form broad- banded fibers		
Type IV (basement membrane collagen)	$[\alpha1(IV)_2\alpha2(IV)$	Major component of all basal laminae.
Type V	$[\alpha1(V)]_2\alpha2(V)$ $[\alpha1(V)]_3$ $[\alpha3(V)]_3$ $\alpha1(V)\alpha2(V)\alpha3(V)$	Minor components of most tissues except cartilage; fiber forms unknown.
Type VI	$[\alpha1(VI)]2\alpha2(VI)$	First identified in aortic intima, but now thought to have a broad, but yet undefined, tissue distribution.
Type VII "long-chain collagen"	$[\alpha1(VII)]_3$	Identified in amniotic membrane and skin; believed to be associated with all stratified epithelia; may be anchoring fibril protein.
Type VIII "endothelial cell collagen"	$[\alpha1(VIII)]_3$	Identified as product of a variety of cell types.
Type IX ("HMW-LMW")	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Cartilaginous tissues.
Type X ("G collagen")		Product of cartilage hypertrophic cells
C. Minor cartilage collagens with unknown subunit compositions		
1α 2α		Compositionally similar to type V; structures, fiber forms, and functions unknown.
3α		Compositionally similar to type II.

amino acid sequence, gly-X-Y, making this domain to be susceptible to a large number of proteolytic enzymes (Bornstein and Sage, 1980).

TYPE V. This term refers to a family of several molecules of genetically distinct chains with similar structure (Burgeson et al, 1976). The chains are designated as α1(V), α2(V), α3(V) and α4(V) with different chain organization. Mayne and Von der Mark (1983) suggested that type V collagen is probably present in all connective tissues with the exception of hyaline cartilage. Gay and associates (1981) observed type V collagen in association with cell surfaces, and, for this reason, it was also described as cytoskeletal collagen. It is suggested that type V collagen may somehow be associated with larger fibrils of types I and II collagens, and may either hold them in position or may have a role in determining their organization (Mayne and Von der Mark, 1983).

C. Minor cartilage collagen with unknown subunit compositions:

Details of their structure and distribution are summarized in Table 2.

Collagen Involvement in Organogenesis:

Because collagen undergoes turnover at significant rates during development, it has been implicated to play a role during development of many tissues and organs. Kleinman et al (1981) suggested that collagen is essential for cell adhesion and migration. The effect of collagen on adhesion could be direct or mediated via collagen-bound factors such as fibronectin and proteoglycans (Klebe, 1974; Pearlstein, 1976; Engvall et al, 1978; Heine et al, 1990). Folkman and Tucker (1980) indicated that collagen influences cell growth via increasing adhesion. In

lung and salivary gland, collagen was implicated in allowing epithelial-mesenchymal interaction and subsequently the branching morphogenesis during development (Grobstein and Cohen, 1965; Wessels and Cohen, 1968; Nakanishi et al., 1986a,b). Fukuda and associates (1988) observed that interstitial collagenase from bovine dental pulp, which degrades types I and III collagen, but not IV or V, inhibited *in vitro* branching of developing mouse submandibular gland epithelium. This observation led them to suggest that the collagen required for cleft initiation could be type I and/or III. Similarly, Nakanishi and his colleagues (1988) suggested that collagen type III may be a key substance for either *in vitro* or *in vivo* cleft initiation of the developing mouse submandibular epithelium. Chen and Little (1987) used anticollagen type IV antibodies on embryonic lung sections to show that type IV collagen may be the extracellular scaffold within which early branching morphogenesis of lung epithelial cells may be accomplished. Recently the role of collagen type I was investigated in the developing mouse cornea by Bard et al (1988) who compared the stromal morphogenesis in normal corneas with those of homozygous Mov13 mice which do not make collagen type I. Their data suggested that collagen type I plays only a structural role in the stromal morphogenesis and that its absence is not compensated for. It is clear from these data that synthesis of different types of collagen may play a significant role in morphogenesis of an organ or structure.

Collagen involvement in the secondary palate development:

Over the past thirty years, considerable efforts have been focused on the possible mechanism of mammalian secondary palate reorientation with a special emphasis on the role of ECM. As already noted above, the involvement of

collagen during the mammalian secondary palate formation was studied only by few investigators (Shapira, 1969; Pratt and King, 1971; Shapira and Shoshan, 1972; Uitto and Thesleff, 1979; Silver et al., 1984). In some of these studies, drugs such as glucocorticoids, which affects collagen synthesis (Shapira and Shoshan, 1972; Uitto and Thesleff, 1979), or β-aminoproprionitrile, which interfere with collagen fibre formation (Steffek et al, 1972; Pratt and King 1972) were used to observe their effects on palate development. Whether collagen played any critical role during the reorientation of mammalian palate, however, was unclear.

on palate development. Whether collagen played any critical role during the reorientation of mammalian palate, however, was unclear.

PURPOSE OF THE STUDY

The preceding analysis of the literature indicates that considerable efforts have been directed toward studying the cellular and biochemical sequence of events and their possible molecular regulation during morphogenesis of secondary palate in mammals. Among the issues that have been discussed in these studies is the mechanism responsible for the reorientation of palatal shelves, a phenomenon unique to mammals. Although an increase in collagen synthesis has been observed during the morphogenesis of mammalian palate, its contribution to the reorientation of shelves remains unclear. Earlier studies were performed either *in vitro,* where shelf reorientation could not be studied (Uitto and Thesleff, 1979), or *in vivo* where collagen synthesis was studied at an interval of 12-24 hours (Shapira, 1969; Pratt and King, 1971; Shapira and Shoshan, 1972; Silver et al., 1984). In the later instance, the interpretation of data after such a prolonged intervals are post-hoc since reorientation of palatal shelf in rodents (mice, rat, hamster, etc.) is rapid occurring in 2-3 hours (Walker and Fraser, 1956; Coleman, 1965; Shah and Chaudhry, 1974a). Also extensive intervals in the experimental design would not allow one to characterize subtle alterations which may occur at specific stages of palate development. Hence, the first purpose of this study is to investigate collagen synthesis during the reorientation stage of the secondary palate morphogenesis in hamster. Since, during avian palatogenesis, shelf reorientation stage is absent, and if the collagen synthesis is involved in regulating the reorientation of palatal shelf in mammals, one would expect that the profile of

collagen synthesis in birds would be different from that seen during mammalian palatogenesis. Therefore, the second purpose of this study is to investigate the hitherto unknown aspect of collagen synthesis during quail palate morphogenesis.

Cleft palate is one of the major malformations induced by 5-FU in mammals (Shah and Wong, 1980; Shah et al., 1984), and bird (Karnofsky et al., 1958; Ruddick and Runner, 1974). Since, unlike mammals, the morphogenesis of 5-FU-induced cleft palate is not studied in birds, the third purpose of the current study is to analyse the effect of 5-FU on palate development in quail. Finally, since 5-FU delays the shelf reorientation stage in hamster (Shah and Wong, 1980; Shah et al., 1984, 1989c), a stage which is absent in birds, the collagen synthesis will be analysed both in hamster and in quail following treatment with 5-FU to further analyse, from a comparative biology viewpoint, the involvement of collagen synthesis during vertebrate palate development.

according to HH staging method and observed a high correlation between the age of quail embryo and the stage of its development. Also, subsequently, this staging method was employed successfully in studying both *in vivo* and *in vitro* palatogenesis in quail by Shah et al (1985a) and Shah and Cheng (1988).

The quail embryonic secondary palate was dissected, and grouped according to the HH stage. An average of 12 ± 2 staged palates were pooled. The pooled palates were subjected to an incubation and post-incubation protocol similar to the one described above for hamster.

The samples of both hamster and quail palates were then used for one of the following assays:

1. Collagen digestion assay.
2. Total protein determination using Biuret method as described by Gornall and associates (1948).
3. High performance liquid chromatography (HPLC).
4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

C. Collagen digestion assay

The solubilized samples (both hamster and quail, as described in B) were dialysed against 0.5 N acetic acid (0-4°C). During dialysis, acetic acid solution was changed four times following a minimum duration of six hours. From each dialysis bag 1ml of sample was pipetted into an eppendorf vial for determination of total protein (described below in D). The remaining samples were dialysed further against 0.5 M Tris-HCl buffer, pH 7.4 (0-4°C). The Tris-HCL buffer was changed four times at six hours intervals.

Following dialysis, 0.4ml of each radiolabelled samples were digested at 37°C for four hours with 100µl of 25 units (0.2mg/ml) bacterial collagenase (Clostridiopeptidase A; EC 3.4.24.3, from *Clostridium histolyticum*; type VII, Sigma Chemicals, St. Louis, Missouri, USA, Catalog # C-0773, Lot # 79F6817) in the presence of 0.1mM N-ethylmalemide. Then, to each sample, 10µl of 10% FCS and 125µl of a mixture of 10% trichloroacetic acid (TCA) and 1% tannic acid (TA) were added, and left overnight at 0-4°C to precipitate the proteins. Subsequently, the samples were spun in a microcentrifuge (Biofuge A, Heraeus-Christ Gmbh, W. Germany) at 13,000 rpm for 40 minutes. The pellets were discarded and 200µl of the supernatant from each sample was counted in a liquid scintillation counter (Phillips, Holland, Model PW 4700). The measurement was corrected for the counting efficiency, and disintegration per minute (DPM) was determined.

D. Measurement of total protein

The total protein was determined by Biuret method described by Gornall and associates (1948). To the acetic acid-dialysed samples in eppendorf vials (described above in C) 250µl of 10% TCA-1%TA mixture was added. The vials were vortexed and left overnight at 0-4°C. The samples were spun in a microcentrifuge for 30 minutes and the supernatant discarded. To the precipitate 1ml of 0.05N NaOH was added, vortexed and left overnight at 0-4°C. Subsequently, 0.1ml of digest was used for the total protein determination using human albumin and globulin in saline (Sigma Diagnostics, Catalog # 540-10, Lot: 48F-6087, St. Louis, Missouri, USA) as a protein standard.

Both the ^{14}C-proline DPM and the total protein measurements were standardized and expressed as DPM/mg protein. Each experiment was repeated 3-5 times, and means ± SD calculated. Both the control and drug-treated data on the rates of collagen synthesis were analyzed by the ANOVA method. The comparison between treated and control groups were evaluated by Student's-t test at a significance level of 0.05.

E. High performance liquid chromatography

I. Equipment

The HPLC system (Waters Associates, Millipore, Massachusetts, USA) consisted of a Model 730 Data processor, a Model 721 chromatography control station, two Model 510 solvent pumps, a Model 440 absorbance detector (wavelength 254 nm), and a Model 710B automatic sample processor. A Pico-Tag column (Waters Associates, Millipore, Massachusetts, USA) was kept at a temperature of 38° ± 0.1°C. A Pico-Tag work station (Waters Associates, Millipore, Massachusetts, USA) was used for vapour hydrolysis of the dried specimens.

Incorporation of proline into procollagen and its subsequent hydroxylation to HYP was measured by HPLC. HYP is an amino acid which is virtually unique to collagen (Miller, 1983). Thus analysis of the amount of HYP can be used to evaluate collagen biosynthesis (Miller, 1983; Svanberg, 1987).

II. Sample preparation

20µl aliquots of the acetic acid digested samples, as described above in B, were brought to dryness in 6 x 50 mm pyrex culture tubes (Corning glass works, Corning, New York, USA) using a Pico-Tag work station. (Up to 12 samples and

one standard in culture tubes can be processed per glass reaction vial). Then, 200µl of 6 N hydrochloric acid containing 0.1% phenol was added to the bottom of the reaction vial. Oxygen was removed from the reaction vial by three successive evacuations interposed by flushing with nitrogen. After the third evacuation, the reaction vials were sealed under vacuum and samples hydrolysed at 110°C for 20-24 hours. After cooling the reaction vial, each sample tube was wiped dry, the reaction vial cleaned and dried, and the sample tubes placed back into the reaction vial and then brought to dryness. Next, 20µl of fresh redrying solution (ethanol:water:triethylamine (TEA), 2:2:1 by volume) was added to each sample which was then vortexed and brought to dryness. Subsequently 50µl of fresh derivatization solution (ethanol:water:TEA:phenylisothiocyanate (PITC), 7:1:1:1, by volume) was added to each sample, vortexed, left for 20 minutes at room temperature and brought to dryness using the Pico-Tag work station. Thereafter, 200µl of sample diluent (0.05 M phosphate buffer) was added to each sample, vortexed for 10 seconds and then passed through Millex-HV4 filters into low volume inserts (Waters Associates, Millipore, Massachusetts, USA).

III. Chromatography

For chromatography, 25µl of the reconstituting sample, was injected onto a C_{18} Pico-Tag Column for separation and determination of PITC derivative of HYP. The flow program for the solvent gradient of buffer A and B are given in Table 3. (Buffer A consists of 19 g of sodium acetate trihydrate, 1000 ml water, 0.05 ml TEA; titrated to pH 6.4 with glacial acetic acid. Then 60 ml of acetonitrile were added to 940 ml of this solution. Buffer B consisted of 60% acetonitrile in 40% water by volume). Each sample was separated over a period of 12 minutes followed by a

gradient washing and re-equilibration for 8 minutes. Chromatographed profile of the sample was obtained (Figures 2 & 3) which was then compared with the standard amino acid profile (Figure 4) to determine the location of HYP peak.

Table 3. The flow program for the solvent gradient of buffer A and B for chromatography:

Time (min.sec.)	Flow (ml/min)	% of buffer A*	% of buffer B*	Curve
Initial	1.0	100	0	5
10.0	1.0	54	46	5
10.5	1.0	000	100	6
11.5	1.0	0	100	6
12.0	1.5	0	100	6
12.5	1.5	100	0	6
20.0	1.5	100	0	6
20.5	1.0	100	0	6

* For composition of buffer see text

Figure 2 Chromatographic profile of sample obtained from a control hamster palatal tissue on day 12:04 of gestation showing hydroxyproline peak.

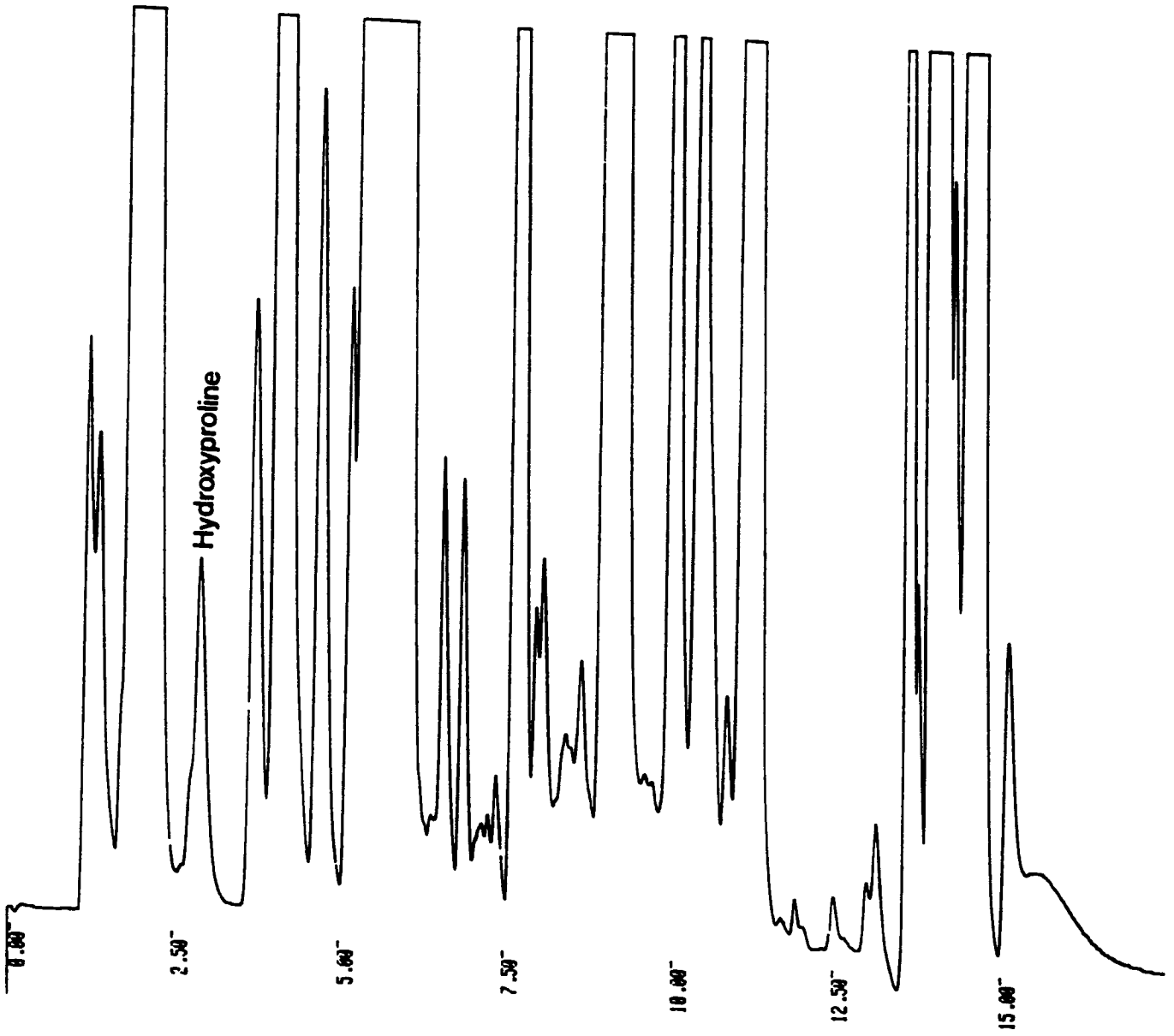


Figure 2

Figure 3 Chromatographic profile of sample obtained from a control quail palatal tissue on day 8 of incubation showing hydroxyproline peak.

