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THE MICROSCOPIC ANATOMY AND DYNAMICS OF AVIAN AND MAMMALIAN MIDDLE EAR DEVELOPMENT

by

Tina Felice Jaskoll

A dissertation submitted to the graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Tina Jaskoll

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Chairman of Examining Committee
Dr. P.F.A. Maderson

August 17, 1978
Date

Executive Officer
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The City University of New York

Abstract

THE MICROSCOPIC ANATOMY AND DYNAMICS OF AVIAN AND MAMMALIAN MIDDLE EAR DEVELOPMENT

by

Tina Felice Jaskoll

Adviser: Dr. P. F. A. Maderson

The development of the chick and mouse middle ear is presented. A detailed morphological description of the development of each middle ear component is provided. The development of the mouse and chick middle ears are similar although the chick's consists of a single ossicle, while the mouse has three ossicles. The formation of the fenestra ovalis region is complex; cellular interactions between the stapedial (columella) footplate and the otic capsule result in the differentiation of the annular ligament and the formation of the fenestra ovalis.

Two types of experimental studies were conducted on the avian middle ear, chorioallantoic (CAM) grafting and in vivo teratogen studies. Three classes of teratogens--drugs which inhibit neural crest cell differentiation,

antimitotic drugs, and other drugs that cause skeletal abnormalities--were injected into the developing chick embryo and the middle ear regions were examined. In both types of experimental studies, three classes of columella abnormalities were observed. Type I: the footplate was fused with the otic capsule and the annular ligament did not differentiate. Type II: the columella shaft was not continuous with the otic capsule and a footplate was absent. Type III: the columella was deformed. The other ear components were infrequently abnormal but no association between the degree of differentiation between middle ear components could be determined. The possible etiology of columella abnormalities and the role of neural crest in middle ear development are discussed.

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INTRODUCTION

A. General Introduction

Interest in the tetrapod middle ear has centered around the evolutionary origin of the ossicles, a subject of controversy since the early nineteenth century (see review Strickland et al., 1962). A stapes (columella) is primarily present in all tetrapods, but in mammals there are in addition the incus and the malleus derived respectively from the quadrate and articular elements of the reptilian suspensorium (Allin, 1975). These three structures, whether located within the middle ear region and/or the jaw apparatus, are derived from the branchial arches (Goodrich, 1930; Hanson et al., 1962). Morphological descriptions of the development of the avian (Romanoff, 1960; Smith, 1905) and mammalian (Anson, 1947; Jenkinson, 1911; Stephens, 1972) middle ear ossicles are available, but nothing is known of the dynamics of the developmental process within the middle ear region. Except for a few experimental studies (Altmann, 1957; Reagan, 1917; Simons, 1974), the middle ear region has been relatively ignored.

B. The Role of Epithelial Mesenchymal Interactions in Development

The differentiated state in many developmental systems is the result of interactions between two cell populations. Spemann (1910) first demonstrated that induction occurs during development by describing the role of the forebrain in the formation of the eye. Following Spemann and Mangold's (1924) study of amphibian primary induction, many authors sought to confirm the involvement of diffusible factors (Saxen and Toivonen, 1962), but this quest has proved futile. Several alternate proposals of the nature of the inducer have been suggested (Grobstein, 1956; Toivonen et al., 1976). In vitro experiments on metanephros differentiation (Grobstein, 1956) and other developing systems (Fleischmayer and Billingham, 1968) indicate the general prevalence of epithelial mesenchymal interactions during development. Based on Grobstein's (1956) transfilter experiments, direct cell contact was not considered necessary and he emphasized the role of extracellular material. Extracellular material, collagen and glycosaminoglycans, have been demonstrated to permit cell migration (Ebendal, 1977; Toole et al, 1972) and organogenesis (Grobstein, 1975; Hay, 1977). Glycosaminoglycans have been identified in the basal lamina of developing epithelia (Banajee et al., 1977). The basal lamina imposes morphological stability on the epithelia. Mesenchyme

may influence epithelial changes by degrading the extracellular material (Banajee et al., 1977). Recent transfilter experiments using nucleopore filters to reinvestigate metanephros development (Wartiovaara et al., 1974; Lehtonen et al., 1975) have demonstrated that differentiation will occur only in the presence of direct cell contacts. Therefore, epithelial mesenchymal interactions may be the result of a long distance effector, direct cell contact, extracellular material and/or a combination of any or all these factors to result in the differentiation of a system.

C. Developmental Interaction in the Tetrapod Ear

1. Investigations on the Tetrapod Inner Ear

a. Amphibian studies

Most of our information has derived from investigation on the development of the amphibian inner ear and surrounding otic capsule (see review Benoit, 1957). The invagination of the surface ectoderm to form the otocyst is induced by a neural factor (Spemann, 1910) first mentioned by Stone (1931) to be the rhombencephalon and further confirmed by Mangold (1937). The neural inducer is unable to completely induce the formation of the otocyst. Dalcq (1933) demonstrated the existence of a mesodermal factor,

apparently derived from the prechordal mesoderm. Harrison (1938) further investigated the role of prechordal mesoderm and found that it is necessary for otocyst formation. Yntema (1950) showed that the development of the otocyst is the result of a cooperative interaction of the two inducers. In transplantation experiments on Ambystoma otic rudiments at various stages of development, he showed that mesodermal induction is active during the gastrula and early neurula stages to induce the initial formation of the optic placode, and a second neural induction during the late neurula stage is necessary for otocyst formation. Trampusch (1941), investigating ear induction, proposed a possible role of neural crest in otocyst induction based on the temporal relationship of crest migration and organogenesis. Other investigators (Dalcq, 1933; Mangold, 1937) have also attributed a possible inductive role to the neural crest.