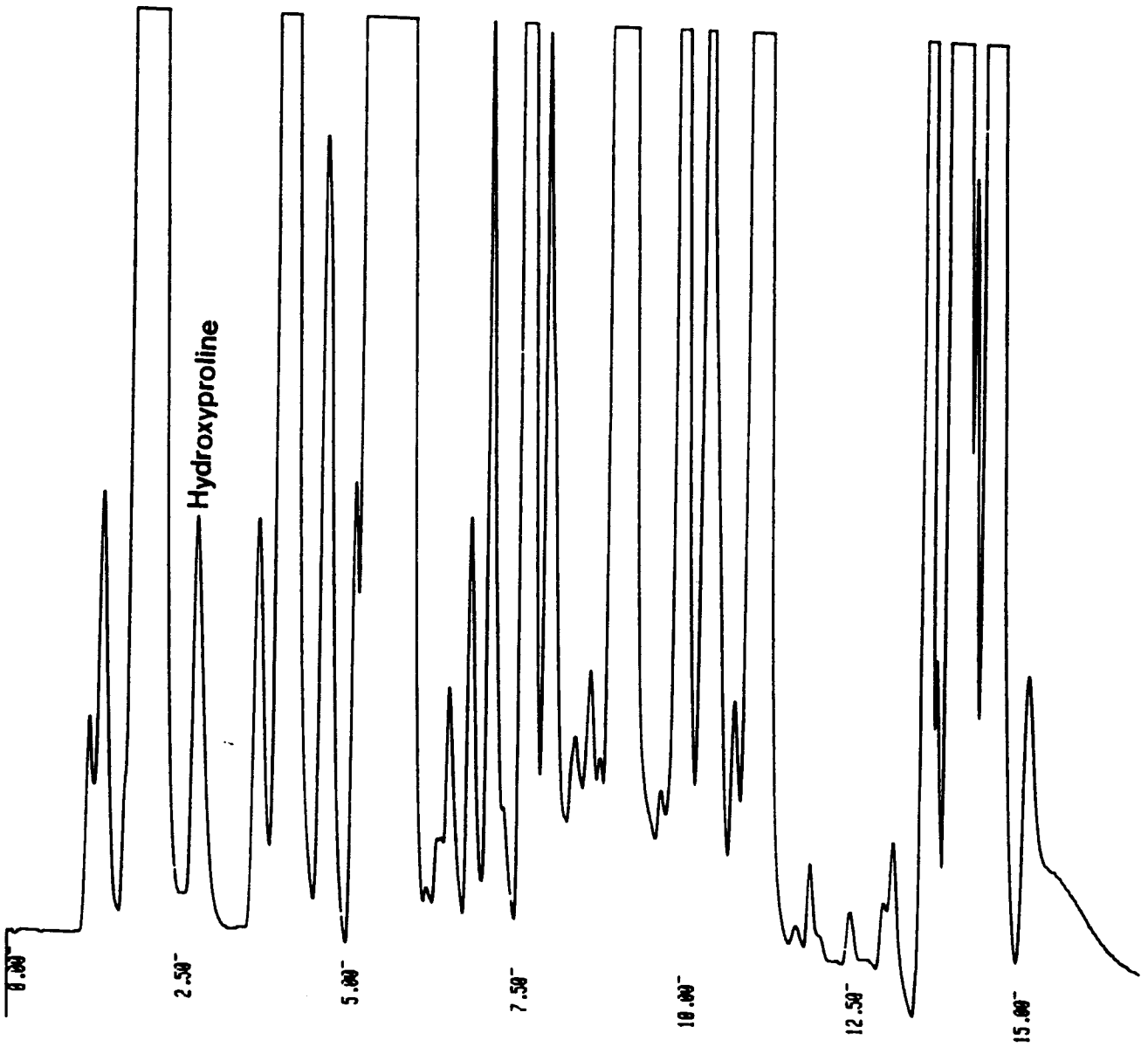


Figure 3

Figure 4 Chromatographic profile of standard amino acids showing hydroxyproline peak.

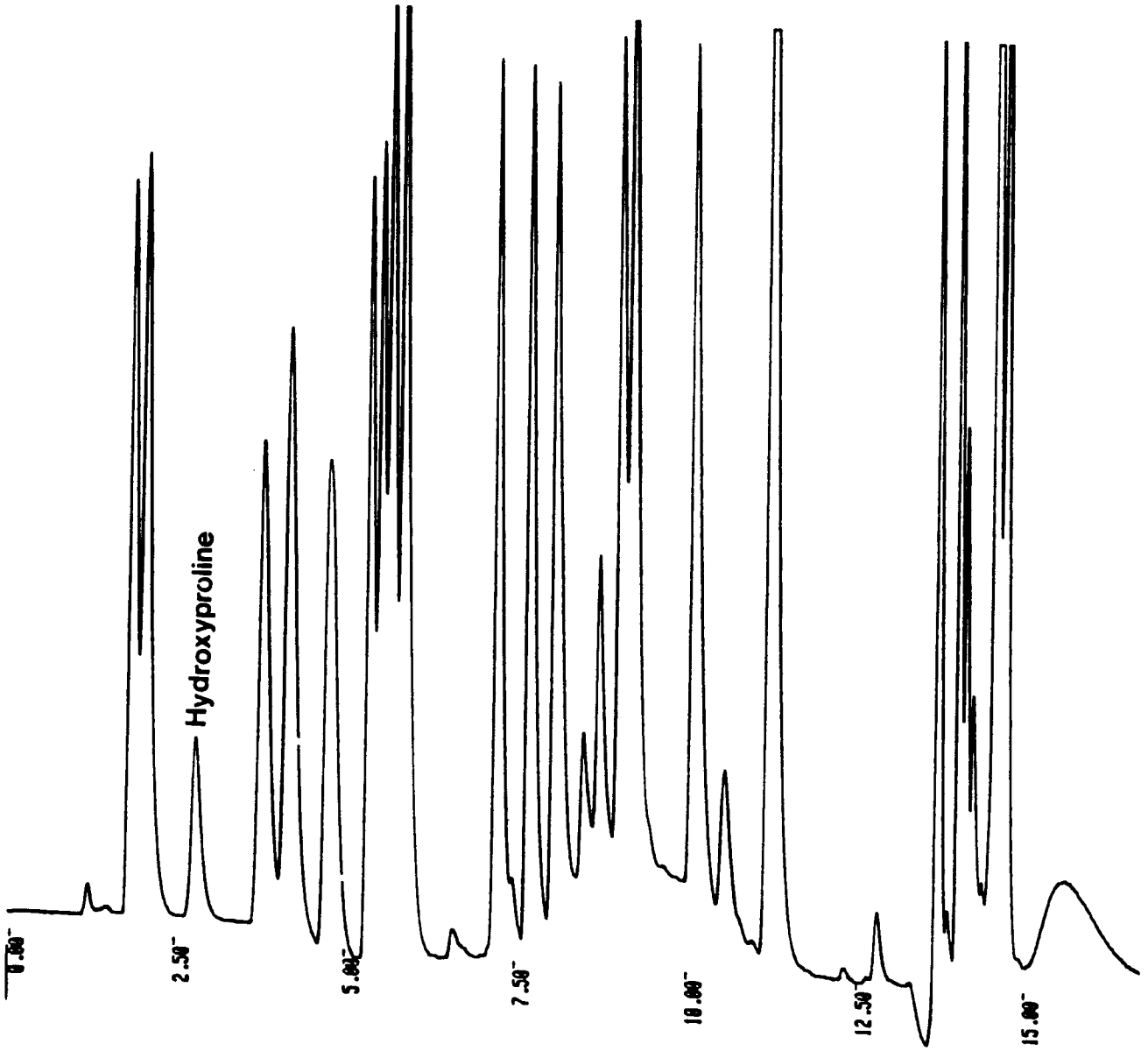


Figure 4

From the profiles, the area of absorbance of HYP in correspondence with retention time of samples were calculated for both the control and 5-FU-treated palates. The control and 5-FU-treated data were compared by student's t-test at the 95% level of significance.

F. Collagen Extraction for SDS-PAGE

The isotypes of collagen were separated using a modified method of Chung and Miller (1974) as described by Narayanan and Page (1976). Because during the initial efforts of identifying isotype of collagen in quail palate was not successful, the quail samples were double-labelled with ^{14}C-proline and ^{14}C-glycine (Specific Activity 250μCi/mmole, New England Nuclear, Boston, Massachusetts, USA). Both the drug-treated and control samples, left for 24-48 hours in 2ml of 0.5N acetic acid as described in B, were digested by 200μg pepsin (100μg/ml) at 4°C for 24 hours. Lathyritic rat skin collagen carrier (5mg) was then added to the samples followed by slow addition of solid NaCl to 1.7M, to help precipitate the collagen. The mixture was allowed to stand overnight at 4°C, and centrifuged (Beckman Instruments, Model L8-70 Ultracentrifuge, Palo Alto, California, USA) at 27,000g for 25 minutes at 4°C to collect the collagen precipitate. The supernatants were discarded and the pellets dissolved in 0.5ml of 0.5N acetic acid containing 10mM EDTA for a minimum of 24 hours at 4°C. Subsequently, the samples were spun in a microcentrifuge at 13,000 rpm for 10 minutes. The pellets were discarded, supernatant lyophilized, and then, reconstituted with 100μl of 0.5N acetic acid.

G. SDS-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

was performed using 7.5% polyacrylamide in SDS Tris-glycine, pH 8.8 (Laemmli, 1970). An aliquot (36µl) of extracted collagen, as described in F, was dissolved in 9µl of "Laemmli" sample buffer (composed of 4ml of distilled water, 1ml of 0.5M Tris-HCl pH 6.8, 0.8ml glycerol, 0.4ml of 2-β mercaptoethanol, 1.6ml of 10% SDS and 0.2ml of 0.05% bromophenol blue), boiled for 5 minutes, and loaded into the SDS-PAGE as described by Laemmli (1970). The electrophoresis was performed at 125V, 75mA for 15 minutes. At this stage, the gel was interrupted by switching off the current and the sample wells filled with 5% v/v 2-mercaptoethanol in sample buffer to separate collagen type I from collagen type III. Mercaptoethanol was allowed to diffuse into the gel for 15 minutes before the current was again switched on to resume electrophoresis for a further period of one hour (Sykes, et al., 1976). Subsequently, the gels were dehydrated in dimethyl sulphoxide and soaked in a solution of 2,5-diphenyloxazol in dimethylsulphoxide. The gels were then dried and each gel was exposed on 8"x10" Cronex X-ray (E. I. DuPont de Nemours, Willington, Delaware, USA) film at -70°C for 15 days. Radiolabelled bands, representing different types of collagen, were identified using ^{14}C-labelled rainbow protein molecular weight marker (Specific Activity 37KBq, 1µCi, Amersham, U.K., Batch 13, Lot 4).

H. Light microscopy

Three control and three 5-FU-treated hamster embryos were obtained randomly from different litters at various gestational times as described in

Materials and Methods, Section B. Three control and three 5-FU-treated quail embryos were also obtained between days 5-9 of incubation. The embryos were immersed in Bouin's solution for 72 hours. They were then dehydrated through an ascending concentration of ethanol, starting at 30%. Upon reaching 100% concentration, the embryos were processed through three changes of chloroform, and embedded in paraffin to procure frontal sections. Seven micron serial sections were obtained and stained with Haematoxylin and Eosin for light microscopic examination of the status of palatal development in hamster and quail embryos.

RESULTS

5-FU Dose-Time Response in Quail

The effects of different concentrations of 5-FU, administered on different days of incubation, on palate development in quail is outlined in Table 4. One may deduce from the table that 5-FU treatment on different days of incubation did not affect the mean fetal weight. Drug treatment on day 4 of incubation caused high mortality rate ($P<0.05$) following a dose of 75-150μg 5-FU. However, only 100-150μg 5-FU increased the width between the palatal shelves ($P< 0.05$).

The width between the palatal shelves remained unaffected following 5-FU treatment on day 5 of incubation, although there was an increase in the mortality rate at a dose range of 125-150μg,. After 5-FU administration on day 6 of incubation neither the mortality rate nor the width between the palatal shelves has affected. On the basis of this analysis, it is clear that an administration of 100μg 5-FU on day 4 of incubation to quail eggs affords the best dose-time regimen to increase the width between the palatal shelves, i.e., cleft palate. Hence, this dose-time regimen was used in the further studies on the effect of 5-FU on quail secondary palate development.

Light Microscopic Observations of the Developing Secondary Palate

Hamster

Control

Light microscopic observations indicated that the individual morphologic events of normal palatal development in hamster can be seen to fall within one of

the following four stages:

Stage 1: The palatal shelves, composed of mesenchyme covered by an epithelium, are vertical alongside the tongue (Figure 5).

Stage 2: The palatal shelves are changing from a vertical position, on the side of the tongue, to a horizontal position above the tongue (Figure 6).

Stage 3: The palatal shelves are horizontal above the tongue (Figure 7).

Stage 4: The horizontal palatal shelves are united with one another thus separating the oral and nasal cavities (Figure 8). The union of the horizontal shelves results in the formation of an epithelial seam (Figure 8), which eventually fragments to establish a mesenchymal continuity between the united palatal shelves (see Figure 13 in Shah and Chaudhry, 1974a).

Between days 11:00 and 12:00 of gestation, the palatal shelves are vertical (Stage 1). During the next four hours, i.e., until day 12:04 of gestation, the palatal shelves reoriented (Stage 2) to a horizontal position (Stage 3) and united (Stage 4).

5-FU-treated

Following 5-FU treatment, only the first three stages of palate development are observed. The palatal shelves are vertical (Stage 1) between days 11:00 and 12:16 of gestation (Figure 9). The reorientation of 5-FU treated shelves, however, is delayed by 12-20 hours. Unlike controls, which reunit between days 12:00 and 12:04 of gestation, the treated shelves reorient (Stage 2) from a vertical to a horizontal position (Stage 3) between days 12:16 and 13:00 of gestation (Figures 10 & 11). Stage 4 (fusion) seen in controls, is not observed in 5-FU-treated fetuses.

Table 4. Effects of 5-fluorouracil on mortality, fetal weight and the palate gap in quail.

Incubation day of injection	Dose (μg)	No. of eggs	Mortality (%)	Mean weight of live fetuses ±SD (gm)	Man palate gap* ±SD (mm)
4	Control	31	0	7.73±0.45	0.59±0.02
	75	15	13**	7.86±0.41	0.63±0.031
	100	31	13**	6.80±0.75	0.71±0.036**
	125	34	13**	7.33±0.59	0.78±0.070**
	150	34	15**	7.79±0.40	0.71±0.054**
5	Control	30	0	7.35±0.47	0.36±0.015
	75	34	0	7.92±1.20	0.36±0.031
	100	28	0	7.90±0.65	0.40±0.012
	125	31	6**	7.81±0.73	0.41±0.026
	150	39	8**	6.91±0.97	0.44±0.025
6	Control	30	6	7.35±0.40	0.40±0.110
	75	36	0	7.76±0.42	0.41±0.130
	100	32	6	6.66±0.72	0.51±0.119
	125	35	5	6.96±0.61	0.49±0.112
	150	32	0	7.68±0.61	0.55±0.108

* Distance between palatal shelves measured on day 15 of incubation.
** $P < 0.05$; Student's t-test.

Quail

Control

The quail palatal shelves develop as horizontal ridges from the beginning of day 5 of incubation (HH stages 29-30). The horizontal ridges form intraorally from the medial aspect of the maxillary processes, dorsal to the tongue (Figure 12). Each ridge (shelf) consist of a core of mesenchyme enveloped by an epithelium.

Between days 6-8 of incubation (HH stages 31-34), the palatal shelves continue to grow horizontally toward one another until they approximate with one another on day 9 (HH stages 35-36; Figures 13, 14 & 15). A distinct osteogenic site is present in the mesenchyme (Figure 14).

On day 9 (HH stages 35-36), when the opposing horizontal shelves approximate, several small epithelial invagination from the oral side extend into the mesenchyme (Figure 15). They represent precursors of minor salivary glands (Shah et al., 1985a). In addition, a distinct, well differentiated osteogenic site is present in the mesenchyme (Figure 15).

5-FU-treated

Following 5-FU administration, the palatal ridges extend horizontally (Figure 16) from the maxillary processes on day 5 of incubation (HH stages 29-30). The structure of a 5-FU-treated palatal ridge resemble that seen in the control.

Subsequently, between days 6-7 of incubation (HH stages 31-33), the horizontal shelves grow toward one another (Figure 17).

On days 8-9 of incubation (HH stages 34-36), although the palatal shelves continue their growth toward one another they never approximate (Figure 18 & 19). Unlike their control counterparts, a wide gap persist between the medial edges

Figure 5 Frontal section through the secondary palate in a control hamster embryo on day 11:18 of gestation. The palatal shelves (P) are vertical (Stage 1) on the sides of the tongue (T). ONC-oronasal cavity. Hematoxylin and Eosin stain. 125X.

Figure 6 Frontal section through the secondary palate in a control hamster embryo on day 12:00 of gestation. The palatal shelves (P) are reorienting (Stage 2) above the tongue (T). ONC-oronasal cavity. Hematoxylin and Eosin stain. 125X.

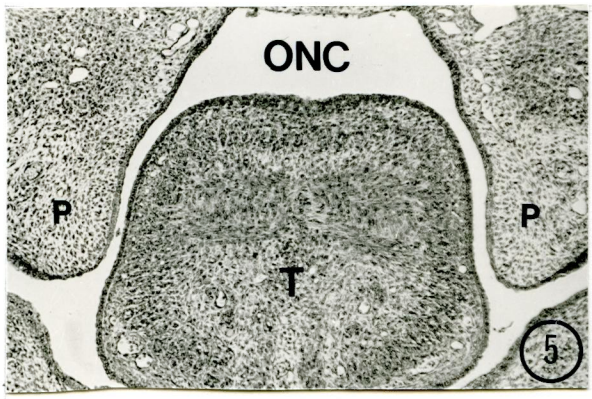
Figure 7 Frontal section through the secondary palate in a control hamster embryo on day 12:02 of gestation. The palatal shelves (P) are horizontal (Stage 3) above the dorsal surface of the tongue (T). Hematoxylin and Eosin stain. 125X.

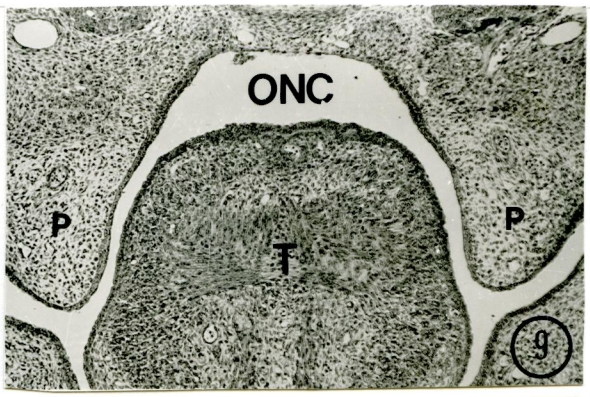
Figure 8 Frontal section through the secondary palate in a control hamster embryo on day 12:04 of gestation. The opposing palatal shelves (P) are united with one another (Stage 4) separating the nasal cavity (NC) from the oral cavity (OC). Hematoxylin and Eosin stain. 125X.

Figure 9 Frontal section through the secondary palate in a 5-FU-treated hamster embryo on day 12:00 of gestation. The palatal shelves (P) are vertical (Stage 1) on the sides of the tongue (T). ONC-oronasal cavity. Hematoxylin and Eosin stain. 125X.

Figure 10 Frontal section through the secondary palate in a 5-FU-treated hamster embryo on day 12:16 of gestation. The palatal shelves (P) are reorienting (Stage 2) above the tongue (T). ONC-oronasal cavity. Hematoxylin and Eosin stain. 125X.

Figure 11 Frontal section through the secondary palate in a 5-FU-treated hamster embryo on day 12:20 of gestation. The palatal shelves (P)are horizontal (Stage 3) above the surface of the tongue (T). Hematoxylin and Eosin stain. 125X.


ONC
P
P
T
5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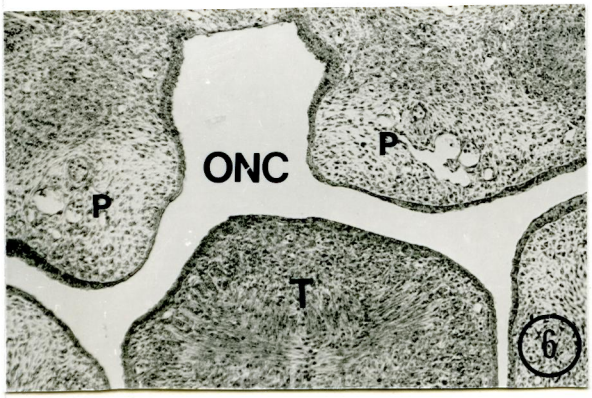
9


ONC
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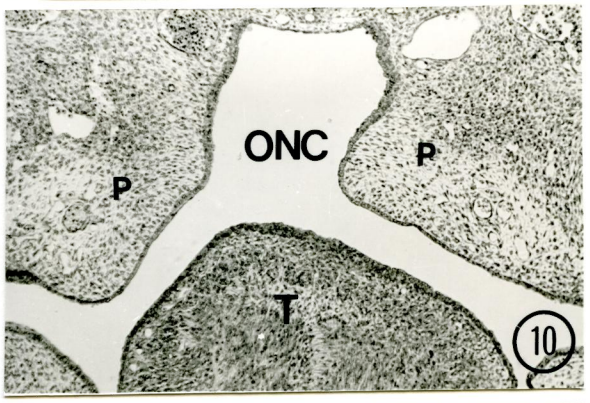
ONC
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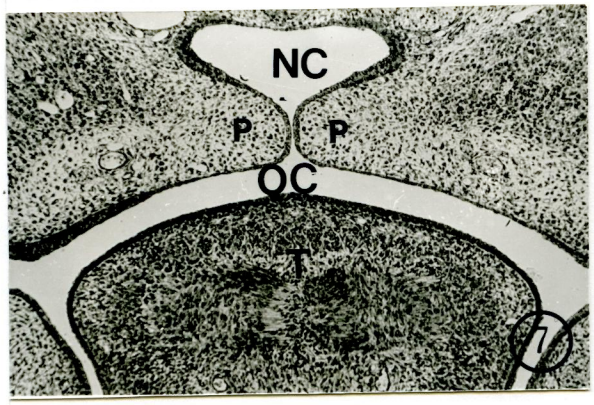
NC
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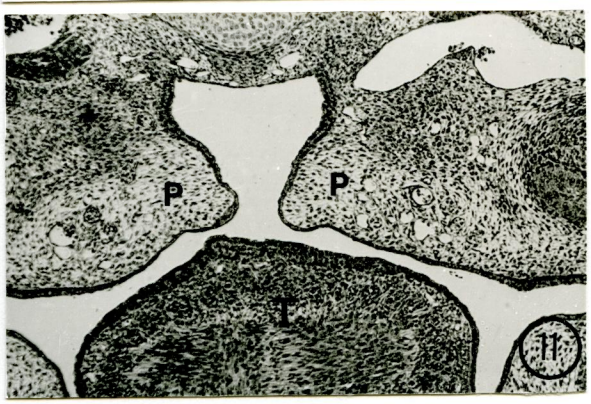
7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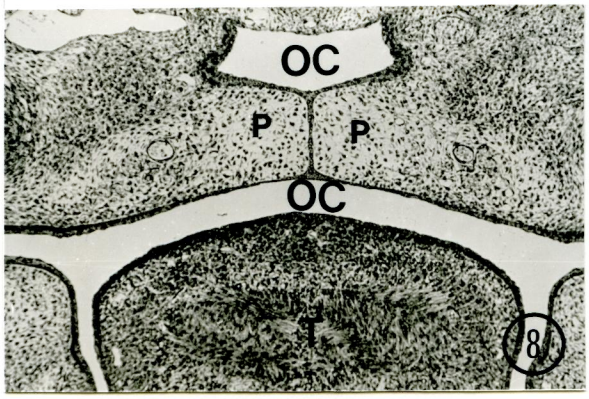
11


OC
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8

of 5-FU treated horizontal palatal shelves (cf. Figure 14 & 18, and 15 & 19). In addition, the precursors of minor salivary glands, seen in control palates on day 9 of incubation, are absent in 5-FU-treated palates (cf. Figures 15 & 19). Also, a condensation of mesenchyme is seen in the treated palatal shelves, but sites of osteogenesis observed in control palates, are absent in 5-FU-treated ones (Figures 18 & 19).

Measurement of Incorporation of ^{14}C-proline into Collagen

Hamster

Control

In hamster, between days 11:00 and 12:00 of gestation, i.e., the period when palatal shelves are growing vertically (Figure 20), the rate of collagen synthesis, remains unchanged (Figure 20). During the next four hours, i.e., between days 12:00 and 12:04 of gestation, when the palatal shelves reorient from a vertical to a horizontal plane and fuse, the rate of collagen synthesis doubles ($P<0.05$). In the ensuing four hours, when the epithelial seam is fragmenting (Shah and Chaudhry, 1974a), the rate decreases. On day 12:08 of gestation, the rate of collagen synthesis is approximately 40% of that seen four hours earlier ($P<0.05$). Subsequently, when the palate closed completely through its length, the rate of collagen synthesis increase approximately 6-fold.

5-FU-treated

Following 5-FU treatment between days 11:04 and 12:02 of gestation, the rate of colagen synthesis in the developing secondary palate of hamster remain unchanged (Figure 20). During this period, however, the rate is lower than the

Figure 12 Frontal section through the secondary palate in a control quail embryo on day 5 of incubation. The palatal primordia (arrows) develop horizontally from the maxillary process (M) above the tongue (T). Hematoxylin and Eosin stain. 100X.

Figure 13 Frontal section through the secondary palate in a control quail embryo on day 7 of incubation. palatal shelves (P) are horizontal above the tongue (T). Hematoxylin and Eosin stain. 100X.

Figure 14 Frontal section through the secondary palate in a control quail embryo on day 8 of incubation. The approximated palatal shelves (P) are above the dorsal surface of the tongue (T). Site of osteogenesis (O) within the palate. Hematoxylin and Eosin stain. 100X.

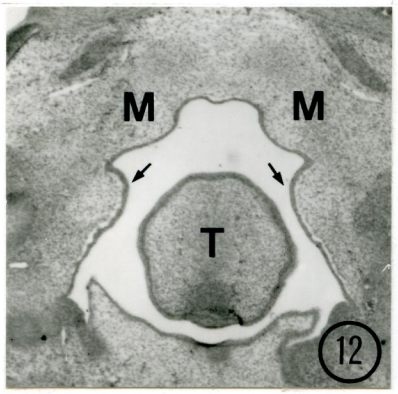
Figure 15 Frontal section through the secondary palate in a control quail embryo on day 9 of incubation. Showing approximated palatal shelves (P) above the tongue (T). Minor salivary gland (G) within the shelves Site of osteogenesis (O). Hematoxylin and Eosin stain. 100X.

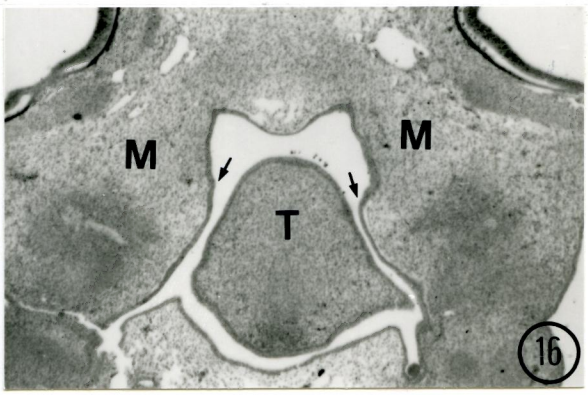
Figure 16 Frontal section through the secondary palate in a 5-FU-treated quail embryo on day 5 of incubation. The palatal primordia (arrows) develop horizontally from maxillary processes (M) above the tongue (T). Hematoxylin and Eosin stain. 100X.

Figure 17 Frontal section through the secondary palate in a 5-FU-treated quail embryo on day 7 of incubation. The palatal shelves (P) are horizontal above the tongue (T). Hematoxylin and Eosin stain. 100X.

Figure 18 Frontal section through the secondary palate in a 5-FU-treated quail embryo on day 8 of incubation. The palatal shelves (P) are horizontal above the dorsal surface of the tongue (T). Site of osteogenesis (O) within the shelves (cf. Figure 13). Hematoxylin and Eosin stain. 100X.

Figure 19 Frontal section through the secondary palate in a 5-FU-treated quail embryo on day 9 of incubation. The palatal shelves (P) are above the tongue (T). Site of osteogenesis (O) (cf. Figure 15). Hematoxylin and Eosin stain. 100X.


M
M
T
12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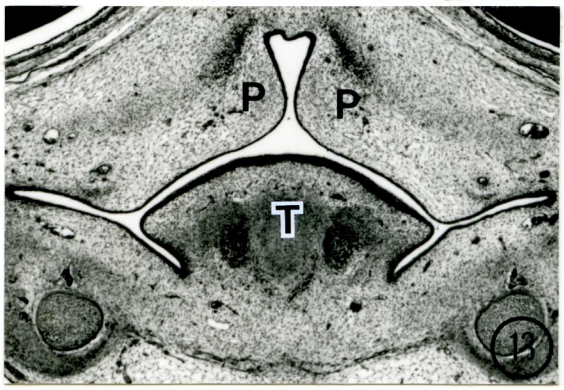
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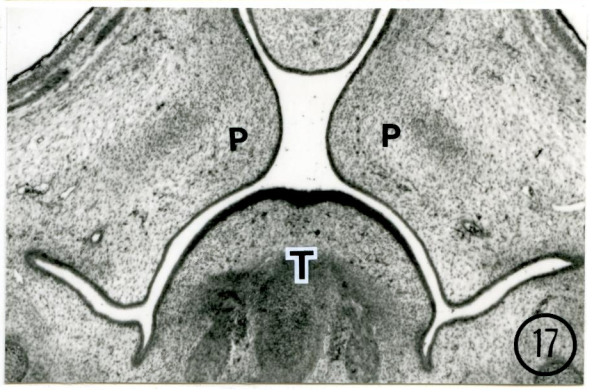
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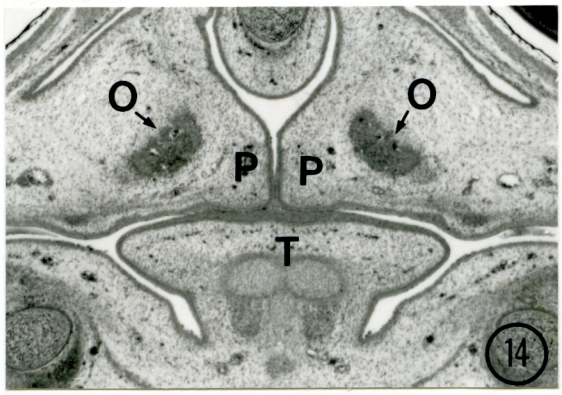
17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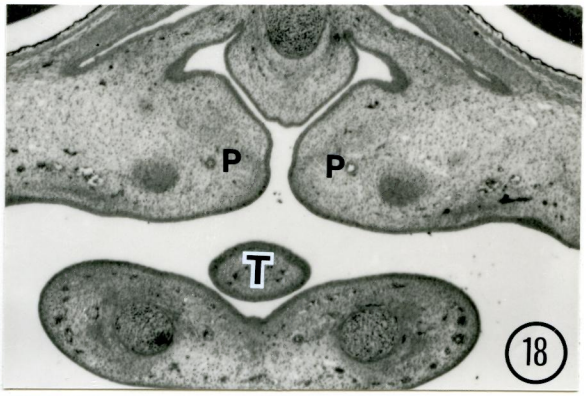
14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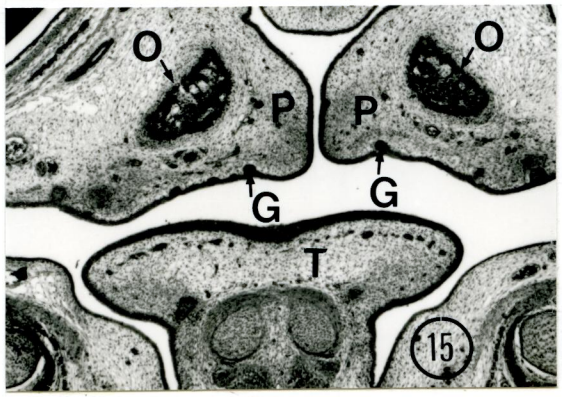
O
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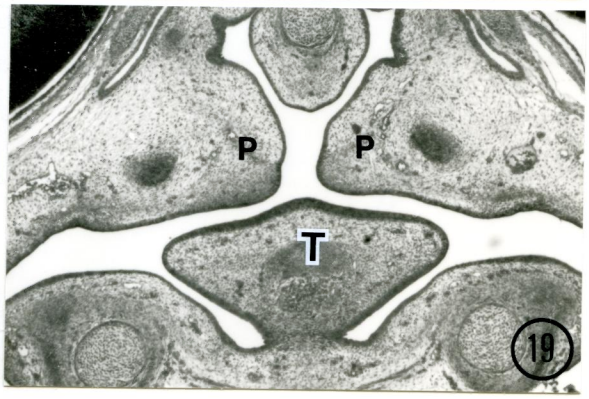
P
P
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19

Figure 20 Rate of collagen synthesis in the control and 5-FU-treated developing secondary palate of hamster embryos between days 11 and 13 of gestation.

Figure 21 Rate collagen synthesis in the control and 5-FU-teated developing secondary palate of quail embryos between days 5 and 10 of incubation.

Figure 20

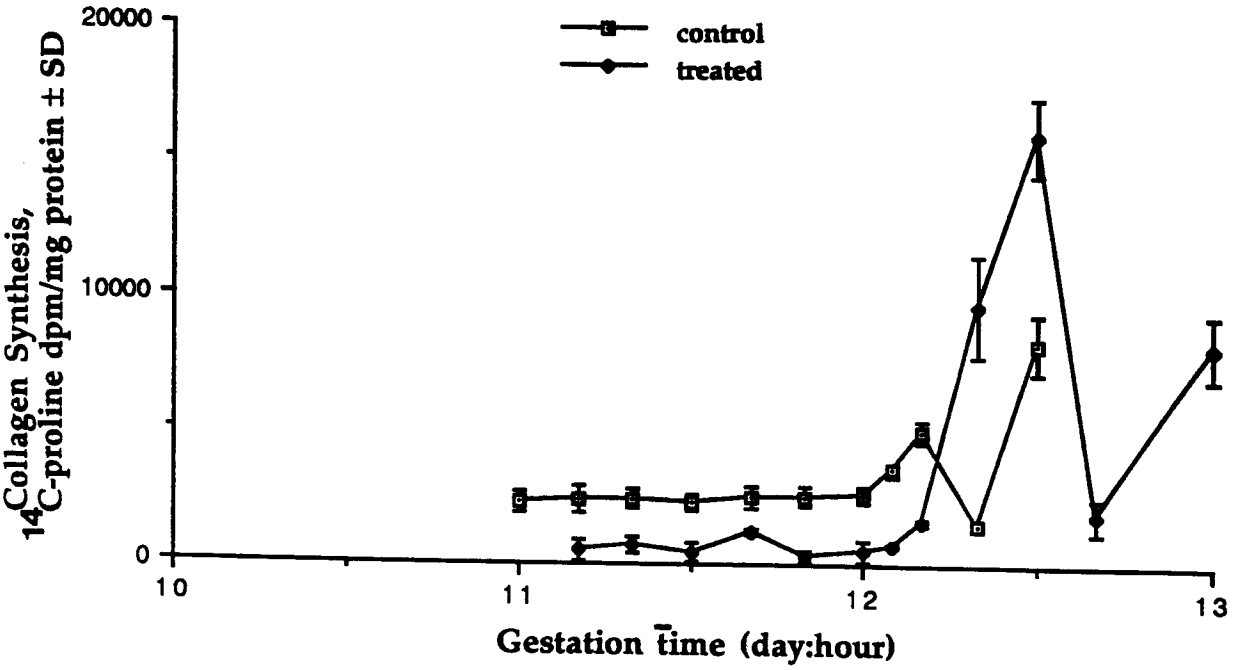
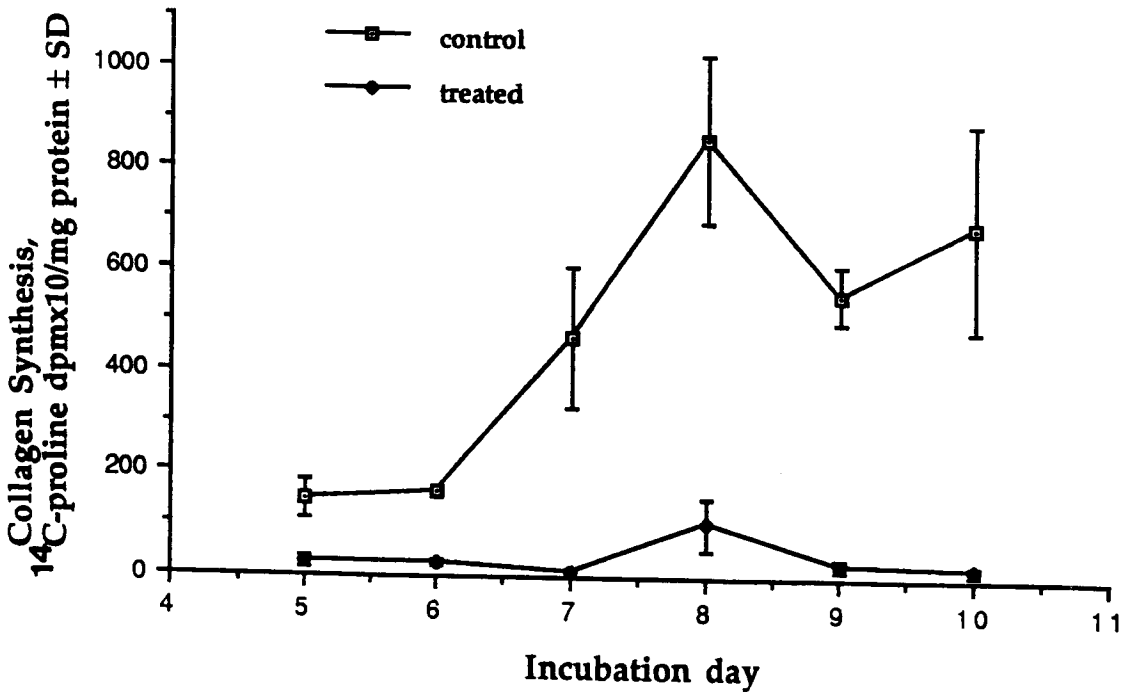


Figure 21



controls ($P<0.01$). It then increases approximately 10-fold to peak at day 12:12 of gestation ($P<0.01$). On day 12:16 of gestation, when reorientation begins in the drug-treated shelf, the rate drops 8-fold before showing a 4-fold increase on day 13:00 of gestation (Figure 20).

Quail

Control

The rate of collagen synthesis in the developing secondary palate of quail, between days 5 and 6 of incubation, remains unchanged (Figure 21). It increases approximately 4-fold between days 6 and 8 of incubation ($P<0.01$). During the next 24 hours, the rate drops 35% ($P<0.01$) before levelling off between days 9 and 10 of incubation (Figure 21).

5-FU-treated

The rate of collagen synthesis in 5-FU-treated secondary palate of quail embryos, between days 5 and 7 of incubation, demonstrates the same pattern of change as that seen in the controls but overall uptake is significantly decreased. It increases 3-fold to peak on day 8 of incubation ($P<0.05$), but drops on day 9 before levelling off on day 10 of incubation (Figure 21).

High Performance Liquid Chromatography

Because in hamster controls the reorientation of palate occurs between days 12:00 and 12:04 of gestation, and in 5-FU-treated between days 12:16 and 13:00 of gestation the amount of HYP was determined at these times.