The otocyst induces the surrounding mesenchyme to condense and chondrify to form the cartilaginous otic capsule (Lewis, 1907; Spemann, 1910). In the absence of the otocyst, the capsule will not form. Non-cephalic mesenchyme is not capable of responding to the otocyst induction to form the capsule (Kaan, 1926, 1930).

b. Avian studies

Fewer studies have been conducted on the avian embryo. The otic placode is formed under the influence of the prechordal mesoderm (Cuevas, 1977; LeviMontalcini, 1946; Waddington, 1937). A second induction by the rhombencephalon is necessary for the complete development of the otocyst (Waddington, 1937). Szepsenwol (1936) proposed that the acoustic ganglion (VIII) was the organizer of the otocyst. Orr (1968) conducted dissociation/reaggregation experiments with extirpated three and one-half-day-old chick otocysts and observed better histodifferentiation of the sensory cells in the presence of the ganglion. The otocyst differentiates in the absence of the VIII ganglion in vitro (Fell, 1928) and on the chorioallantoic membrane (Hoadley, 1924).

Meier (1978 a, b), investigating the initial development of the otic placode, correlated the migration of neural crest cells under the surface epithelium with the simultaneous formation of the placode. He proposed that the epithelium overlying these neural crest cells represents the portion that will form the placode. Extracellular material accumulates between the neural crest cells and the placode epithelium. Meier concluded that tissue interaction between the neural crest cells, extracellular material, and overlying epithelium may be necessary for placode formation.

Similar to the induction of the cartilaginous capsule by the otocyst in amphibians, the avian otic capsule is also induced by the otocyst (Benoit, 1960; Reagan, 1917; Simons, 1974; Yntema, 1950). Reagan (1917) cauterized the otocyst on one side of the embryo and observed that in the absence of the otocyst, the capsule did not form. Benoit and Schowing (1970), repeating this experiment, confirmed the role of otocyst in capsule induction. They also investigated the specificity of the inducer. When they replaced the otocyst with fragments of the medulla and notochord, cartilage formed. They concluded that the otic epithelium is not the only inducer of the cartilaginous capsule. Simons (1974) repeated Reagan's (1917) experiment and confirmed that the capsule did not form in the absence of the otocyst.

c. Mammalian studies

Based on a series of experiments, investigators have concluded that the otic vesicle in amphibians and birds is induced by the prechordal mesoderm and the neural tube (Waddington, 1937; Yntema, 1950). It is not possible to conduct similar experiments on mammalian development for technical reasons. The use of mutant mice provides one approach for investigating ear development. Hertwig (1944) first produced and described Kreisler mice

which have inner ear and brain abnormalities. The most common features are rhombencephalic degeneration and a defective capsule through which the membranous labyrinth may form extracapsular cysts. Hertwig (1944) concluded from the fact that the otic cup is more lateral than normal, it is too far removed from the inducing influence of the brain. Deol (1964a) described the development of the inner ear of the Kreisler mouse and concluded that the initial induction of the otic placode by the prechordal mesoderm occurs, but the subsequent neural induction is faulty. Inner ear abnormalities are directly dependent on neural abnormalities. Deol (1964b, 1966) investigated the Dreher (Deol, 1964b), Splotch, and Looptail (Deol, 1966) mice and determined that the inner ear abnormalities are also associated with neural tube abnormalities. Studies on mouse mutants (Hertwig, 1944; Deol, 1966) therefore indicate that the neural tube influences otic differentiation in mammals.

Experimental investigations provide a second approach for investigating ear development. Van Der Borden (1967) induced synotia dorsalis by X-irradiating mice. The otic abnormalities are dependent on the degree of rhombendephalon underdevelopment. This experiment also indicates that the mesoderm also influences the development of the otocyst. In the normal mouse, mesoderm is adjacent to the lateral

ectoderm. In the X-irradiated mouse, the parachordal mesoderm and possibly the neural crest is in contact with the dorsal ectoderm. Therefore, in the treated mouse, the lateral and dorsal ectoderm are induced to form otic epithelium. Altmann (1957) observed middle and inner ear abnormalities in X-irradiated and Trypan blue treated rats. Based on his observations, he concludes that otic developmental processes in mammals are similar to those of amphibians (Yntema, 1950).

Numerous in vitro studies on the mammalian otocyst have been conducted. Maximow (1925) reported the first cultivation of the inner ear in vitro, but the otocyst did not differentiate. Lawrence and Merchant (1953) observed slight differentiation of the sensory cells in the cultured otocyst. Van De Water and Ruben (1971) cultured the mouse otocyst and observed morphogenesis and histodifferentiation. Van De Water (1976) observed normal differentiation of the otocyst in the absence of the VIII ganglion, and concluded that an interaction between these tissues does not exist, in contrast to Orr's (1968) conclusion.

Chorioallantoic membrane grafting experiments (Waterman, 1938) of the rabbit otocyst demonstrated the role of the otocyst in capsule formation. Friedmann (1969) confirmed these results. Investigators (Deol, 1964a, 1966; Hertwig, 1944) observed that the otic capsule is abnormal

in mouse mutants. These observations provide additional evidence that the otocyst induces the otic capsule. Grobstein and Holtzer (1955) investigated the ability of the otocyst to induce cartilage in foreign mesenchyme. They observed cartilage in the axial mesenchyme when the otocyst was transplanted to that region, concluding that the otocyst can induce cartilage in non-cephalic mesenchyme. Transplanted mouse otocyst in the chick embryo was capable of inducing the formation of the otic capsule (Pugin, 1972).

d. Summary

Amphibian studies provide the majority of our data on ear induction, although some evidence is available from avian and mammalian studies. The formation of the otic placode is the result of the induction of cephalic mesoderm, possibly including neural crest cells. The subsequent formation of the otocyst requires a second induction by the rhombencephalon. After the formation of the otocyst, it will induce the surrounding mesenchyme to chondrify into the cartilaginous otic capsule.

2. Investigations on the Tetrapod Middle Ear

a. Amphibian studies

Stone (1926, 1929) demonstrated that the skeletal tissue of the face and visceral cartilages are

derived from the neural crest. Horstadius and Sellman (1946) published the first fate map of the cephalic neural crest. Chibon (1967) traced neural crest migration in Pleurodeles waltii by labeling the crest cells. He studied and mapped the cranial and visceral skeletal derivatives. Holtfreter and his co-workers (1968) investigated the differentiation of cephalic neural crest in vitro. Cartilage formed only when the neural crest was cultured with pharyngeal endoderm. Other in vitro studies (Drews et al., 1972; Epperlein, 1974; Epperlein and Lehmann, 1975) investigated the induction of cartilage in cephalic neural crest. Neural crest cells lose their motility upon contact with pharygeal endoderm and subsequently differentiate into cartilage. Cartilage was not observed when neural crest was cultured alone. Holtfreter (1968) investigated the specificity of this induction. He showed that tissues that induce cartilage in somitic mesoderm, i.e., notochord, could not induce cartilage in the crest cells, but equally trunk neural crest cultured with pharyngeal endoderm formed no cartilage. He concluded that cephalic neural crest alone has the ability to respond to the inductive influence of the pharyngeal endoderm to form cartilage. Pharyngeal endoderm also induces the surface ectoderm to invaginate to form pharyngeal clefts (Holtfreter, 1968). It appears that the pharyngeal endoderm is the "inducer" for the

initial stages of middle ear development.

Helff (1928, 1931, 1940) investigated the differentiation of the tympanic membrane in metamorphosizing anura. He demonstrated that the tympanic membrane would not differentiate in the absence of the annular tympani (not homologous with the mammalian structure), columella, or other components of the visceral cartilages. He emphasized the importance of contact between the inducer and the presumptive tympanic membrane; differentiation did not begin until contact was made. Helff's experiments showing that the presumptive tympanic epithelium transplanted to the flank region do not differentiate, emphasize the importance of the environment for normal development. Helff states that he was not able to fully evaluate the type of induction that resulted in the differentiation of the tympanic membrane.

b. Avian studies

Neural crest derivatives in the avian embryo are probably one of the most comprehensively studied aspects of development. Hammond and Yntema (1964) observed deficiencies of the nasal septum and visceral skeleton after neural crest extirpation. Weston (1963) mapped the fate of trunk neural crest cells using tritiated thymidine labeling, but could not follow all their derivatives due to

dilution of the label. Johnston (1966) showed that the neural crest cells contribute extensively to the mesenchyme of the upper facial region and visceral cartilages, demonstrating that labeled crest cells participate in cartilage formation. Le Lievre (1974) used quail-chick biological marking to show that the visceral cartilages and facial bone are derived from neural crest. Noden (1975) followed the migration of cephalic neural crest cells and determined their time of migration into the derivatives. All these studies indicated that neural crest contributions to the head were much more extensive than previously thought. Neural crest contributes, at least in part, to the connective tissue of the lower jaw, tongue, and the ventrolateral portion of the neck; glands of the tongue and pharynx; dermis; adipose tissue; ganglia; and the striated muscles of the branchial arches. Neural crest cells do not contribute to the endothelium of blood vessels (Le Lievre and Le Douarin, 1975; Le Douarin et al., 1977).

Based on the amphibian studies, it is concluded that pharyngeal endoderm induces cartilage formation in cephalic neural crest. In the presence of other embryonic tissues, cephalic neural crest cells will form cartilage. When quail mesencephalon and anterior rhombencephalon were grafted into the chick trunk, a chimaera of quail-chick cells is observed in the host vertebra. The cephalic neural crest is therefore

thought to be sensitive to the vertebral morphogenetic field, like mesodermal cells, to form cartilage (Le Douarin and Teillet, 1974; Le Douarin et al., 1977). Cartilage formation in crest cells was also induced by chick retinal epithelium (Newsome, 1976).

Reagan (1917), investigating the induction of the otic capsule by the otocyst, observed that the columella was present in his experimental animals but that the columella footplate was absent. He concludes that the footplate is derived from the otic capsule while the columella is derived from the second branchial arch. Simons (1974, 1975, 1976) recently repeated Reagan's (1917) experiments and confirmed these results; the columella footplate is absent in the absence of the capsule. Friedmann et al. (1977) cultured the chick otocyst in vitro and reported the presence of the stapedial component in many of his grafts. This supports the conclusion that the footplate receives a capsular component. Le Lievre (pers. com.) showed that the entire columella is derived from neural crest. This does not exclude the possibility that the footplate receives a capsular contribution as the capsule could also be derived, in part, from neural crest (Le Lievre, pers. comm.).

c. Mammalian studies

The classification of mammalian neural crest derivatives is based on amphibian studies. Preliminary studies on rodents suggest that facial mesenchyme is derived from neural crest (Johnston and Pratt, 1975). Most investigations concerning the neural crest have centered around tooth development (Koch, 1967; Kollar, 1972a). Johnston and Pratt (1975) have suggested that neural crest abnormalities are probably involved in producing facial abnormalities.