In quail, because the profile of collagen synthesis changes between days 6 and 9 of gestation, the amount of HYP was measured during these periods.

Figure 22 Hydroxyproline content in the control and 5-FU-treated developing secondary palate of hamster embryos at their respective periods of reorientation. (No comparable controls was obtained on days 12:16 and 13:00 of gestation because in control the palate was already closed).

Figure 23 Hydroxyproline content in the control and 5-FU-treated developing secondary palate of quail embryos between days 6 and 9 of incubation.

Figure 22

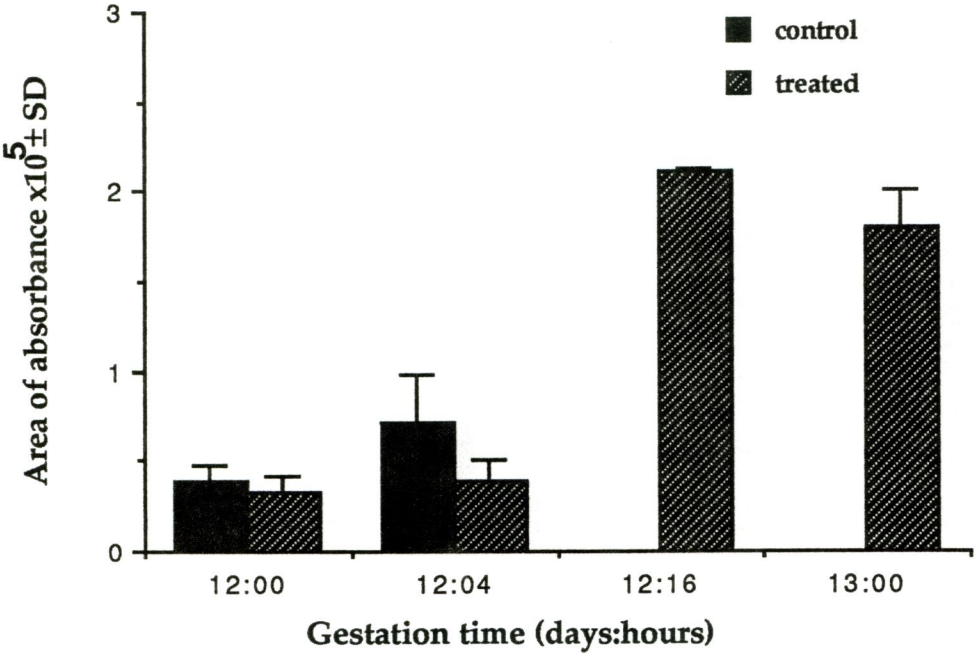


Figure 23

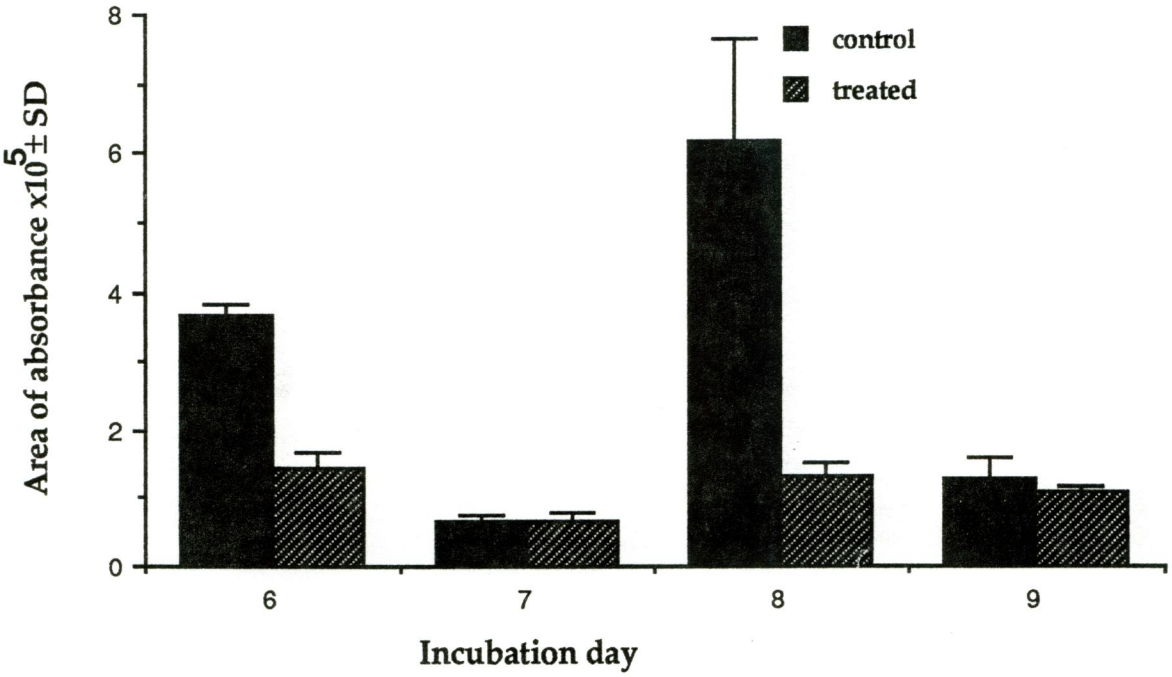


Figure 24 Total protein content in the control and 5-FU-treated developing secondary palate of hamster embryos between days 11:04 and 13:00 of gestation

Figure 25 Total protein content in the control and 5-FU-treated developing secondary palate of quail embryos between days 5 and 10 of incubation.

Figure 24

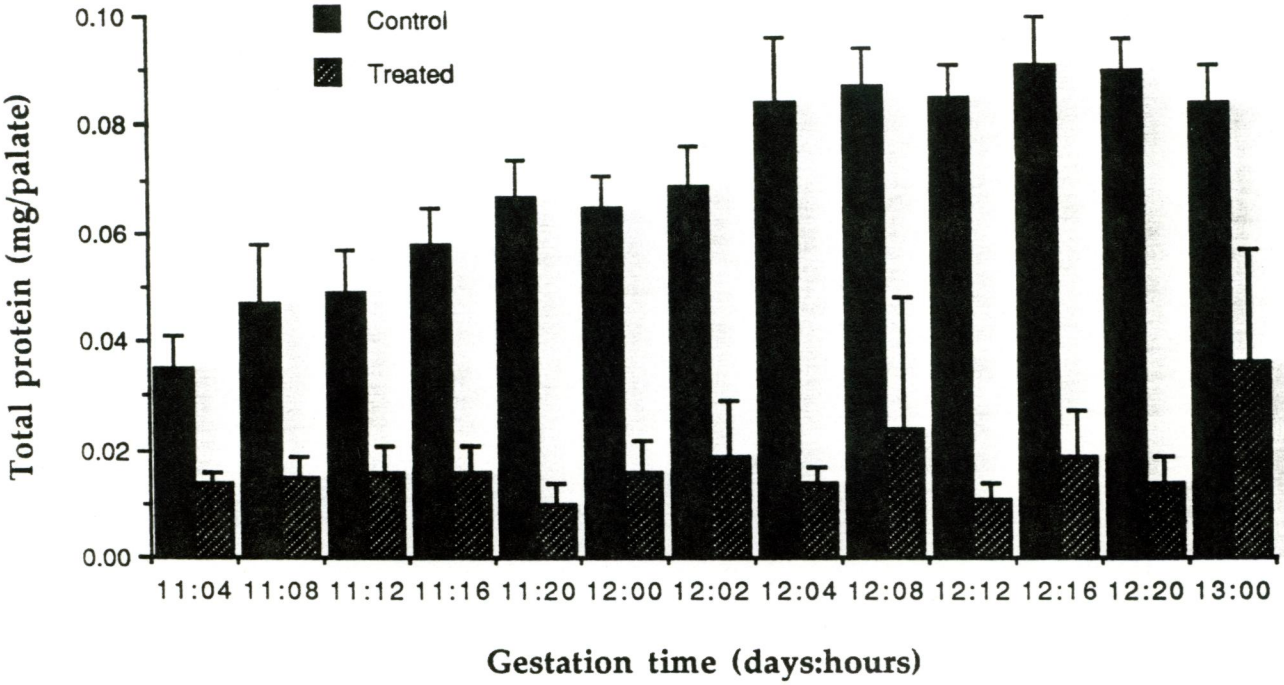
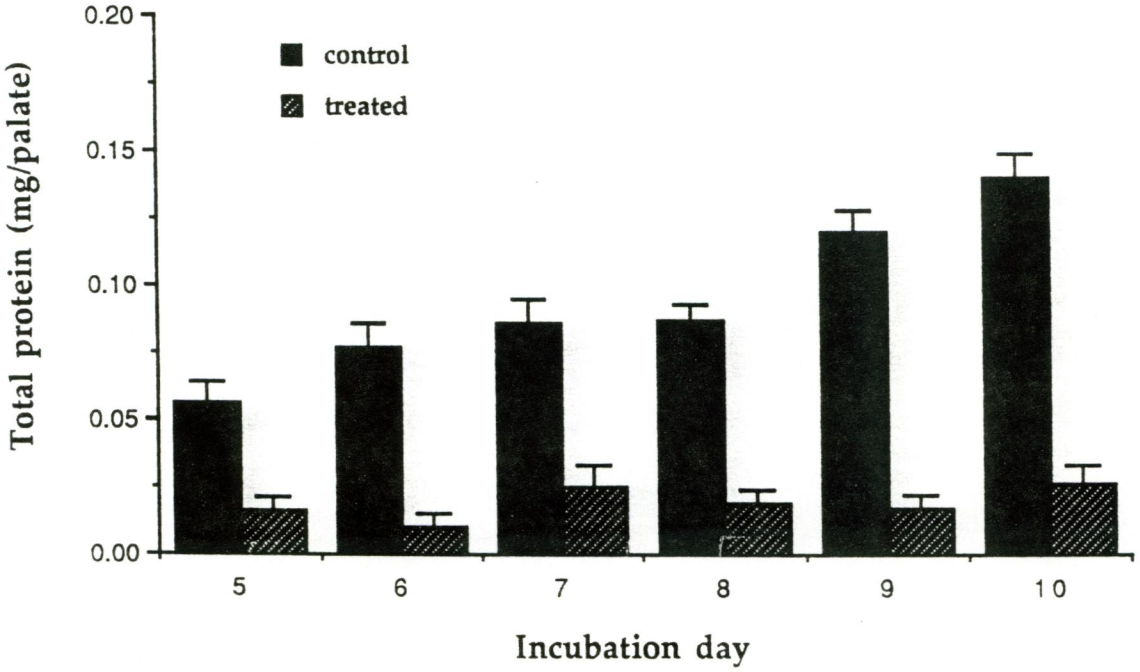


Figure 25



Hamster

Control

The result on HYP content in the developing secondary palate of hamster embryos are summarized in Figure 22. The results show that the amount of HYP, as represented by the area of absorption on the chromatogram, remains unchanged between days 12:00 and 12:04 of gestation ($P>0.05$). This observation is in contrast to the increase in the rate of collagen synthesis observed during this period in the control palate of hamster (Figure 20), suggesting that only a portion of ^{14}C-proline is utilized for hydroxylation to HYP, and hence for collagen synthesis. The spurt in ^{14}C-proline uptake, thus may reflect synthesis of both collagenous and non-collagenous proteins.

5-FU-treated

The observation on HYP content in the 5-FU-treated developing secondary palates of hamsters are outlined in Figure 22. The data indicates that in spite of a significantly low uptake of ^{14}C-proline between days 12:00 and 12:04 of gestation in 5-FU-treated palates, the amount of HYP accumulated is comparable to the controls. This would indicate that, in comparison to controls, proportionally a large amount of HYP is accumulated in 5-FU-treated palates, perhaps utilizing much of the ^{14}C-proline for collagen synthesis. On day 12:16 of gestation, when the reorientation begin in 5-FU-treated palates, and when the rate of ^{14}C-proline uptake is comparable to that seen in control palates on day 12:00 of gestation, the HYP accumulation is five-fold higher possibly reflecting accumulated HYP following high uptake of ^{14}C-proline on day 12:12 of gestation. Subsequently, however, even though the ^{14}C-proline uptake increase approximately four-fold,

the HYP level remains unchanged. This suggests that perhaps much of the ^{14}C-proline may have been utilized for non-collagenous protein synthesis.

Quail

Control

The data on HYP content in the developing secondary palate of quail embryos are summarized in Figure 23. One may infer from the data that the amount of HYP in the control is 50% higher on day 8 than on day 6 of incubation ($P<0.05$). These data are consistent with the observations that the uptake of ^{14}C-proline in the developing palate of quail is 4-fold higher on day 8 than on day 6 of incubation (Figure 21). The data further show that although the rate of ^{14}C-proline uptake is higher on days 7 and 9 than on day 6 (Figure 21), the HYP content are significantly lower ($P< 0.05$) on days 7 and 9 than on day 6 of incubation. This would indicate that, in comparison to day 6, much of the ^{14}C-proline uptake on days 7 and 9 is associated with the synthesis of non-collagenous, rather than collagenous, proteins.

5-FU-treated

The results of HYP content in the 5-FU-exposed secondary palate of quail embryos are summarized in Figure 23. The data indicates that, in comparison to controls, 5-FU treatment of quail results in a reduction in the amount of HYP in the developing palate on days 6 and 8 of incubation ($P<0.05$). Although the reduction in HYP content is more severe on day 8 (75%) than on day 6 (50%), the total amount of HYP on both days in 5-FU-treated quail palate is similar, perhaps suggesting that much of the ^{14}C-proline uptake on day 6 may be related to the synthesis of non-collagenous proteins. In addition, although the rate of ^{14}C-

proline uptake in 5-FU treated palates is similar on days 6, 7 and 9 (Figure 21), the amount of HYP is lower on day 7 than on days 6 and 9 of incubation. This may indicate a higher rate of accumulation of non-collagenous proteins on day 7 than on days 6 and 9.

Measurement of Total Protein

Hamster

Control

The data on the total protein content of the developing secondary palate in hamster are outlined in Figure 24. One may infer from the figure that, between days 11:04 and 12:04 of gestation, i.e., during the period when the shelves completed their vertical growth and reoriented to a horizontal plane and fused, the amount of total protein in the developing secondary palate doubles ($P<0.05$). In the ensuing 20 hours the amount of total protein levell-off.

5-FU-treated

The results of the measurement of total protein content following 5-FU treatment are summarized in Figure 24. In general, between days 11:04 and 13:00 of gestation, the total protein contents in 5-FU treated palates is approximately 50-75% lower than control palates ($P<0.05$). Also, unlike controls, the amount of total protein in 5-FU treated palates is unchanged between days 11:04 and 13:00 of gestation, i.e., the period during which the cleft palate was forming.

Quail

Control

The data on the amount of total protein in the developing secondary palate

of quail are summarized in Figure 25. The data show that in the control palates of quail embryos there is a gradual increase in the amount of total protein between days 5 and 10 of incubation ($P<0.05$). Also, the amount of total protein on day 10 is twice the amount seen on day 5 of incubation.

5-FU-treated

The results of total protein content in 5-FU treated palates are outlined in Figure 25. One may deduce from the figure that the total protein contents in 5-FU treated palates is 50-70% lower than their control counterparts between days 5 and 10 of incubation ($P<0.05$). Furthermore, as observed in the 5-FU-treated developing palate of hamster, the amount of total protein content remains unchanged during the period of observation.

SDS-Polyacrylamide Gel Electrophoresis

Hamster

The results on SDS-Polyacrylamide Gel Electrophoresis to separate different collagen isotypes in the developing palate of hamster embryos are presented in Figure 26. Collagen isotypes were determined in both the control and drug-treated animals at the time of shelf reorientation. In control hamster embryos on day 12:02 of gestation only type I collagen is detected by SDS-PAGE. Similarly after 5-FU administration, only type I collagen is seen on SDS-PAGE of embryonic palates at the time of delayed shelf reorientation, i.e., on day 12:16 of gestation. In both controls and drug-treated palates, a delayed reduction was performed using 2-mercaptoethanol to separate type I isomers from type III. The results indicated that either type III collagen is present in such a small quantity that it was not possible to

detect by SDS-PAGE method in the present study or that it does not exist *in vivo* in the developing secondary palate of hamster.

Quail

The observations on SDS-Polyacrylamide Gel Electrophoresis in developing quail secondary palate are documented in Figure 27. In both the control and drug-treated quail palates collagen types were determined on day 8 of incubation because there is an increase in collagen synthesis at this time. Like hamster, in both the control and 5-FU treated quail palates only collagen type I is detected on SDS-PAGE electrophoresis. When delayed reduction was performed using 2-mercaptoethanol to separate type I from type III, type I was the only isomer of collagen identified.

Figure 26 SDS-PAGE showing type I collagen in the control and 5-FU-treated developing secondary palate of hamster embryos on days 12:02 and 12:16 of gestation.