There are various reports in the clinical literature of human middle ear abnormalities (Altmann, 1957; Anson and Donaldson, 1973; Hough, 1963; Klein, 1967; Warkany, 1971). In most abnormal ears, the stapes shows the highest incidence of abnormalities while the other ossicles are less frequently abnormal (Altmann, 1957). Altmann (1955, 1957) observed otic abnormalities, including the middle ear, when pregnant rats were injected with Trypan blue (Altmann, 1955) or irradiated (Altmann, 1957). The stapes and stapedial muscle were absent, the incus was only partially developed, and other components were abnormal. In trying to understand the etiology of these abnormalities as well as those observed clinically, Altmann (1957) concluded that the developmental processes during mammalian otic development are similar to those observed in amphibians. Therefore, these abnormalities are due to abnormal influences of the otocyst in otic

capsule formation since in the absence of the capsule, the footplate is absent. Abnormalities of the visceral cartilages, derived from the neural crest, would be dependent on the development of the neural tube. Since the development of the otocyst is dependent on neural tube formation, all the structures within this region are associated with primary induction.

The development of human middle ear components has been described in detail (Anson et al., 1960; Hanson et al., 1962). The malleus and incus are derived from the first visceral arch, except that the malleus manubrium and the long crus of the incus are derived from the second arch. The anterior process of the malleus is an independent ossification. The stapes is derived from the second visceral arch with the footplate also receiving a contribution from the otic capsule (Anson et al., 1948).

The lamina stapedialis is derived from the capsule and differentiates into the annular ligament, the basal perichondrium, and the stapedial rim on the vestibular surface. Marovitz and his co-workers (Marovitz and Porubsky, 1971; Marovitz and Shapiro, 1971) confirmed this dual origin of the footplate using histochemical techniques. The distribution of phosphorylase (Marovitz and Shapiro, 1971) and acid phosphatase (Marovitz and Porubsky, 1971) activity indicate an affinity between the vestibular portion of the

footplate and the otic capsule, while the other regions of the stapes show staining characteristics similar to those of the other ossicles.

d. Summary

The visceral cartilages are derived from cephalic neural crest, therefore, the ossicles are derived from neural crest. Cephalic neural crest will only differentiate into cartilage in the presence of pharyngeal endoderm and possibly other embryonic structures. The external auditory meatus (first visceral cleft) invaginates due to pharyngeal endoderm induction. In anura, the visceral cartilages induce the differentiation of the tympanic membrane. Based mainly on amphibian studies, there appears to be a series of tissue interactions during the development of the middle ear.

D. Proposed Investigations of the Middle Ear

The tetrapod middle ear is extremely complex. Based on the evidence of interactions during the development of the inner ear and capsule (Reagan, 1917; Waddington, 1937) and middle ear (Altmann, 1957; Helff, 1928, 1940; Simons, 1975), it is logical to propose a series of interactions in the middle ear. An investigation of the development of the avian and mammalian middle ear is of interest

for three reasons. First, to determine the exact sequence of development in the middle ear region in light of our present concepts of development. Second, to possibly explain the etiology of the clinical abnormalities observed in humans. Third, to use the middle ear as a region for the investigation of epithelial mesenchymal interactions. In this thesis I will describe the normal development of the chick and mouse middle ear, describe a series of experiments conducted on the developing chick middle ear, and discuss the implications of these studies.

SECTION II

THE DEVELOPMENT OF THE AVIAN AND MAMMALIAN MIDDLE EAR AND TYMPANUM

A. General Introduction

The complexity of the avian and mammalian middle ear region, including the tympanum, with structures of diverse embryonic origin--all three germ layers and neural crest being represented--makes it an intriguing system of developmental study. In this section, I will present a detailed histological account of its development in the chick and mouse, discuss differences observed between the two animals, and address three specific questions. First, how does the pharyngeal pouch expand to form the tympanic cavity and what is the fate of the mesenchyme originally present within this region? Second, is there any significance to the differences observed in the rate of development of middle ear components in the two animals? Third, what is the precise sequence of histogenic events during the differentiation of tissues in the region of the fenestra ovalis? To facilitate comparison and discussion I will use the term "fenestra ovalis" throughout this thesis for this region in both the chick and the mouse, although in

the chick the term fenestra vestibularis is the accepted one according to the Nomina Anatomica 65: Anatomica Vetermacia 68: Nomina Anatomica Avium 1977.

B. Materials and Methods

1. The Development of the Avian Middle Ear

Eggs of the common fowl Gallus gallus (Shamrock Farms, New Jersey) were incubated at 37° C. Embryos were recovered from the fourth day of incubation and staged according to Hamburger and Hamilton (1951). Whole embryos (early stages) or heads alone (later stages) of stages 22-46 were fixed in Bouin's fixative or 10 per cent formalin for twenty-four hours, dehydrated in ethyl alcohol, and embedded in 56° C paraplast. Older embryos were decalcified in RDO Rapid Bone Decalcifier (Du Page Kinetic Lab) according to directions. The embryos were serially sectioned at 10 μ. The sections were cut transverse to the head axis rather than the body axis to maintain a constant overall relationship in the ear region. The sections were stained in hematoxylin and eosin, aniline blue/orange green, or Hematoxylin-Alcian Blue 8GX-Chlorantine fast red 5B (Lison, 1954). Embryos or the heads of HH stages 34-46 and hatchlings were cleared and stained for bone with Alizarin red (Dawson, 1926) or cartilage and bone with

Alcian Blue 8GX-Alizarin red (Wasserzug, 1976); they were examined to determine the general skeletal relationships during skull development. Otic regions of hatchlings, and adult roosters and hens, were dissected and serially sectioned to examine the spatial relationships of various tissue components.

2. The Development of the Mouse Middle Ear

A breeding colony of CBA/J (kindly donated by Dr. T. Van De Water, Albert Einstein College of Medicine, and subsequently enhanced by purchases from Jackson Laboratories, Maine) was maintained in a controlled environment, fed Purina Breeding Chow and provided with water ad libitum. Mature females were placed in the males' cage in the afternoon, plugged the next morning, and the pregnant females were separated from the other females and recorded, the day of plug equals day 0. Pregnant females were sacrificed by cervical dislocation on days 9-19 of pregnancy and the embryos removed from the uterus, staged according to Gruneberg (1943), and fixed in 10 per cent formalin for twenty-four hours. Newborn through 18-day-old mice were sacrificed, their bodies (for younger animals) or heads alone (the older animals) were fixed in 10 per cent formalin and subsequently skinned. Older heads were decalcified in RDO Rapid Bone Decalcifier according to directions. The

heads were processed for histology as described on page Whole embryos were cleared and their skeleton stained as previously described (page 19).

C. The Normal Development of the Avian Middle Ear and Tympanum

1. General Anatomy and Development

The otic region of the avian skull is topographically complex and varies considerably from one genus to another (Saiff, 1974). I here present a generalized account based mainly on studies of the late embryo, and newly hatched Gallus material, to facilitate description of the earlier stages of organogenesis.

The avian middle ear is hidden beneath the posteroventrally directed external auditory meatus (fig. 1). It is clearly demarcated by the quadrate, squamosal, exoccipital, and basiparasphenoid (Jollie, 1957), all bordering the tympanic membrane, a partition which separates the external meatus from the middle ear cavity. The columella, the single avian ear ossicle, inserts onto the tympanic membrane.

An idealized cross-section through a late embryo illustrates the general relationships of the components of the middle ear region (fig. 2). The size and shape of the middle ear cavity, and its related structures alter continuously throughout development due to both cranial growth and intrinsic changes, resulting especially in sequential

changes in the orientation of the columella relative to the axes of the skull. However, the relationships of the otic components to each other remain relatively constant. The columella, projecting antero-ventro-laterally from the fenestra ovalis towards the tympanic membrane, is suspended within the middle ear cavity. The columella is composed of the proximal footplate, the rod (shaft), and the distal extracolumella. Figure 2 also indicates the location of the unique avian paratympanic organ (Vitali, 1911, 1914; see page 33) which is embedded in connective tissue medial to the quadrate-squamosal articulation, close to the jugular vein, external ophthalmic artery, and a branch of the facial (VII) cranial nerve.

Avian middle ear development spans Hamburger-Hamilton horizons 25-46. However, at any one stage, while some component(s) may show significant histological differences from the preceding or subsequent stage, others may not. To facilitate description and discussion, I provide first an account of the overall anatomical changes which occur (figs. 3-10), and follow this by detailed analyses of the histogenesis of the individual components.

The first pharyngeal pouch and the presumptive mesenchymal components of the middle ear are recognizable at stage 25 (Romanoff, 1960). By stages 29-30 (fig. 3A),

the columella is distinctly represented by a cartilaginous condensation (fig. 4) surrounded by mesenchymal cells. The laterally-directed pouch extension (tubo-tympanic sulcus), which represents the primordium of the middle ear cavity, is directed towards this columella condensation. The primordium of the external auditory meatus is represented by a depression in the head ectoderm (fig. 4).

By stages 32-34, the cartilaginous columella spans the middle ear region from the otic capsule to the presumptive tympanic tissues (fig. 3B). The distal portion of the first pharyngeal pouch has expanded towards the ventral aspect of the columella and external auditory meatus, thus forming the ventral portion of the tympanic membrane (figs. 5, 6, and 7). Anteriorly directed diverticula of the pouch envelop the anterior aspect of the columella, resulting in the ossicle remaining surrounded by mesenchyme on its dorsal and posterior aspects. It is not until the posterior and anterior pouch diverticula meet around the columella that the dorsal tympanic membrane can be recognized (figs. 8 and 9, and p. 30).

During stages 35 and 36, the pharyngeal pouch diverticula continue to enlarge around the columella (fig. 3C), producing cavities dorsal to the extracolumella and anterior and posterior to the shaft (fig. 8). This process permits identification of the dorsal tympanic

membrane, and eventually the dorsally placed squamosal-columellar (Platner's) ligament (fig. 9).

The continued expansion of the pouch diverticula results in the columella being "suspended" by stage 37 (fig. 3D). The points of fusion between the expanding diverticula form "mesenteries" which subdivide the region. The disappearance of most of these mesenteries forms the definitive middle ear cavity, but one remains as the primordium of the squamosal-columella ligament, inserting at the rod-extracolumella junction (fig. 10). Sub-pouches continue to expand posteriorly and anteriorly under the squamosal and otic capsule, and will eventually give rise to pneumatized spaces.

2. The Development of the Columella

The columella is first seen at stages 25-27 as a mesenchymal condensation clearly separate from the otic capsule whose cells show a slight affinity for alcian blue. By stage 28, the cells of the columella show a comparable affinity for alcian blue, so that both the capsule and the ossicle may be described as consisting of chondroblasts (fig. 4). However, although the two structures are closer than previous, there remains a distinct region of undifferentiated cells between them. At stage 30, matrix accumulation has continued in both ossicle and capsule populations,

but the undifferentiated region between them persists (fig. 11). While this region shows a slight alcian blue affinity, the constituent cells are not as densely packed, nor as rounded, as those in the two adjacent populations (fig. 11).