Track 1. Molecular weight Marker (MW 14300-200000).
Track 2. Standard type I collagen showing α1 and α2 chains.
Track 3. Control secondary palate of hamster embryo (12:02).
Track 4. 5-FU-treated secondary palate of hamster embryo (12:02).
Track 5. Control secondary palate of hamster embryo (12:16).
Track 6. 5-FU-treated secondary palate of hamster embryo (12:16)

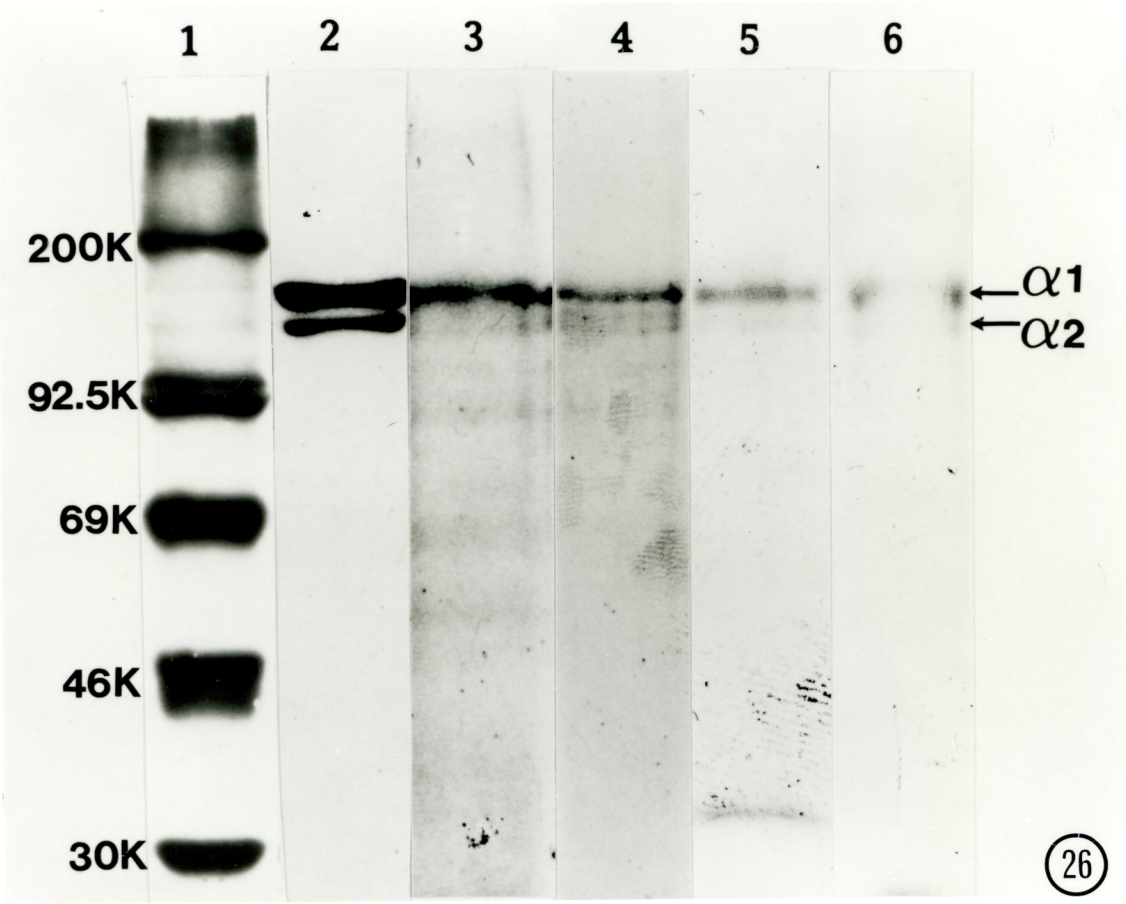
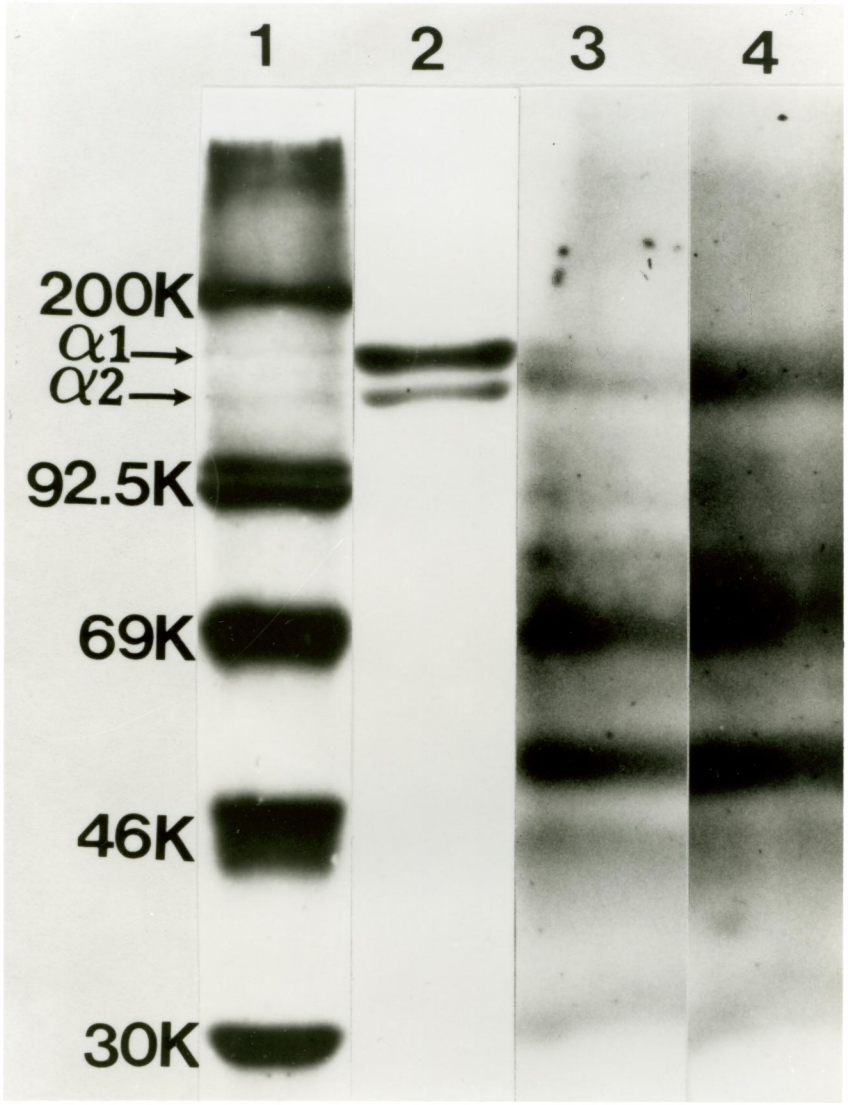

1
2
3
4
5
6
200K
92.5K
69K
46K
30K
α1
α2
26

Figure 27 SDS-PAGE showing type I collagen in the control and 5-FU-treated developing secondary palate of quail embryos on day 8 of incubation.

Track 1. Molecular weight Marker (MW 14300-200000).
Track 2. Standard type I collagen showing α1 and α2 chains.
Track 3. Control secondary palate of quail embryo (Day 8).
Track 4. 5-FU-treated secondary palate of quail embryo (Day 8).



(27)

DISCUSSION

In the initial study, a dose-time relationship of the effect of 5-FU on palate development in quail was determined because this information was not available in the literature, and was crucial for subsequent studies on the effect of 5-FU on collagen synthesis in the developing palate of quail. The data of the present study show that following 5-FU treatment of quail eggs on day 4 of incubation, the rate of mortility in embryos increased. This may be due to a variability in the embryonic growth rates during early phases of development or it could be due to a possible genetic variability of susceptibility of embryos to 5-FU as well. During the first four days of incubation, the variability in HH stages, implying variabilities in growth rates of quail embryos is greater than at the later times of incubation (Pedgett and Ivey, 1960; Sato et al., 1971). Thus, developmentally older embryos may be relatively less vulnerable to the 5-FU assault than younger embryos on day 4 of incubation. Following various experimental manipulations during early phases of development, an increased rate of mortility of avian embryos has also been noted in other studies (Landauer, 1954, 1976; Karnofsky et al., 1958; Singh and Gupta, 1972; Ruddick and Runner, 1974; Grubb and Montiegel, 1975; Mann and Persaud, 1978; Jelinek and Kistler, 1981; Gilani and Chatzinoff, 1983). It has been indicated that during the early phases of development, embryos are generally highly vulnerable to the effects of environmental agents because rapidly dividing embryonic cells are more susceptible than non-dividing cells to an assault by an agent (Saxén, 1976; Scott, 1979). Perhaps this may explain an increase in mortility

rate following 5-FU administration on day 4 of incubation observed in the present study.

Results of the present study further showed that the treatment of quail eggs with 100μg 5-FU/egg on day 4 of incubation caused an increase in the distance between palatal shelves, eg, increased clefting of the palate. The drug showed little or no adverse effect on fetal weight or distance between palatal shelves when injected on day 5 or 6 of incubation. These observations are consistent with findings by Karnofsky et al (1958) and Ruddick and Runner (1974) who also observed increased cleft palate in chick embryos following 5-FU treatment. Hence, injection of 100μg/egg 5-FU on day 4 of incubation was used in the present study on subsequent experiments on quail.

The light microscopic observations of palate development in control hamster showed that the palatal shelves were vertical until day 12:00 of gestation. They reoriented and fused between days 12:00 and 12:04 of gestation. These observations of the development of hamster secondary palate in the present study are similar to those described earlier in normal hamster by Shah and Chaudhry (1974a), Shah and Travill (1976a), Shah (1979b), Shah and Wong (1980) and Kiso et al (1984). Thus, the temporal reproducibility of events of normal palate development is once again reconfirmed.

In the present study, like controls, the 5-FU-treated palatal shelves were vertical on day 12:00 of gestation. Subsequently, the reorientation of hamster embryonic palatal shelves occurred between days 12:16 and 13:00 of gestation, i.e., it was delayed by approximately 16-20 hours. Fusion between the shelves never occurred and a cleft palate formed. These observations are consistent with earlier

data on 5-FU induced cleft palate in hamster reported by Shah and Wong (1980) and Shah et al (1984). Also, on the basis of their study in mice, Arvystas and Cohen (1971) showed that 5-FU affects reorientation of shelves to induce cleft palate. A morphological mechanism of cleft palate formation by delayed reorientation of shelves has been observed following administration of different environmental agents in various mammalian species i.e., mice, rats, hamster, etc., (Table 1). On the other hand the morphological mechanism by which 5-FU-induces cleft palate in hamster differs from the mechanism of cleft palate development in hamster following treatment with other agents such as hydrocortisone, which prevent fusion between the palatal shelves (Shah and Travill, 1976a), and 6-mercaptopurine, hadacidine and bromodeoxyuridine, which affect vertical development of the shelves (Burdett and Shah, 1988; Burdett et al., 1988; Shah et al., 1990; 1991). Finally, treatment of mice with cortisone over a four day period (Walker and Fraser, 1957), or of hamster with a single dose of 5-FU (present study) produces cleft palate by delaying reorientation of palatal shelves while administration of a single dose of hydrocortisone to pregnant hamsters affects the fusion between shelves in embryos (Shah and Travill, 1976a, b). These analyses suggest that differences amongst species, the nature of teratogenic chemicals, and treatment schedule are some of the factors which may affect the morphological mechanism of cleft palate development in mammals.

The light microscopic observation of the development of secondary palate in control quail in the present study showed that, unlike hamster, the quail palatal shelves develop horizontally *ad initium* on day 5 of incubation, and grow toward one another until approximation on day 8 of incubation. Thereafter, the shelves

never fuse with one another. These observations of quail secondary palate development are similar to those described earlier by Shah et al (1985a). A similar mode of palate development was also observed in other birds, e.g., chick (Shah and Crawford, 1980; Koch and Smiley, 1981), duck (Shah et al., 1987), and pigeon (Shah et al., 1988).

Treatment of quail eggs with 5-FU resulted in an increased gap between the two horizontal palatal shelves. Numerous teratogenic agents which increases palate clefting in birds, have been identified (Landauer, 1954; Karnofsky et al., 1958; Ruddick and Runner, 1974; Verrett et al., 1980; Tamarin et al.,1984). There are, however, no studies in the literature which analyzed the morphological mechanism of teratogen-induced cleft of secondary palate in any species of bird. Consequently, the light microscopic morphological results of the present study on 5-FU-induced cleft palate in quail are difficult to compare. Nevertheless, it is clear that 5-FU affects growth of the shelves towards one another leading to widening of the palate cleft.

In the present study, a changing profile of uptake of ^{14}C-proline, reflecting collagen synthesis (discussed below), in the normally developing secondary palate of hamster embryos was observed. Specifically, at the time of shelf reorientation in hamster, the rate of ^{14}C-proline uptake showed a two-fold increase. Earlier Shapira (1969) and Shapira and Shoshan (1972) observed a two-fold increase of ^{3}H-proline uptake in the normally developing palatal shelves of mice **before shelf reorientation**. In their study on rats, Pratt and King (1971), noted that the amount of collagen doubled **at the time of palate closure**. Hassell and Orkin (1976) observed 82% increase in the rate of incorporation of radiolabelled proline in the

vertical shelf, **prior to shelf reorientation**. One of the drawbacks of these earlier studies on mice and rats is that the collagen synthesis was analyzed at 12-24 hour intervals. In such experimental circumstances, subtle changes in the rates of radiolabelled proline uptake, **during the shelf reorientation** (which in mammals occur very rapidly), are not localized. Nevertheless these observations suggest that, at least during mammalian palatogenesis, a spurt in the uptake of radiolabelled proline may be related to the reorientation of the palatal shelves.

Because collagen synthesis is generally measured by incorporation of proline into procollagen, and its subsequent hydroxylation to HYP, an amino acid which is virtually unique to collagen (Bornstein and Traub, 1979), determination of levels of HYP is considered to be an useful measure of collagen synthesis (Svenberg, 1987). Also, it is generally accepted that both synthesis and degradation of collagen in a tissue can be monitored by the release of HYP (Svenberg, 1987). One may note that in interpreting the experimental results of a study on collagen turnover, under normal circumstances, the actual amount of collagen present in any structure at a given time is the result of its synthetic and degradative rates (Neblock and Berg, 1987). In the steady state circumstances, the rate of synthesis equals the rate of degradation (Laurent, 1986). Degradation of collagen molecules can occur at both intracellular and extracellular sites (Laurent, 1986) and can be assessed from the appearance of radiolabelled HYP (Laurent, 1986; McAnulty and Laurent, 1987). Neblock and Berg (1987) suggested that 10-50% of all newly synthesized collagen could be degraded by cells within a short period of time. Thus, it is clear that the amount of HYP could be used as a measure of degradation and/or synthesis of collagen.

In the present study, HPLC was used to determine HYP content in the developing secondary palate. The results on control hamster palate showed that the amount of HYP between days 12:00 and 12:04 of gestation, eg., during the period of palatal shelf reorientation remained unchanged. Since, the uptake of ^{14}C-proline also doubles during the period of shelf reorientation, the simultaneous doubling in the HYP content could be interpreted to reflect the hydroxylation of proline to HYP at the time of reorientation thereby suggesting, in line of the previously held view (Svenberg, 1987; Neblock and Berg, 1987), an increase synthesis of new collagen. Earlier Shapira and Shoshan (1972) and Hassell and Orkin (1976), also suggested a synthesis of new collagen in mice and rats prior to achieving the reorientation of palatal shelf.

The quantitative observations of the present study on the normal palate development in quail showed a 4-fold increase in the rates of collagen synthesis between days 6 and 8 of incubation. This increase in the rate of collagen synthesis occurred in spite of the fact that the reorientation stage is absent during the development of palate in quail, as well as in other birds (Shah and Crawford, 1980; Koch and Smiley, 1981; Greene et al., 1983; Shah et al., 1985a, 1987, 1988).

HPLC data obtained from the developing secondary palate of quail indicated that, in contrast to the 4-fold increase observed in the rate of collagen synthesis, HYP content increased by only two-fold between days 6 and 8 of incubation. This may indicate that part of the synthesized collagen is rapidly degraded. No efforts were made during the course of present investigation to either determine the types and amounts of non-collagenous proteins or analyze their roles in quail palate development. It is, however, clear that, at the molecular level, there are

substantial differences in the types and amounts of proteins synthesized during avian and mammalian palate morphogenesis which, in turn, may relate to the shelf reorientation in mammals, or its lack in birds. This issue needs further investigation. Also, it is interesting that during palate development in both mammals and birds the increase in the amount of HYP was similar, eg., two-fold. This could, perhaps, suggest that proportionally, the actual amount of collagen synthesized in the developing palate of birds and mammals may be similar.

The foregoing analysis, along with the issue of palatal shelf reorientation discussed in the INTRODUCTION segment of this thesis, raises an interesting question: Does a spurt in the synthesis of new collagen in the developing secondary palate of mammals causes the shelf to reorient?

In the present study, this question was evaluated using a teratological approach. In the hamster, 5-FU delays the reorientation of shelves to induce a cleft palate (Shah and Wong, 1980; Shah et al., 1984; present study). It was thought that, if 5-FU affected collagen synthesis and if the collagen synthesis is critical for shelf reorientation, then a spurt in the collagen synthesis would be delayed to correspond with the 5-FU-induced delayed reorientation of the shelves.

Like controls, a changing profile of collagen synthesis was also observed in 5-FU-treated developing palate of hamster. Specifically, in the drug treated palates initially the proline uptake was suppressed. Subsequently, however, a 10-fold increase (cf. control 2-fold increase) in the rate of proline uptake was observed on day 12:12 of gestation (Figure 20), eg., at least four hours prior to the period of delayed reorientation. In an earlier study, it was shown that within six hours of drug administration, 5-FU injured a significant proportion of cells in the vertically

developing palate of hamster (Shah et al., 1984). In addition, following drug treatment, the expansion of the shelf volume was delayed (Shah et al., 1989c). The reorientation of shelves occurred only after the resolution of cellular damage on day 12:06 and subsequent restoration of shelf volume on day 12:12 of gestation. In their recent work, Young et al (1991b) have shown that following 5-FU administration, initial alteration in the synthesis of glycosaminoglycans was quickly restored prior to the time of normal shelf reorientation in hamster, and thus it did not appear to be related to the delayed reorientation. Hence, it is likely that altered cytodifferentiation due to 5-FU-induced cell injury (Shah et al., 1984) would in turn, affect collagen synthesis (present study), and consequently the expansion of the shelf volume (Shah et al., 1989c). A delay in the spurt of collagen synthesis following 5-FU treatment may thus be one of the event of the recovery phase of palate morphogenesis. During such a phase, a "catch-up" growth in the rate of collagen synthesis could contribute to procuring the volume of the developing shelf and prepare it for reorientation in the 5-FU assaulted embryos. There are no reports in the literature on the effects of 5-FU on collagen synthesis in any developing system. Also there is a paucity of studies analyzing drug-induced alterations in collagen synthesis during palate development. In the only *in vivo* study (Shapira and Shoshan, 1972), where drug (cortisone)-induced alterations in the rate of collagen synthesis were observed, the data showed a two-fold increase in collagen synthesis prior to the period of delayed reorientation. In the presence of glucocorticoids, an increased rate of collagen synthesis observed in cultured palatal tissue explants (Uitto and Thesleff, 1979), but the rate was suppressed when palatal cells were isolated and cultured (Sasaki and Kurisu, 1983). Notwithstanding the

different observations, which may be attributed to variations in *in vivo* and *in vitro* experimental approaches, one may suggest, on the basis of limited evidence available in the literature and along with the data of the present study, that perhaps a certain minimum amount of collagen accumulation in the developing palate may be necessary before reorientation of the palatal shelf could occur.

An accumulation of HYP was also observed in 5-FU-treated hamster palate. Interestingly, between days 12:00 and 12:04 of gestation, even though collagen synthesis in 5-FU-treated palate was depressed and the shelf reorientation delayed, the HYP levels, indicative of collagen content, were comparable to that of the controls. Subsequent spurt in collagen synthesis preceeded HYP accumulation and initiation of shelf reorientation. Indeed, in the drug treated palates, the spurt in HYP content corresponded with decrease in collagen synthesis. A high level of HYP thus could imply either an increased rate of synthesis of new collagen, or a decreased degradation of collagen (Laurent, 1986; Neblock and Berg 1987) due to drug treatment during palate development. In such circumstances, although a certain amount of collagen synthesis could still occur further morphogenesis of palate may be thwarted due to degradation of proteins reflected in high HYP content.

In comparison to that seen in the hamster, the profile of collagen synthesis in 5-FU-treated developing quail palate showed less dramatic changes than their control counterpart. Following drug treatment, a three-fold increase in the rate of collagen synthesis was observed in quail palate between days 7 and 8 of incubation. Also, the overall rate of collagen synthesis was lower in 5-FU-treated than in the control quail palates. Unlike recovery in the rate of collagen synthesis within 36

hours of 5-FU assault of hamster palate, however, the recovery in collagen synthesis, over a 5 day period, in the drug-treated quail palate was low. This could be due to a lack of placenta in birds. In the absence of placenta, since the quail embryo was directly exposed to the drug the resultant cellular response was probably severe and hence the recovery less pronounced than observed in hamster. Nevertheless, when the data on the rate of collagen synthesis and that of HYP accumulation in quail are analyzed together, it is clear that in spite of a significant reduction in the incorporation of ^{14}C-proline in the drug-treated quail palates the relative amount of HYP remained high in 5-FU exposed quail palates. As indicated for the hamster, the high level of HYP, in light of reduced ^{14}C-proline incorporation in the 5-FU-treated quail palate, may be interpreted to reflect increased degradation of collagen (Laurent, 1986, 1987; Neblock and Berg, 1987). This, however, need to be varified.