By stage 32, the extracolumella is clearly distinguishable (fig. 5): matrix deposition is well advanced in its proximal portion, but distally the cells merge imperceptibly with the presumptive dermal cells of the tympanum. The columella cells (figs. 5, 12, and 13) are typical chondrocytes staining darkly with alcian blue, and some embryos show discreet boundaries with the surrounding mesenchyme, indicating the differentiation of perichondral tissues. At this time, the capsule and columella cell populations are contiguous (figs. 12 and 13). Figure 12 clearly demonstrates the greater affinity for alcian blue of the columella chondrocytes relative to the chondroblasts of the adjacent regions of the otic capsule, as well as a regionalization of the capsule (fig. 12, arrows). Within this region several different degrees of alcian blue affinity are visible (fig. 12 and 13), particularly a light region proximal to the columellar chondrocytes.

By stage 34, a definitive "footplate" is discernbile from the surrounding otic capsule, due to the appearance of a discreet discoidal cell population (fig. 14).

The edges of the disc are circumscribed by rounded or ovoid cells with only a slight affinity for alcian blue as compared to the capsule cells: these rounded cells are here designated presumptive annular ligament cells. While matrix accumulation around the capsule chondrocytes is still less advanced than around columellar cells, the light staining region proximal to the latter is less apparent than previous (compare figs. 13 and 14).

By stage 35, the columella assumes its adult antero-ventro-lateral orientation (figs. 7 and 8). It consists of equally well differentiated chondrocytes throughout its distal (extracolumella), medial (shaft) and proximal (footplate) regions, and is nearly completely surrounded by a distinct perichondrium. The perichondrium is not as distinct where the footplate abuts the capsule, but the histology of these cells (presumptive annular ligament) resembles that of individual perichondral cells. The uniform chondrocyte population of the footplate directly abuts the perichondrium (fig. 8) and there is no region of relatively undifferentiated cells between them.

The annular ligament is fully differentiated by stage 36 (fig. 9), so that a definitive insertion of the footplate into a fenestra ovalis may be discerned.

The shape of the columella alters from a slender rod to conical, and it inserts by three separate processes

of the extracolumella onto the tympanic membrane (fig. 9). The columella continues to elongate and becomes more slender as growth continues into stage 38 (fig. 10). Ossification begins at stage 41 and continues through and after hatching. The extracolumella remains cartilaginous into adult life, but the orientation of its three processes changes after hatching, so that their adult locations on the tympanic membrane differ from those of the embryo.

3. The Development of the Middle Ear Cavity and the Histogenesis of the Endodermal Epithelia

By stages 22-23, the lumen formed by the earlier breakthrough of the first pharyngeal pouch to the external ectoderm is identified as the tubo-tympanic sulcus (Romanoff, 1960). Its proximal and distal portions represent the primordia of the Eustachian tube and middle ear cavity respectively. The middle ear cavity remains continuous with the external body surface until the primordium of the paratympanic organ (see page 33) is pinched off and sinks into the dorsal mesenchyme at stage 26.

At stages 22-23, the dorsal and ventral epithelia of the Eustachian tube are clearly distinct from one another. Not only is this distinction maintained throughout development (figs. 4, 5, 6 and 7) but the cytological characteristics are indistinguishable between the early embryo

(fig. 15) and hatchling chicks. The dorsal tissues comprise only cuboidal epithelial cells resting on a poorly defined basement membrane. The ventral epithelium has a very well-defined basement membrane staining intensely with alcian blue. The basal cells are columnar and there are one to three layers of ovoid to flattened supra-basal cells to form a stratified cuboidal epithelium. The epithelial lining of the extreme distal pouch extensions (figs. 4, 5, and 6), which will enlarge to form the middle ear cavity, has cytological characteristics intermediate between these two extremes.

During the process of distal pouch expansion around the columella occurring during stages 28-38, the gross form of the epithelium remains relatively regular (figs. 4-10), but the cytology of the cells is significantly different from the pseudostratified epithelium seen earlier. There is a single layer of extremely flattened cells which have a distinct affinity for alcian blue, although no basement membrane is distinguishable in preparations so stained (figs. 13, 14, and 16). This extreme simplicity of epithelial structure remains through hatching, indeed further thinning is apparent in some areas (fig. 17).

Since the elaboration of the pouch involves the reduction of mesenchyme (see below, page 29), all the

constituent elements of the middle ear region--columella, columella-squamosal ligament, annular ligament, and inner aspect of the tympanic membrane--become covered with this simple squamous epithelium (fig. 10).

4. The Fate of the Mesenchyme During Middle Ear Cavity Expansion

The presumptive middle ear region is initially occupied by typical undifferentiated mesenchyme (figs. 4 and 5). As development continues, the cells become stellate in appearance and more loosely packed. By stage 34 small blood vessels draining into the jugular vein ramify throughout the mesenchyme (fig. 6). There are numerous macrophages within the lumina of these vessels, and in the mesenchyme, but there is little evidence of mesenchymal cell death.

Figures 18 and 19 illustrate the variety and number of spaces which may be seen in the mesenchyme at stage 37. Each is bounded by but a single layer of cells. Study of serial sections has revealed that there are three types of space present. One type may be shown to contain blood cells and/or be continuous with the jugular vein, and may thus be identified as vascular sinuses (figs. 18 and 19 number 3). A second type may be shown to be continuous with the main portion of the middle ear cavity, and may thus be identified as diverticula from it (figs. 18 and 19

number 1). The third type is a space completely enclosed by an epithelium, continuous with no other spaces: since such are completely surrounded by mesodermal cells, they must be defined as celomic spaces (figs. 18 and 19 number 2). Throughout stages 34-40, celomic spaces and vascular sinuses may be detected in regions previously occupied by homogeneous mesenchyme, always before middle ear cavity intrusions can be detected.

In summary, the history of any specific region of middle ear mesenchyme involves the sequential appearance of vascular and celomic spaces, and finally complete obliteration as diverticula from the middle ear cavity continue their processes of expansion. The significance of this sequence of events will be discussed later (pages 46-53).

5. The Histogenesis of the Tympanic Membrane and the External Auditory Meatus

The mature tympanic membrane consists of three components, an inner epithelium of endodermal origin, a connective tissue component of mesodermal origin, and an external epithelium of ectodermal origin continuous with the epidermis of the external auditory meatus. Since the inner epithelium has been treated in the context of the middle ear cavity, this section deals with the tympanum and the external auditory meatus.

At stage 26, a depression in the ectoderm ventral to the opening of the first pharyngeal pouch represents the primordium of the external auditory meatus, the innermost aspect of which will form the tympanic membrane. The ectodermal epithelium throughout consists of cuboidal germinal cells overlain by rounded peridermal cells (Sengel, 1976); on the tympanic membrane the mesenchymal cells are denser than in the external auditory meatus (fig. 20).

By stages 29-32, the external auditory meatus is a distinct depression of the head of the embryo (figs. 4 and 5), and there are distinct differences in integumentary structure between it and the tympanic membrane. While the ectoderm of the external auditory meatus has an histology similar to that shown in Figure 20, the subjacent tissue lacks the pronounced mesenchymal densification characterizing the tympanic membrane. Histogenesis of the tympanic membrane has continued: there are now four to six mesenchymal cell layers lying parallel to the ectoderm, there are alcian blue positive fibers around them, and the basement membrane is prominent. An essentially similar picture is seen through stage 35 (figs. 7, 8, and 16) except for a gradual increase in the numbers of layers of dermal cells.

By stage 35, the external auditory meatus has increased in length, slanting posteroventrally away from the

surface, with the tympanic membrane at its base. Thus, serial sections cut transverse to the head axis show the external auditory meatus and/or tympanum in an apparently closed space (figs. 7-10). By stage 36, the histological differences between the tympanic ectoderm and that of the external auditory meatus are very pronounced (figs. 9 and 21). Over the tympanic membrane there is a basal layer of cuboidal cells on a pronounced basement membrane, with one to two layers of very flattened supra-basal cells (fig. 21). Throughout the external auditory meatus, however, the germinal cells are columnar, and there are two to three layers of ovoid supra-basal cells and a periderm (fig. 21). There has been further accumulation of dermal cells on the tympanic membrane, and collagen fibers stain characteristically with chlorantine fast red (fig. 21). The dorsal tympanic membrane is differentiated later than the ventral portion due to the pattern of growth of the middle ear cavity (see page 23). Nevertheless, the histogenesis of both the ectodermal and mesodermal components of the dorsal region is more advanced than that of the ventral region at stage 36 (fig. 9).

While the ectodermal face of the tympanic membrane remains relatively straight, the integument of the external auditory meatus may show pronounced infoldings by stage 38 (fig. 10). The tympanic membrane is considerably thinner

at stage 38 (fig. 10) than previous (fig. 9), apparently due to a differentiation of the mesodermal elements.

Keratinization of the ectodermal epithelia begins around stage 41. However, at this time through hatching, there is a conspicuous difference in epidermal thickness between the tympanic membrane (fig. 17) and the folded integument of the external auditory meatus (fig. 22).

6. The Paratympanic Organ

This is a banana-shaped, vesicular structure approximately 1.0 mm long, embedded in connective tissue. Its long axis parallels the antero-postero head axis. It is innervated by the seventh cranial nerve, and the sensory epithelium is restricted to the medial wall (fig. 23). This characteristic asymmetry is seen from the time of its first appearance as a lateral diverticulum from the epibranchial placode adjacent to the geniculate ganglion at stages 22-23 (Vitali, 1911, 1914). The primordium is located near the seventh nerve, external opthalmic artery and jugular vein. At stage 29, the paratympanic organ is still continuous with the middle ear cavity (fig. 4), but the first indications of a characteristic mucopolysaccharide material are seen within its lumen. The structure loses its connection with the middle ear cavity at stage 32 (fig. 24), and is displaced medio-dorsally by the enlarging

cavity. By stage 37 its adult orientation is established (figs. 10 and 25), and by hatching its cytology is indistinguishable from the adult condition (Jaskoll, unpublished).

D. The Normal Development of the Mammalian Middle Ear and Tympanum

1. General Anatomy and Development

The mammalian otic region is more complex than that of the chick. The morphology of the mammalian middle ear is generally more familiar and therefore simpler to describe because of the numerous studies on the middle ear of humans and other mammals (Anson and Donaldson, 1973; McClain, 1939; Richany et al., 1954; Stephens, 1972). Therefore, only a very short general description will be provided.