Since proline is an amino acid ubiquitous to most proteins (Miller, 1983), and since in developing tissues a great amount of proline gets incorporated into collagen via hydroxylation to HYP, a question, whether the profile of total proteins accumulation would follow that of collagenous protein, was examined. In the present study, we evaluated only the accumulation of total protein in both the control and 5-FU-treated developing palate of quail and hamster. The results indicated that although in the control palates of both species the protein accumulation increased during palate morphogenesis, it remained suppressed following 5-FU treatment in both vertebrates. Using a dose-time regimen for 5-FU treatment similar to the one employed in the present study, Ruddick and Runner (1974) also observed a suppression of protein synthesis in homogenates of chick

embryo. Unlike the observation of the present study, in which 5-FU treated palates did not show an increase in the protein accumulation, Ruddick and Runner (1974) observed a continuous but slow accumulation of protein in chick embryo homogenates. Clearly, following 5-FU treatment, individual structures, such as the palate, may show a different profile of protein accumulation in comparison to the whole embryo. Indeed, our results are consistent with the previously noted effect of 5-FU on reduced protein accumulation in various individual organs and tissues (Anand and Han, 1975; Cohen and Glazer, 1984; Sandborg and Siegel, 1990). Also, it is interesting that a recovery in collagen synthesis occurred in both the reorienting palate of hamster and non-reorienting palate of quail. This would reinforce the proposition made earlier that an accumulation of certain minimum amount of collagen in the developing palate may be one of the features preceeding shelf reorientation.

The mechanism by which 5-FU induces suppression of protein synthesis, or enhance its degradation is, however, not clear. At least during palate development in hamster 5-FU does not affect DNA synthesis (Shah et al., 1989c). A series of recent observations on various tissues, however, have shown that 5-FU may inhibit production of cytoplasmic ribosomal RNA (rRNA) which in turn could affect the protein synthesis (Hadjiolova et al., 1981; Cohen and Glazer, 1984; Iwata, 1986; Greenhalgh and Parish, 1989; Sandborg and Siegel, 1990). Hence, it would be interesting to examine if 5-FU assault of the developing secondary palate of vertebrates (a) suppresses the synthesis of rRNA and consequently of proteins to subsequently induce the cellular injury observed by Shah et al (1984), and (b) whether a recovery in rRNA synthesis precedes the "catch-up" growth observed

during hamster and quail palate morphogenesis. In such circumstances post-translational accumulation of newly synthesized collagen, as well other proteins, would be crucial for the advancement of palate morphogenesis.

The analysis of the results of the present study, along with the data from the literature, clearly suggest that since (a) in birds, a spurt in collagen synthesis occurs in the absence of shelf reorientation, (b) following 5-FU assault a recovery of collagen synthesis occurs four hours prior to initiation of shelf reorientation, and a reduction occurs at the time of reorientation, a spurt in collagen synthesis may not be critical for causing mammalian palatal shelf reorientation. On the other hand, it is plausible that an increasing collagen synthesis during vertebrates' palate morphogenesis may play a role hitherto not considered in the literature.

Although collagen has been implicated to play a role in the development of many structures and organs, its precise role during morphogenesis has begun to emerge only during the recent years. For example, it has been indicated that both types I and III collagen acts as stabilizing molecules during initiation and maintenance of the branching pattern of the salivary glands (Kallman and Grobstein, 1966; Spooner and Faubion, 1980; Nakanishi et al., 1988). On the other hand, Kratochwil et al (1986) and Chen and Little (1987) suggested that collagen type I has no role in the branching morphogenesis since type I can be fully replaced by type III, IV and V collagen to act as an extracellular scaffold within which a glandular parenchyme may branch. Also, during initial healing of wound type III collagen is synthesized which, as the subsequent maturation process of wound progresses, is replaced by type I collagen (Gay et al., 1978; Alvarez, 1987). In this circumstances, type I collagen provides a lattice network to stabilize the wound and

thus could facilitate proliferation and migration of endothelial and fibroblastic cells (Gay et al., 1978; Maciag et al., 1982; Kramer et al., 1983; Alvarez, 1987). During the morphogenesis of the limb, a structure which, like the secondary palate, grows outward from the body surface, increasing synthesis of type I collagen has been related to cell orientation and arrangement (Trelstad and Hayshi, 1979; Hurle et al., 1989) Synthesis of type II collagen on the other hand appears to be related to cartilage development (Devlin et al., 1988). As observed in the present study, as well as noted by previous investigators (Shapira, 1969; Pratt and King, 1971; Shapira and Shoshan, 1972; Silver et al., 1984) the developing secondary palates of mammals show type I collagen during their vertical growth and reorientation stages. Type III collagen was observed *in vivo* only in the vertical shelf (Silver et al., 1981), or in *in vitro* circumstances (Uitto and Thesleff, 1979; Sasaki and Kurisu, 1983). It thus appears that type I is the predominent collagen isoform synthesized in the developing palate of both mammals and bird (present study). Since type I collagen is important for providing rigidity to the ECM (Bornstein and Sage, 1980), and in the developing structures such as salivary gland, limb, lung (references cited above) and during wound healing for providing a stabilizing network on which cells can proliferate, migrate and rearrange themselves, it is plausible that during the formation of vertebrate secondary palate, the type I collagen may contribute both to the shelf volume, as well as to the rigidity of the substratum on which cells may perform their various functions. In this manner, a continued synthesis of collagen would serve an important biological function during the morphogenesis of the vertebrates' secondary palate.

SUMMARY AND CONCLUSION

1. A single injection of 100μg 5-FU in 0.1ml saline into the air sac of quail eggs on day 4 of incubation increased the gap between the two palatal shelves to increase palatal clefting
2. A single intramuscular injection of 81mg/kg 5-FU in 1ml saline on day 11:00 of gestation in hamster delayed the reorientation of palatal shelves by 16-20 hours and induced cleft palate.
3. The rate of collagen synthesis changed during vertebrate palatogenesis. During morphogenesis of the palate in hamster control, the rate of collagen synthesis peaked between days 12:00 and 12:04 of gestation, i.e., during shelf reorientation. 5-FU treatment initially suppressed the collagen synthesis. Later, during the recovery phase, it peaked in the drug treated palates on day 12:12 of gestation, eg., just before reorientation. The shelf reorientation in 5-FU-treated palates began on day 12:16 of gestation at which time the collagen synthesis was low.
4. During morphogenesis of palate in control quail, in spite of an absence of the reorientation stage, the rate of collagen synthesis peaked on day 8 of incubation. Also, following 5-FU treatment, the rate of collagen synthesis peaked on day 8 of incubation but, unlike hamster, the recovery was incomplete.
5. HPLC results showed that the rates of conversion of proline to HYP, indicating the content of collagen in both the control and 5-FU-treated hamster palates were similar and remained unchanged between days 12:00

and 12:04 of gestation. However, in 5-FU-treated palates it increased substantially on day 12:16 and 13:00 of gestation, when shelves were reorienting.

6. In control palate of quail the amount of HYP was higher on day 8 than on day 6 of incubation which was consistent with the rate of collagen content. Low accumulation of HYP on days 7 and 9 of incubation, relative to the rate of collagen synthesis, may suggest significant synthesis of non-collagenous proteins during quail palate development. In 5-FU treated quail palates, the amount of HYP is reduced on both day 6 and 8, but not on days 7 and 9 of incubation.

7. The amount of total protein in the developing secondary palate in hamster doubled between days 11:04 and 12:04 of gestation, i.e., during the period when shelf completed their vertical growth and reoriented to a horizontal plane and fused. The total protein content in 5-FU treated hamster embryonic palates were approximately 50-75% lower than control palates .

8. In the control palates of quail embryos, between days 5 and 10 of incubation there was a gradual increase in the amount of total protein. In comparison, the total protein content in 5-FU treated palates was reduced by 50-70%.

9. Using SDS-PAGE, type I collagen was identified in the developing secondary palates of both control and 5-FU-treated hamster and quail.

10. The analysis of the results of the present study, along with the data from the literature, indicates that since (a) in birds, a spurt in collagen synthesis occurs in the absence of shelf reorientation, (b) an equal amount of new collagen was synthesized in both the control and 5-FU-treated hamster embryos

during the period of normal reorientation, and (c) in 5-FU-treated hamster embryos, a recovery in collagen synthesis occurs prior to, and a reduction occurs at the time of delayed shelf reorientations, the results suggest that collagen synthesis may not cause shelf reorientation in mammals.

11. Synthesis of type I collagen during vertebrate's palatogenesis may contribute toward the volume of the developing shelf and may provide structural rigidity to maintain the shelf for further morphogenesis.

REFERENCES

Ads, A.H., Piddington, R., Goldman, A.S., and Herold, R., 1983. Cortisol inhibition of development of various lysosomal enzymes in cultured palatal shelves from mouse embryos. Archs. Oral Biol.,28: 1115-1119.

Alvarez, M. O., 1987. Pharmacological and Environmental Modulation of Wound Healing. In: "Connective Tissue Disease, Molecular Pathology of Extracellular Matrix." (J. Uitto and A. J. Perejda, eds.), Dekker, New York, pp. 267-397.

Anand, C., and Han, S., 1975. Effects of 5-fluorouracil on exocrine glands: III. Fine structure of brunner's glands of rats. J. Anat., 119: 1-17.

Andersen, H., and Matthiessen, M., 1967. Histochemistry of the early development of the human central face and nasal cavity with special reference to the movements and fusion of the palatine processes. Acta Anat., 8: 473-508.

Arvystas, M.G., and Cohen, M.M., 1971. Effects of 5-Fluorouracil on secondary palate development of 129/J mice. J. Dent. Res., 50: 66-71.

Asling, C.W., Nelson, M.M., Dougherty, H.D., Wright, H.V., Evans, H.M., 1960. Development of cleft palate resulting from maternal pterylglutamic (folic) acid deficiency during the latter half of gestation in rats. Surg. Gynec. Obst., 111: 19-28.

Babiarz, B.S., Allenspach, A.L., and Zimmerman, E.F., 1975. Ultrastructural evidence of contractile systems in mouse palates prior to rotation. Dev. Biol., 47: 32-44.

Bard, J.B., Bansal, M.K., and Ross, A.S., 1988. The extracellular matrix of the developing cornea: diversity, deposition and function. Development, 103 (Suppl): 195-205.

Baxter, H., and Fraser, F.C., 1950. The production of congenital defects in the offspring of female mice treated with cortisone. McGill Med. J., 19: 245-249.

Bekhor, I., Mirell, C., and Anne, L., 1978. Induction of cleft palate by triamcinolone acetonide: Re-examination of the problem. Cleft Palate J., 15: 220-232.

Bhatnagar, R.S., and Rapaka, R.S., 1975. Polypeptide models of collagen: Properties of (Pro-Pro-betaA1a)n. Biopolymers, 14: 597-603.

Bornstein, P., and Sage, H., 1980. Structurally distinct collagen types. Ann. Rev. Biochem., 49: 957-1003.

Bornstein, P., and Traub, W., 1979. The chemistry and Biology of collagen. In: The Proteins. (H. Neurath and R.L. Hill, eds.) Vol. 4. Academic Press, New York, pp. 411-432.

Brinkley, L., 1980. *In vitro* studies of palatal shelf elevation. In: Current Research Trends in Prenatal Craniofacial Development. (R.M. Pratt and R.C. Christiansen, eds.), Elsevier/North-Holland, New York, pp. 203-220.

Brinkley, L., and Morris-Wiman, J., 1984. The role of extracellular matrices in palatal shelf closure. Curr. Top. Dev. Biol., 19: 17-36.

Burdett, D.N., and Shah, R.M., 1988. Gross and cellular analysis of 6-mercaptopurine-induced cleft palate in hamster. Am. J. Anat., 181: 179-194.

Burdett, D.N., Waterfield, J.D., and Shah, R.M., 1988. Vertical development of the secondary palate in hamster embryos following exposure to 6-mercaptopurine. Teratology, 37: 591-597.

Burgeson, R.E., 1982. Genetic heterogeneity of collagens. J. Invest. Dermat., 79 (Suppl 1): 25s-30s.

Burgeson, R.E., 1988. New collagens, new concepts. Ann. Rev. Cell Biol., 4: 551-577.

Burgeson, R.E., El Adli, F.A., Kaitila, I.I., and Hollister, D.W., 1976. Fetal membrane collgens: identification of two new collagen alpha chains. Proc. Nat. Acad. Sci. (USA), 73: 2579-83.

Burgeson, R.E., and Morris, N.P., 1987. The Collagen Family of Proteins. In: "Connective Tissue Diseases, Molecular Pathology of Extracellular Matrix." (J. Uitto and A.J. Perejda, eds.), Dekker, New York, pp. 3-28.

Callas, G., and Walker, B.E., 1963. Palate morphogenesis in mouse embryos after x-irradiation. Anat. Rec., 145: 61-71.

Carrico, C.K., and Glazer, R.I., 1979. Augmentation by thymidine of the incorporation and distribution of 5-fluorouracil into ribsomal RNA. Biochem. Biophys. Res. Comm., 87: 664-670.

Chabner, B.A., Myers, C.E., Coleman, C.N., and Johns, D.G., 1975. The Clinical pharmacology of antineoplastic agents (First of two parts). New Engl. J. Med., 292: 1107-1113.

Chabner, B.A., 1982. Pyrimidine antagonists. In: Pharmacological principles of cancer treatment. (Chabner, B.A., ed.) W.B. Sanders Co., Philadelphia, pp. 183-212.

Chaudhry, A.P., and Shah, R.M., 1973. Palatogenesis in hamster. II. Ultrastructural observations on the closure of palate. J. Morph., 139: 329-350.

Chaudhry, A.P., and Shah, R.M., 1979. Light and electron microscopic observations on closure of the secondary palate with the primary palate and the nasal septum. Acta Anat., 103: 384-394.

Chaudhuri, N.K., Montag, B.J and Heidelberger, C., 1958. Studies on fluorinated pyrimidines III. The metabolism of 5-fluorouracil-2-C^{14} and 5-fluoroorotic-2-C^{14} acid in vivo. Cancer Res., 18: 318-328.

Chepenik, K.P., Ho, W.C., Waite, B.M., and Parker, C.L., 1984. Arachidonate metabolism during chondrogenesis in vitro. Calcified Tissue Internat., 36: 175-181.

Chen, J.M., and Little, C.D., 1987. Cellular events associated with lung branching morphogenesis including the deposition of collagen type IV. Dev. Biol., 120: 311-21.

Chung, E., and Miller, E.J., 1974. Collagen polymorphism: Characterization of molecules with the chain composition $[\alpha 1(III)]_3$ in human tissues. Science, 183: 1200-1201.

Cleaton-Jones, P., 1976. Radioautographic study of mesenchymal cell activity in the secondary palate of the rat. J. Dent. Res., 55: 437-40.

Cohen, M., and Glazer, R., 1984. Cytotoxicity and the inhibition of ribosomal RNA processing in human colon carcinoma cells. Molec. Pharm., 27: 308-313.

Coleman, R.D., 1965. Development of the rat palate. Anat. Rec., 151: 107-118.

Dagg, C.P., 1960. Sensitive stages for the production of developmental abnormalities in mice with 5-fluorouracil. Am. J. Anat., 106: 89-96.

Dagg, C.P., Schlager, G., and Doerr, A., 1966. Polygenic control of the teratogenicity of 5-fluorouracil in mice. Genetics, 53: 1101-1117.

Danenberg, P.V. and Danenberg, K.D., 1978. Effect of 5,10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2'-deoxyuridylate from thymidylate synthetase: evidence for an ordered mechanism. Biochemistry, 17: 4018-4024.

DeVita, V.T., 1985. Principles of Chemotherapy. In: "Cancer, Principles and Practice of Oncology". (DeVita, V. T., Hellman, S., Rosenberg, S. A,. eds.), 2nd ed., JB Lippincott Company, Philadelphia, pp. 257-258.

Diasio, R.B., and Harris, B.E., 1989. Clinical Pharmacology of 5-fluorouracil. Clin. Phamacokinetics 16: 215-237.

Diewert, V.M., 1976. Graphic reconstructions of craniofacial structures during secondary palate development in rats. Teratology, 13: 291-314.

Diewert, V.M., 1979. Correlation between mandibular retrognathia and induction of cleft palate with 6-aminonicotinamide in the rat. Teratology, 19: 213-227.

Diewert, V.M., and Pratt, R.M., 1979. Selective inhibition of mandibular growth and induction of cleft palate by diazo-oxo-norleucine (DON) in the rat. Teratology, 20: 37-52.

Dostal , M., and Jelinek, R., 1970. The morphogenesis of cleft palate induced by exogenous factors. II. Induction of cleft by cortisone in random bred mice. Acta Chirurg. Plast., 12: 206-208.

Dostal, M., and Jelinek, R., 1972. Morphogenesis of cleft palate induced by exogenous factors V. Quantitative study of the process of palatal closure of different strains of mice. Folia Morph., 20: 362-374.

Dursy, E., 1869. Zur Entwicklungsgeschichte des kopfes des menshen und der hoheren wirbeltiere. Verlag der H. Lauppschen Buchhandlung, Tubingen.

Duschinsky, R., and Pleven, E., 1957. The synthesis of 5-fluoropyrimidines. J. Am. Chem. Soc., 79: 4559-4560.

Engvall, E., Ruoslahti, E., and Miller, E.J., 1978. Affinity of fibronectin to collagens of different genetic types and to fibrinogen. J. Exp. Med., 147: 1584-95.

Erickson, R., Butley, M., and Sing, C., 1979. H-2 and non-H-2 determined strain variation in palatal shelf and tongue adenosine 3′ : 5′ cyclic monophosphate. J. Immunogenet., 6: 253-262.

Evans, A.N., Brooke, O.G., and West, R.J., 1980. The ingestion by pregnant women of substances toxic to the fetus. Practitioner, 224: 315-319.

Ferguson, M.W.J., 1977. The mechanism of palatal shelf elevation and the pathogenesis of cleft palate. Virchows Arch. A. Path. Anat. Histol., 375: 97-113.

Ferguson, M., 1978. Palatal shelf elevation in the Wistar rat. J. Anat., 125: 555-577.

Ferguson, M. W. J., 1981. The structure and development of the palate in alligator mississippiensis. Arch. Oral Biol., 26: 427-443.

Fessler, J.H., and Fessler, L.I., 1978. Biosynthesis of procollagen. Ann. Rev. Biochem., 47: 129-162.

Fitzpatrick, D.R., Denhez, F., Kondaiah, P. and Akhurst, A. J., 1990. Differential expression of TGF-β isoforms in murine palatogenesis. Development, 109: 585-595.

Fleischmajer, R., Pearlish, J.S., Krieg, T., and Timpl, R., 1981. Variability of collgen and fibronectin synthesis by scleroderma fibroblasts in primary culture. J. Invest. Dermat., 76: 400-403.

Folkman, J., and Tucker, R. W., 1980. Cell configuration, substratum and growth control. In: "the Cell Surface: Mediator of Developmental Processes." (S. Subtelny and N. K. Wessells, eds.), Academic Press, New York, pp. 259-275.

Forsthoefel, P.F., Blend, M.J., and Snow, J.W., 1978. Biochemical aspects of the interactions of the teratogens 5-fluorouracil and 5-fluorodeoxyuridine with normal nucleic acid precursors in mice selected for low and high expression of strong's luxoid gene. Teratology, 18: 269-276.

Frommer, J., and Monroe, C.W., 1969. Further evidence for the absence of elastic fibers during movement of the palatal shelves in mice. J. Dent. Res., 48: 155.

Fukuda, Y., Masuda, Y., Kishi, J., Hashimoto, Y., Hayakawa, T., Nogawa, H., and Nakanishi, Y., 1988. The role of interstitial collagens in cleft formation of mouse embryonic submandibular gland during initial branching. Development, 103: 259-267.

Gay, S., Rhodes, R.K., Gay, R.E., and Miller, E.J., 1981. Collagen molecules comprised of a 1(V)-chains (b-chains): an apparent localization in the exocytoskeleton. Collagen Relat. Res., 1: 53-58.

Gay, S., Viljanto, J., Raekallio, J., and Pentinen, R., 1978. Collagen types in early phases of wound healing in childern. Acta Chir. Scand., 144: 205-211.

Gehris, A.L., D'angelo, M., and Greene, R.M., 1991. Immunodetection of the transforming growth factors β_1 and β_2 in the developing murine palate. Int. J. Dev. Biol., 35: 17-24.

George, M, and Chepenik, K., 1985. Phospholipase A activities in embryonic palate mesenchyme cells *in vitro*. Biochem. Biophys. Acta., 836: 45-55.

Gilani, S.H., and Chatzinoff, M., 1983. Embryopathic effects of cyclophosphamide. Environmental Res., 31: 296-301.

Goldman, A.S., Herold, R., and Piddington, R., 1981. Inhibition of programmed cell death in the fetal palate by cortisol. Proc. Soc. Exp. Biol. Med., 166: 418-24.

Goldman, A., Shapiro, B., and Katsumata, M., 1978. Human fetal palatal corticoid receptors and teratogens for cleft palate. Nature (London), 272: 464-466.

Gornall, A.G., Bordawill, C.J., and David, M.M., 1948. Determination of serum protein by means of the biuret reagent. J. Biol. Chem., 177: 751-766.

Goss, A.N., 1977. Post-fusion cleft of the fetal rat palate. Cleft palate J., 14: 131-139.

Graham, D.L., and Meier, G., 1975. Standard morphological development of the quail, *Coturnix coturnix japonica* embryo. Growth, 39: 389-400.

Greene, R. M., 1989. Signal transduction during craniofacial development. Crit. Rev. Toxic., 20: 137-152.

Greene, R., and Garbarino, M., 1984. Role of cyclic AMP, Prostaglandins and Catecholamines during normal palate development. In: "Current Topics in Developmental Biology". (Zimmerman, E., ed.), Academic Press, New York, 65-79.

Greene, R.M., LIoyd, M.R., and Nicolaou, K.C., 1981. Agonist-specific desensitization of prostaglandin stimulated cyclic AMP accumulation in palatal mesenchymal cells. J. Craniofac. Genet. Dev. Biol., 1: 261-272.

Greene, R.M., Linask, K.K., Pisano, M.M., and LIoyd, M.R., 1989. Characterization of soluble cyclic adenosine monophosphate-dependant protein kinase isozymes in murine embryonic palatal tissue. J. Craniofac. Genet. Devel. Biol., 9: 207-222.

Greene, R., McAndrew, V., and LIoyd, M., 1982. Stimulation of palatal glycosaminoglycan synthesis by cyclic AMP. Biochem. Biophys. Res. Comm., 130: 232-238.

Greene, R.M., and Pratt, R.M., 1979. Correlation between cyclic AMP levels and cytochemical localization of adenylate cyclase during development of the secondary palate. J. Histochem. Cytochem., 27: 924-931.

Greene, R.M., Shah, R.M., Lloyd, M.R., Crawford. B.J., Suen, R., Shanfeld, J.L., and Davidovitcch, Z., 1983. Differentiation of the Avian Secondary Palate. J. Exp. Zool., 225: 43-52.

Greenhalgh, D.A., and Parish, J.H., 1989. Effects of 5-fluorouracil on cytotoxicity and RNA metabolism in human colonic carcinoma cells. Cancer Chemoth. Pharmac., 25: 37-44.

Grobstein, C., and Cohen, J., 1965. Collagenase: effect on the morphogenesis of embryonic salivary epithelium in vitro. Science, 150: 626-628.

Gross, J., 1974. Collagen biology: structure, degradation, and disease. Harvey Lectures, 68: 351-432.

Grubb, R.B., and Montiegel, E.L., 1975. The teratogenic effects of 6-mercaptopurine on chick embryos in ovo. Teratology, 11: 179-185.

Gulamhusein, A.P., and England, M.A., 1982. The developing ferret palate. A scanning electron microscope study: 1. Primary palate and secondary palatal shelves. J. Craniofacial Genet. Dev. Biol., 2: 107-123.

Gupta, C., Katsumata, M., Goldman, A.S., Herold, R., and Piddington, R., 1984. Glucocorticoid-induced phospholipase A2-inhibitory proteins mediate glucocorticoid teratogenicity in vitro. Proc. Nat. Acad. Sci. (USA), 81: 1140-1143.

Hadjiolova, K.V., Naydenova, Z.G., and Hadjiolov, A.A., 1981. Inhibition of ribosomal RNA maturation in Friend erythroleukemia cells by 5-fluorouridine and toyocamycin. Biochemical Pharmacology, 30: 1861-1863.

Hamburger, V., and Hamilton, H., 1951. A series of normal stages in the development of the chick embryo. J. Morph., 88: 49-92.

Hassell, J.R., 1975. An ultrastructural analysis of the inhibition of epithelial cell death and palate fusion by epidermal growth factor. Dev. Biol., 45: 90-102.

Hassell, J.R., and Orkin, R.W., 1976. Synthesis and distribution of collagen in the rat palate during shelf elevation. Dev. Biol., 49: 80-88.

Hassell, J.R., and Pratt, R.M., 1977. Elevated level of cAMP alter the effect of epidermal growth factor *in vitro* on programmed cell death in secondary palatal epithelium. Exp. Cell Res., 106: 55-62.

Heidelberger, C., 1983. Initiation, promotion, transformation, and mutagenesis of mouse embryo cell lines. Progress Clinic. Biol. Res., 132B: 83-89.

Heidelberger, C., Chaudhuri, N.K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R.J., Pleven, E., and Scheiner, J., 1957. Fluorinated pyrimidines, a new class of tumor-inhibitory compounds. Nature, 179: 663-666.

Heidelberger, C., Danenberg, P.V., and Morgan, R.G., 1983. Fluorinated pyrimidines and their Nucleosides. Adv. Enzym. Relat. Areas Molec. Biol., 54: 58-119.

Heine, U., Munoz, E., Flanders, K., Ellingsworth, L., Lam, H-Y., Thompson, N., Roberts, A., And Sporn, M., 1987. Role of transforming growth factor-β in the development of the mouse embryo. J. Cell Biol., 105: 2861.

Herold, R.C., and Futran, N., 1980. Effect of cortisol on medial edge epithelium of organ-cultured single palatal shelves from steroid-susceptible mouse strains. Arch. Oral Biol., 25: 423-429.

His, W., 1901. Beobachtungen zur Geschichte der Nasen-und Gaumenbildung beim menschlichen Embryo. Abhdlng Math-phys Class Konig Sachs Gesellsch Wissenschaft, 27: 349-389.

Holmstedt, J.O.V., and Bagwell, J.N., 1977. Morphogenesis of the secondary palate in the mongolian gerbil (*Meriones unguiculatus*). Acta Anat., 97: 443-449.

Hudson, C., and Shapiro, B.L., 1973. A radioautographic study of deoxyribonucleic acid synthesis in embryonic rat palatal shelf epithelium with reference to the concept of programmed cell death. Arch. Oral Biol., 18: 77-81.

Hughes, L.V., Furstman, L., and Bernick, S., 1967. Prenatal development of the rat palate. J. Dent. Res., 46: 373-379.

Humphrey, T., 1971. Development of oral and facial motor mechanisms in human fetuses and their relation to craniofacial growth. J. Dent. Res., 50: 1428-1441.

Hurle, J. M., Hinchliffe, J. R., Ros, M. A., Critchlow, M. A., and Genis-Galvez, J. M., 1989. The extracellular matrix architecture relating to myotendinous pattern formation in the distal part of the developing chick limb: an ultrastructural. histochemical and immunocytochemical analysis. Cell Differ. Dev., 27: 103-120.

IARC, 1981. IARC monographs on the evaluation of carcinogenic risk of chemicals to humans. Vol. 26. 5-Fluorouracil, pp. 217-235.

Imagawa, S., Tsuge, K., and Watari, S., 1979. Morphogenesis of 5-fluorouracil induced symbrachydactyly in mice. Teratology, 20: Abstract 155.

Ingraham, H.A., Tseng, B.Y., and Goulian, M., 1980. Mechanism for exclusion of 5-fluorouracil from DNA. Cancer Res., 40: 998-1001.

Inouye, M., 1912. Die Entwickelung der Sekundaeren Gaumens Einiger Saeugetiere. Anat. Hefte, 46: 5-187.

Iwata, T., Watanabe, T., and Kufe, D. W., 1986. Effects of 5-fluorouracil on globin mRNA synthesis in murine erythroleukemia cells. Biochemistry, 25: 2703-2707.

Jackson, D.S., 1979. Cell proliferation and collagen metabolism. Agents and Actions (Suppl.), 5: 9-23.

Jacobs, R.M., 1964. Histochemical study of morphogenesis and teratogenesis of the palate in mouse embryos. Anat. Rec., 149: 691-698.

Jacobson, B., and Shah, R.M., 1981. The glycosaminoglycan composition of fetal palate from normal and hydrocortisone treated hamster. Teratology, 23: 42a-43a.

Jelinek, R., and Dostal, M., 1974. Morphogenesis of cleft palate induced by exogenous factors. VII. Mitotic activity during the formation of the mouse secondary palate. Folia Morph., 22: 94-101.

Jelinek, R., and Kistler, A., 1981. Effect of retinoic acid uppon the chick embryonic morphogenetic systems. I. The embryotoxicity dose range. Teratology, 23: 191-195.

Kallman, F., and Grobstein, C., 1966. Localization of glucosamine-incorporating materials at epithelial surfaces during salivary epithelial-mesenchymal interactions in vitro. Dev. Biol., 14: 52-67.

Karnofsky, D.A., Murphy, M.L., and Lacon, C.R., 1958. Comparative toxicologic and teratogenic effects of 5-fluoro-substituted pyrimidines in the chick embryo and pregnant rat. Proc. Am. Assoc. Cancer Res., 2 (Abstr.): 312.

Kessel, D., 1980. Cell surfsce alterations associated with exposure of leukemia L1210 cells to fluorouracil. Cancer Res., 40: 322-324.

Kessel, D., Hall, T.C., and Wodinsky, I., 1966. Nucleotide formation as a determinant of 5-fluorouracil response in mouse leukemias. Science, 154: 911-913.

Kiso, Y., Nishida, T., and Mochizuki, K., 1984. Morphogenesis of cleft palate induced by cortisone in hamster. Jap. J. Vet. Sci., 46: 115-118.

Kitamura, H., 1966. Epithelial remnants and pearls in the secondary palate in the human abortus: A contribution to the study of the mechanism of cleft palate formation. Cleft Palate J., 3: 240-256.

Kitamura, H., 1991. Evidence for cleft palate as a postfusion phenomenon. Cleft Palate Craniofac. J., 28: 195-210.

Klebe, R.J., 1974. Isolation of a collagen-dependent cell attachment factor. Nature (London), 250: 248-251.

Kleinman, H.K., Klebe, R.J., and Martin, G.R., 198a. Role of collagenous matrices in the adhesion and growth of cells. J. Cell Biol., 88: 473-485.

Kleinman, H,K., McGarvey, M.L., Hassell, J.R., Star, V.L., Cannon, F.B., Laurie, G.W., and Martin, G.R., 1986. Basement membrane complexes with biological activity. Biochemistry, 25: 312-318.

Koch, W., and Smiley, G.R., 1981. In vivo and in vitro studies of the development of the avian secondary palate. Arch. Oral Biol., 26: 181-189.

Kochhar, D.M., and Johnson, E.M., 1965. Morphological and autoradiographic studies of cleft palate induced in rat embryos by maternal hypervitaminosis A. J. Embryol. Exp. Morphol., 14: 223-238.

Kramer, R., Davison, P., and Karasek, M., 1983. Characterization of a basement membrane matrix synthesized by Cultured human microvascular endothelial cells. J. Invest. Dermatol., 8: 341 (abstract).

Kratochwil, K., Dziadek, M., Löhler, J., and Harbers, K., 1986. Normal epithelial branching morphogenesis in the absence of collagen I. Dev. Biol., 117: 596-606.

Krawczyk, W.S., and Gillon, D.G., 1976. Immunofluorescent detection of actin in non-muscle cells of the developing mouse palatal shelf. Arch. Oral Biol., 21: 503-508.

Kromka, H. and Hoar, R.M., 1973. Use of guinea pigs in teratological investigations. Teratology, 7: 21-22 (Abstract).

Kurisu, K., Ohsaki, Y., Nagata, K., Kukita, T., Yoshikawa, H., and Inai, T., 1987. Immunocytochemical demonstration of simultaneous synthesis of type I, III and V collagen and fibronectin in mouse embryonic palatal mesenchymal cells in vitro. Collagen Rel. Res., 7: 333-340.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680-685.

Landauer, W., 1954. On chemical production of developmental abnormalities and of phenocopies in chicken embryos. J. Cell Comp. Physiol. (suppl. 1), 43: 261-305.

Landauer, W., 1975. Cholinomimetic teratogens. II. Studies with chicken embryos. Teratology, 12: 125-146.

Landauer, W., 1976. Cholinomimetic teratogens. III. Interaction with amino acids known as neurotransmitters. Teratology, 13: 41-46.

Larsson, K.S., 1962. Studies on the closure of the secondary palate. III. Autoradiographic and histochemical studies in the normal mouse embryo. Acta Morph. Neer. Scand., 4: 349-367.

Lassard, J., Wee, E., and Zimmerman, E.F., 1974. Presence of contractile proteins in mouse fetal palate prior to shelf elevation. Teratology, 9: 113-126.

Laurent, G.J., 1986. Lung collagen: more than scaffolding. Thorax, 41: 418-428.

Lazzaro, C., 1940. Sul meccanismo dichiusura del palato secondario. Monit. Zool. Ital., 51: 249-273.

LeCluyse, E.L., Frost, S.K., and Dentler, W.L., 1985. Development and ciliation of the palate in two frogs, Bambina and Zenopus; comparative study. Tissue Cell, 17: 853-864.

Liss, R.H., and Chadwick, M., 1974. Correlation of 5-fluorouracil (NSC-19893) distribution in rodents with toxicity and chemotherapy in man. Cancer Chemotherap. Rep., 58, 777-786.

Luke, D.A., 1989. Cell proliferation in palatal processes and Meckel's cartilage during development of the secondary palate in the mouse. J. Anat., 165: 151-158.

Maciag, T., Radish, J., Wilkins, L., Sternerman, M. B., and Weinstein, R., 1982. Cell Biol., 94: 511-520.

Mandel, H. G., 1969. Summary of informal discussion on the basis for future approach to cancer chemotherapy. Cancer Res., 29: 2475-2477.

Mann, R.A., and Persaud, T.V., 1978. Teratogenic effect of windowing eggs at early stages of avian development. Experimentelle Pathologie, 15: 324-334.

Martinez-Hernandez, A., and Amenta, P.S., 1983. The basement membrane in pathology. Lab. Invest., 48: 656-77.

Mato, M., Aikawa, E., and Katahira, M., 1966. Appearnce of various types of lysosomes in the epithelium covering lateral palatine shelves during a secondary palate formation. Gunma J. Med. Sci., 15: 46-56.

Maybaum, J., Klein, F.K., and Sadee, W., 1980. Determination of pyrimidine ribotide and deoxyribotide pools in cultured cells and mouse liver by high-performance liquid chromatography. J. Chromato., 25: 149-58.

Mayne, R., and Von Der Mark, K., 1983. Collagens of cartilage. In: Cartilage (B. K. Hall, ed.) Vol. 1, Academic Press, New York, pp. 181-214.

McAnulty, R.J., and Laurent, G.J., 1987. Collagen synthesis and degradation in vivo. Evidence for rapid rates of collagen turnover with extensive degradation of newly synthesized collagen in tissues of the adult rat. Collagen & Related Res., 7: 93-104.

Miller, E., 1971. The metabolism and pharmacology of 5-fluorouracil. J. Surg. Oncol., 3: 309-315.

Miller, E.J., 1976. Biochemical characteristics and biological significance of the genetically-distinct collagens. Molecular and Cellular Biochem., 13: 165-192.

Miller, E. J., 1983. The structure of collagen. Monog. Pathol., 24: 4-15.

Miller, E.J., and Matukas, V.J., 1974. Biosynthesis of collagen. Fed. Proc., 33: 1205-1218.

Montenegro, M.A., Cubillo, P., and Palomino, H., 1976. Induction of cleft palate in ball mice: the comparative effect of 5-fluorouracil, phenylbutazone and water and food deprivation. Revista Medica De Chile, 104: 606-609.

Montenegro, M.A., and Paz de la Vega, Y., 1982. Light and electron microscopic study on the effect of phenylbutazone on developing mouse palatal epithelium in vitro. Arch. Oral Biol., 27: 771-775.

Mott, W.J., Toto, P.D., and Hilgers, D.C., 1969. Labelling index and cellular density in palatine shelves of cleft palate mice. J. Dent. Res., 48: 263-265.

Mukherjee, K.L. and Heidelberger, C.,1960. Studies on fluorinated pyrimidines IX. The degradation of 5-fluorouracil-6-C^{14}. J. Biol. Chem., 235: 433-437.

Nakanishi, Y., Nogawa, H., Hashimoto, Y., Kishi, J., and Hayakawa, T., 1988. Accumulation of collagen III at the cleft points of developing mouse submandibular epithelium. Development, 104: 51-59.

Nakanishi, Y., Sugiura, F., Kishi, J., and Hayakawa, T., 1986a. Collagenase inhibitor stimulates cleft formation during early morphogenesis of mouse salivary gland. Dev. Biol., 113: 201-206.

Nakanishi, Y., Sugiura, F., Kishi, J., and Hayakawa, T., 1986b. Local effects of implanted Elvax chips containing collagenase inhibitor and bacterial collagenase on branching morphogenesis of mouse embryonic submandibular glands in vitro. Zool. Sci., 3: 479-486.

Nanda, R., 1971. The role of sulfated mucopolysaccharides in cleft formation. Teratology, 3: 237-244.

Nanda, R., and Romeo, D., 1975. Differential cell proliferation of embryonic rat palatal processes as determined by incorporation of tritiated thymidine. Cleft Palate J., 12: 436-443

Narayanan, A.S., and Page, R.C., 1976. Biochemical characterization of collagens synthesized by fibroblasts derived from normal and diseased human gingiva. J. Biol. Chem., 251: 5464-5471.

Neblock, D.S., and Berg, R.A., 1987. Intracellular degradation as a modulator of collagen production. In: "Connective Tissue Diseases, Molecular Pathology of Extracellular Matrix." (J. Uitto and A.J. Perejda, eds.), Dekker, New York, pp. 233-246.

Nexø, E., Hollenberg, M., Figueroa, A., Pratt, R., 1980. Detection of epidermal growth factor-urogastrone and its receptor during fetal mouse development. Proc. Nat. Acad. Sci. (USA), 77: 2782-2787.

Olson, F., and Massaro, E., 1980. Developmental pattern of cAMP, adenyl cyclase, and cAMP phosphodiesterase in the palate, lung, and liver of the fetal mouse: Alterations resulting from exposure to methylmercury at levels inhibiting palate closure. Teratology, 22: 155.

Palmer, G., Palmer, J., Waterman, R., and Palmer, S., 1980. In vitro activation of adenylate cyclase by norepinephrine, parathyroid hormone, calcitonin, and prostaglandins in the developing maxillary process and palatal shelf of the golden hamster. Ped. Pharm., 1: 45-54.

Pearlstein, E., 1976. Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. Nature, 262: 497-500.

Pedgett, C.S., and Ivey, W., 1960. The normal embryology of the *coturnix* quail. Anat. Rec., 137: 1-11

Pelton, R.W., Hogan, B.L.M., Miller, D.A., and Moses, H.L., 1990. Differential expression of genes encoding TGF-β1, β2, and β3 during murine palate formation. Dev. Biol., 141: 456-460.

Piddington, R., Herold, R., and Goldman, A.S., 1983. Further evidence for a role of arachidonic acid in glucocorticoid teratogenic action in the palate. Proc. Soc. Exp. Bio. Med., 174: 336-342.

Pisano, M.M., and Greene, R.M., 1986. Hormone and growth factor involvement in craniofacial development. IRCS Med. Sci., 14: 635-640.

Pisano, M.M., and Greene, R.M., 1987. Epidermal growth factor potentiates the induction of ornithine decarboxylase activity by prostaglandins in embryonic palate mesenchymal cells. Effects on cell proliferation and glycosaminoglycan synthesis. Dev. Biol., 122: 419-431.

Polzl, A., 1904. Zur Entwicklungsgeschichet des menschlichen Gaumens. Anat. Hefte., 27: 224-283.

Pons-tortella, E., 1937. Uber die Bildungsweise des sekundaren Gaumens. Anat. Anz., 84: 13-17.

Pratt, R.M., Goggins, J.F., Wilk, A.L., and King, C.T.G., 1973. Acid mucopolysaccharide synthesis in the secondary palate of the developing rat at the time of rotation and fusion. Dev. Biol., 32: 230-237.

Pratt, R.M., Kim, C., and Grove, R.I., 1984. Role of glucocorticoids and epidermal growth factor in normal and abnormal palate development. Curr. Topics Dev. Biol., 19: 81-102.

Pratt, R.M., and King, C.T.G., 1971. Collagen synthesis in the secondary palate of the developing rat. Arch. Oral Biol., 16: 1181-1185.

Pratt, R.M., and King, C.T.G., 1972. Inhibition of collagen cross-linking associated with β-aminoproprionitrile-induced cleft palate in the rat. Dev. Biol., 27: 322-328.

Pratt, R.M., and Martin, G.R., 1975. Epithelial cell death and cyclic AMP increase during palatal development. Proc. Nat. Acad. Sci. (USA), 72: 874-877.

Prior, M.J.W., Maxwell, R.J., and Griffiths, J.R., 1990. In vivo ^{19}F NMR spectroscopy of the antimetabolite 5-fluorouracil and its analogues: an assessment of drug metabolism. Biochem. Pharmacol., 39: 857-863.

Prockop, D.J., Kivirikko, K.I., Tuderman, L., and Guzman, N.A., 1979a. The biosynthesis of collagen and its disorders. The New England J. Med., 301: 13-23.

Prockop, D.J., Kivirikko, K.I., Tuderman, L., and Guzman, N.A., 1979b. The biosynthesis of collagen and its disorders. The New England J. Med., 301: 77-85.

Ruddick, J., and Runner, M., 1974. 5-FU in chick embryos as a source of label for DNA and a depressant of protein synthesis. Teratology, 10: 39-46.

Rudy, B.C., and Senkowski, B.Z., 1973. Fluorouracil. In: "Analytical Profiles of Drug Substances." (Florey,K., ed.), Vol. 2, New York, Academic Press, pp.222-244.

Salomon, D.S., and Pratt, R.M., 1979. Involvement of glucocorticoids in the development of secondary palate. Differentiation, 13: 141-154.

Sandborg, R., and Siegel, I., 1990. Effects of 5-fluorouracil on protein synthesis and secretion of the rat parotid gland. Archs. Oral Biol., 35: 991-1001.

Sanelli, G., Valeriote, F., 1978. In vivo enhancement of 5-fluorouracil cytotoxicity to AKR leukemia cells by thymidine in mice. J. Nat. Cancer Inst., 61: 843.

Sasaki, S., and Kurisu, K., 1983. Effect of triamcinolone acetonide on proliferation and collagen and glycosaminoglycan synthesis in palatal mesenchymal cells from the mouse fetus. J. Craniofac. Genet. Dev. Biol., 3: 351-369.

Sato, K., Hoshino, T., Mizuma, Y., and Nashida, S., 1971. A series of normal stages in the early development of Japanese quail, *Coturnix coturnix japonica*, embryo. Tohoku J. Agric. Res., 22: 80-95.

Saxén, L., 1976. Mechanisms of teratogenesis. J. Embryol. Exp. Morphol., 36: 1-12.

Schmitt, F. O., Hall, C. E., and Jakus, M. A., 1942. Electron microscope investigations of the structure of collagen. J. Cell. Comp. Physiol., 20: 11-33.

Schorr, G., 1907. Zur Entwicklungsgeschichte der sekundaren gaumens bei cinigen saugetieren und beim menschen. Anat. Anz., 30:24.

Scott, W., 1979. Physiological cell death in normal and abnormal limb development. In: "Advances in the Study of Birth Defects." (T.V.N. Persaud, ed.) Vol. 3, MTP Press, Lancaster, pp. 69-84.

Shah, R.M., 1979a. Current concepts on the mechanisms of normal and abnormal palate formation. In: "Advances in the Study of Birth Defects." (T. V. N.,Persaud, ed.) Vol. 1, MTP Press, Lancaster. pp. 69-84.

Shah, R.M., 1979b. A cellular mechanism for the palatal shelf reorientation from a vertical to a horizontal plane in hamster: light and electron microscopic study. J. Embryol. Exp. Morph., 58, 1-13.

Shah, R. M., 1979c. Cleft palate development in hamster embryos following triamcinolone treatment. J. Anat., 129: 531-539.

Shah, R.M., 1984. Morphological, cellular, and biochemical aspects of differentiation of normal and teratogen-treated palate in hamster and chick embryos. Curr. Top. Dev. Biol., 19: 103-135.

Shah, R.M., 1990. Differentiation of cyclophosphamide-treated hamster secondary palate: ultrastructural and biochmical observations. Am. J. Anat., 187: 1-11.

Shah, R.M., Arcadi, F., Suen, R., and Burdett, D.N., 1989a. Effects of cyclophosphamide on the secondary palate development in golden syrian hamster: teratology, morphology, and morphometry. J. Craniofac. Genet. and Dev. Biol., 9: 381-396.

Shah, R. M., and Burton, A. F., 1980. Binding of radioactive glucocorticoids in the palatal tissues of hamster. Teratology, 21: 68 (Abstract).

Shah, R.M., and Chaudhry, A.P., 1974a. Light microscopic and histochemical observations on the development of palate in golden hamsters. J. Anat., 117: 1-15.

Shah, R.M., and Chaudhry, A.P., 1974b. Ultrastructural observations on closure of the soft palate in hamsters. Teratology, 10: 17-30.

Shah, R.M., Chen, Y.P., and Burdett, D.N., 1989b. Growth of the secondary palate in the hamster following hydrocortisone treatment: shelf area, cell number, and DNA synthesis. Teratology, 40: 173-180.

Shah, R.M., Chen, Y.P., and Burdett, D.N., 1989c. Palatal shelf reorientation in hamster embryos following treatment with 5-fluorouracil. Histol. Histopath., 4: 449-456.

Shah, R. M., and Cheng, K. M., 1988. In vitro differentiation of the japanese quail secondary palate. Cleft palate J., 25: 43-47.

Shah, R.M., Cheng, K.M., Mackay, R., and Wong, A., 1987. Secondary palate development in the domestic duck (Khaki Campbell). An electron microscopic, histochemical, autoradiographic and biochemical study. J. Anat., 154: 245-258.

Shah, R.M., Cheng, K.M., Suen, R., and Wong, A., 1985a. An ultrastructural and histochemical study of the development of secondary palate in Japanese quail, *Coturnix coturnix japonica*. J. Craniofac. Genet. Dev. Biol., 5: 41-57.

Shah, R.M., and Crawford, B.J., 1980. Development of the secondary palate in chick embryo: A light and electron microscopic and histochemical study. Invest. Cell Pathol., 3: 319-328.

Shah, R. M., Crawford, B. J., Greene, R. M., Suen, R. S., Burdett, D., King, K. O., and Wong, D. T., 1985b. In vitro development of the hamster and chick secondary palate. J. Craniofac. Genet. Devel. Biol., 5: 299-314.

Shah, R.M., Donaldson, E.M., and Scudder, G.G.E., 1990. Toward the origin of the Secondary Palate. A Possible Homlogue in the Embryo of Fish, Onchorhynchus kisutch, With Description of Changes in the Basement Membrane Area. Am. J. Anat., 189: 329-338.

Shah, R.M., and Ferguson, M.W.J., 1988. Histological evidence of fusion between the posterior palatal shelves and the floor of the mouth in Alligator mississippiensis. Arch. Oral Biol., 33: 769-771.

Shah, R.M., and Kilistoff, A., 1976. Cleft palate induction in hamster fetuses by glucocorticoid hormones and their synthetic analogues. J. Embryol. Exp. Morphol., 36: 101-108.

Shah, R.M., and Mackay, R. A., 1978. Teratological evaluation of 5-fluorouracil and 5-bromo-2-deoxyuridine on hamster fetuses. J. Embryol. Exp. Morph., 43:47-54.

Shah, R.M., Ogasawara, D.M., and Cheng, K.M., 1988. Embryogenesis of the secondary palate in pigeons. Poultry Sci., 67: 865-870.

Shah, R. M., King, K. O., and Feeley E. J. E., 1991. Pathogenesis of bromodeoxyuridine-induced cleft palate in hamster. Am. J. Anat., 190: 219-230.

Shah, R.M., Schuing, R., Benkhaial, G., Young, A.V., and Burdett, D., 1991b. Genesis of hadacidin-induced cleft palate in hamster: Morphogenesis, Electron Microscopy, and Determination of DNA synthesis, cAMP, and Enzyme acid Phosphatase. Am. J. Anat., 192: 55-68.

Shah, R.M., and Travill, A.A., 1976a. Morphogenesis of the Secondary Palate in Normal and Hydrocortisone-treated Hamster. Teratology, 13: 71-84.

Shah, R.M., and Travill, A.A., 1976b. Light and electron microscopic observations on hydrocortisone induced cleft palate in hamsters. Am. J. Anat., 145: 149-166.

Shah, R.M., and Wong, D.T.W., 1980. Morphological study of cleft palate development in 5-fluorouracil-treated hamster fetuses. J. Embryol. Exp. Morph., 57: 119-128.

Shah, R. M., Wong, D. T. W., and Suen, R. S. K., 1984. Ultrastructural and cytochemical observations on 5-fluorouracil induced cleft-palate development in hamster. Am. J. Anat., 170: 567-580.

Shapira, Y., 1969. An autoradiographic study of ^{3}H-proline uptake in the palate of normal mice and in the palate of mice treated with hydrocortisone. J. Dent. Res., 48: 1039-1041.

Shapira, Y., and Shoshan, S., 1972. The effect of cortisone on collagen synthesis in the secondary palate of mice. Archs. Oral Biol., 17: 1699-1703.

Shoshan, S., and Gross, J., 1974. Biosynthesis and metabolism of collagen and its role in tissue repair processes. Israel J. Med. Sci., 10: 537-561.

Silver, M.H., Foidart, J.M., and Pratt, R.M., 1981. Distribution of fibronectin and collagen during mouse limb and palate development. Differentiation, 18: 141-149.

Silver, M.H., Murray, J.C., and Pratt, R.M., 1984. Epidermal growth factor stimulates type-V collagen synthesis in cultured murine palatal shelves. Differentiation, 27: 205-208.

Singh, S., and Gupta, P.K., 1972. Lethality and teratogenicity of cyclophosphamide (Endoxan-Asta) in chick embryos. Cong. Anom., 12: 61-72.

Skalko, R. G., and Jacobs, D. M., 1978. The effect of 5-fluorouracil on [3H] nucleoside incorporation into the DNA of mouse embryos and maternal tissues. Exp. Molec. Pathol., 29: 303-315.

Spooner, B. R., and Faubion, J. M., 1980. Collagen involvement in branching morphogenesis of embryonic lung and salivary gland. Dev. Biol., 77: 84-102.

Stadler, H. E., and Knowles, J., 1971. Fluorouracil in pregnancy : effect on the neonate. J. Am. Med. Assoc., 217: 214-215.

Stark, R.B., and Ehramann, N.A., 1958. The development of the centre of the face with particular reference to surgical correction of bilateral cleft lip. Plast. Reconstruct. Surg., 21: 177-191.

Steffek, A.J., Verrusio, A.C., and Watkins, C.A., 1972. Cleft palate in rodents after maternal treatment with various lathyrogenic agents. Teratology, 5: 33-40.

Stephens, J.D., Golbus, M.S., Miller, T.R., Wilber, R.R., and Epstein, C.J., 1980. Multiple congenital anomalies in a fetus exposed to 5-fluorouracil during the first trimester. Am. J. Obstet. Gynecol., 137: 747-749.

Stevens, A.N., Morris, P.G., Iles, R.A., Sheldon, P.W., and Griffiths, J.R., 1984. 5-Fluorouracil metabolism monitored in vivo by ^{19}F NMR. Brit. J. Cancer, 50: 113-117.

Svanberg, G.K., 1987. Hydroxyproline determination in serum and gingival crevicular fluid. J. Periodont. Res., 22: 133-138.

Sykes, R., and Puddle, B., Francis, M., and Smith, R., 1976. The estimation of two collagens from human dermis by interrupted gel electrophoresis. Biochem. Biophys. Res. Commun., 72: 1472-1480.

Tamarin, A., Crawley, A., Lee, J., and Tickle, C., 1984. Analysis of upper beak defects in chicken embryos following treatment with retinoic acid. J. Embryol. Exp. Morph., 84: 105-123.

Taylor, R. G., and Harris, J. W. S., 1973. Growth and spatial relationships of the cranial base and lower jaw during closure of the secondary palate in hamster. J. Anat., 115: 149-150.

Timpl, R., and Martin, G.R., 1982. Components of basement membranes. In "Immunochemistry of the Extracellular Matrix" (H. Furthmayr, ed.) Vol. 2, CRC Press, Boca Raton, Florida, pp. 119-150.

Trelstad, R. L., and Hayashi, K., 1979. Tendon fibrillogenesis: Intracellular collagen subassemblies and cell surface changes associated with fibril growth. Dev. Biol., 71: 228-242.

Trinkaus, J.P., 1984. Cells into Organs. The forces that shape the embryo. Prentice-Hall Inc., USA, Second ed.

Tuchmann-Duplessis, H., 1975. Drug effects on the fetus: A survey of the mechanisms and effects of drugs on embryogenesis and fetogenesis (H. Tuchmann-Duplessis, ed.) Sydney, 106-107.

Turley, E.A., Hollenberg, M.D., and Pratt, R.M., 1985. Effect of epidermal growth factor/urogastrone on glycosaminoglycan synthesis and accumulation in vitro in the developing mouse palate. Differentiation, 28: 279-285.

Uchida, M., Dah Hsi, W.Ho, Kamiya, K., Yoshimura, T., Sasaki, K., Tsutani, H., and Nakamura, T., 1989. Transport and intracellular metabolism of fluorinated pyrimidines in cultured cell lines. Adv. Exp. Med. Biol., 253(B): 321-326.

Uitto, V.J., and Thesleff, I., 1979. Effect of hydrocortisone on collagen synthesis in cultured mouse palatal explants. Arch. Oral Biol., 24: 575-583.

Veau, V., 1931. Division palatine: Anatomie, Chirurgie Phonetique. Paris, Masson.

Venkatasubramanian, K., and Zimmerman, E.F., 1983. Palate cell motility and substrate interaction. J. Craniofac. Genet. Dev. Biol., 3: 143-157.

Verrett, M.J., Scott, W.F., Reynaldo, E.F., Alterman, E.K., and Thomas, C.A., 1980. Toxicity and teratogenicity of food additive chemicals in the developing chicken embryo. Toxic. Appl. Pharmac., 56: 265-273.

Walker, B.E., 1969. Correlation of embryonic movement with closure in mice. Teratology, 2: 191-197.

Walker, B.E., 1971. Palate morphogenesis in the rabbit. Arch. Oral Biol., 16: 275-286.

Walker, B.E., and Crain, B., 1960. Effects of hypervitaminosis A on palate development in two stains of mice. Am. J. Anat., 107: 49-58.

Walker, B.E., and Fraser, F.C., 1956. Closure of the secondary palate in three strains of mice. J. Embryol. Exp. Morph., 4: 176-189.

Walker, B.E., and Fraser, F.C., 1957. The embryology of cortisone induced cleft palate. J. Embryol. Exp. Morphol., 5: 201-209.

Waterman, R.E., Palmer, G.C., Palmer, S.J., and Palmer, S.M., 1976. Catecholamine-sensitive adenylate cyclase in the developing golden hamster palate. Anat. Rec., 185: 125-37.

Weinstock, M., and Leblond, C.P., 1974. Formation of collagen. Fed. Proc., 33: 1205-1218.

Wessells, N. K., and Cohen, J. H., 1968. Effects of collagenase on developing epithelium in vitro: lung, ureteric bud and pancreas. Dev. Biol., 18: 294-309.

Williams, J.M., Robinson, R.A., and Solursh, M., 1991. Immunohistochemical localization of TGF-β_1 during morphogenetic movements of the developing mouse palate. J. Craniofac. Genet. Dev. Biol., 11: 139-145.

Wilson, J.G., 1971. Use of rhesus monkeys in teratological studies. Fed. Proc., 30: 104-109.

Windholz, M., ed., 1976, The Merck Index, 9th ed., Rahway, NJ, Merck and Co., p.541.

Yoneda, T., and Pratt, R., 1981. Mesenchymal cells from the human embryonic palate are highly responsive to epidermal growth factor. Science, 213, 563-565.

Yoshikawa, H., Tashiro, H., and Kurisu, K., 1986. Effect of glucocorticoid on glycosaminoglycans synthesis in cultured mouse embryonic palatal mesenchymal cells. J. Craniofac. Genet. Dev. Biol. 6: 235-244.

Young, A. V., Feeley, E., and Shah, R. M., 1991a. Does the tongue play a role in the initial development of vertical palatal shelf in hamster ? J. Craniofac. Genet. Dev. Biol., 11: 146-155.

Young, A. V., Hehn, B., and Shah, R. M., 1991b. Effects of 5-fluorouracil on glycosaminoglycans during the secondary palate development. (Unpublished observation; Manuscript in preparation).

Zimmerman, E.F., and Wee, E.L., 1984. Role of neurotransmitters in palate development. Curr. Topics Dev. Biol., 19: 37-63.