The mouse middle ear, contained within the temporal bone, is separated from the external ear by the tympanic membrane. The middle ear cavity is boundared anteriorly by the carotid canal, posteriorly by the mastoid bone, medially by the otic capsule, laterally by the tympanic membrane, dorsally by the tegmen tympani, and ventrally by the canal of the jugular vein. The tympanic cavity proper corresponds to the vertical extent of the tympanic membrane; the dorsal epitympanic recess is continuous with the posterior tympanic antrum. The middle ear cavity

communicates with the nasopharynx by the antero-medially directed Eustachian tube and with the posterior mastoid air spaces. The tympanic membrane is attached to the annulus tympani, an incomplete ring of membrane bone (Anson and Donaldson, 1973).

An idealized cross-section through the seven- to nine-day-old mouse (fig. 26) illustrates the general relationships within the middle ear. The size and shape of the middle ear cavity and its related structures alter continuously throughout development due to both cranial growth and intrinsic changes. However, the relationships of the otic components to each other remain relatively constant. The ossicular chain, projecting antero-laterally from the fenestra ovalis to insert onto the tympanic membrane, is not yet completely suspended within the middle ear cavity. The three ossicles--the malleus, incus, and stapes--connect the external and inner ear. The malleus, composed of the head, lateral process, short anterior process, and malleus manubrium, inserts onto the tympanic membrane by the malleus manubrium. Its head articulates with the body of the incus by the incudomalleolar joint. The short process of the incus projects posteriorly within the middle ear cavity. The long process of the incus articulates by the lenticular process with the head of the stapes at the incudostapedial joint. The stapes

inserts by its footplate into the fenestra ovalis of the inner ear, thus connecting the external and inner ear. The anterior and posterior crura attach to the footplate. The persisting stapedial artery passes through the obturator foramen.

Mouse middle ear development spans a prolonged period, from 11 days post-coitum to 16 days after birth. However, at any one stage, while some component(s) may show significant histological differences from the preceding or subsequent stage, others may not. To facilitate description and discussion, I will first present an account of the overall anatomical changes which occur and follow this with a detailed description of the histogenesis of the individual components.

The first pharyngeal pouch and presumptive mesenchymal components of the middle ear are recognizable on day 11 post-coitum. By day 13, the ossicles can be identified surrounded by mesenchymal cells (fig. 27). The first pharyngeal pouch projects laterally toward the ventrally projecting malleus manubrium. The external auditory meatus is represented by a small depression on the surface of the head (fig. 28). The ossicles continue to differentiate (figs. 28 and 29) so that by day 17 post-coitum, a definitive footplate is suspended within the fenestra ovalis by a presumptive annular ligament (fig. 30). The

ossicular chain spans the middle ear region from the otic capsule to the presumptive tympanic membrane. The middle ear cavity continues to enlarge toward the ossicles, although at this stage the ossicles are still surrounded by mesenchyme (fig. 30). The external auditory meatus grows deeper toward the medial aspect of the embryo.

From days 1-14 after birth (figs. 31 and 32), the overall relationship of the middle ear components remain constant. The external auditory meatus is ventral to the middle ear cavity (fig. 31) until the middle ear cavity and external auditory meatus enlarge. The cavity continues to enlarge towards, anterior, and around the ossicles (fig. 32). The region adjacent to the ossicles is not completely devoid of mesenchyme until 16 days after birth (figs. 33 and 34). The ossicles are suspended within the middle ear cavity by ligaments (Proctor, 1964) (figs. 33 and 34). The middle ear cavity continues to expand and become continuous with the mastoid air spaces (Anson and Donaldson, 1973).

2. The Development of the Ossicles

The middle ear ossicles are first observed as mesenchymal condensations on day 11 post-coitum; by day 12 three precartilaginous condensations can be identified as the presumptive malleus, incus, and stapes. The stapedial

condensation, pierced by the stapedial artery, is adjacent to but not continuous with the otic capsule; the two cell populations are separated by a narrow light staining band. By day 13, the more densely packed prechondroblasts of the ossicles show a greater affinity for alcian blue than the otic capsule cells (figs. 27 and 35) and the cells of the stapedial condensation are no longer separated from the latter (fig. 27). The ossicles are positioned so that they span the middle ear region as a chain from the inner ear region to the presumptive tympanic membrane; the stapes abuts the incus which in turn abuts the malleus (fig. 27). The malleus is continuous with Meckel's cartilage. The chondrifying tissues of each ossicle are separated from the other by mesenchymal cells showing slight affinity for alcian blue; these mesenchymal cells differentiate into the ossicular joints (fig. 27). The distal portion of the malleus, the malleus manubrium, inserts ventrally into the endodermal aspect of the presumptive tympanic membrane. In the 15-day mouse embryo, a definitive stapedial footplate can be identified (figs. 28 and 29). The cells of the stapedial condensation are not completely continuous with those of the otic capsule. Medial to the stapedial condensation, the inner layer of the otic capsule (presumptive lamina stapedialis) can be identified by its different staining characteristics (fig. 29, light arrow).

Lateral to the footplate, the presumptive annular ligament cells show no affinity for alcian blue, although at this stage both the ossicles and otic capsule stain darkly (figs. 28 and 29, dark arrow). The presumptive annular ligament cells have similar staining characteristics to those of the presumptive perichondral tissue. The cells adjacent to the presumptive annular ligament and the ossicles joints remain chondroblasts; those of the ossicles and otic capsule are chondrocytes by day 17 (fig. 30). The malleus is still continuous with Meckel's cartilage. In the 17-day embryo, the footplate is suspended within the fenestra ovalis (fig. 30). There are no longer two distinct cellular regions within the footplate (compare figs. 29 and 30). A perichondrium has differentiated around the chondrifying tissue. The annular ligament will continue to differentiate so that in the two-day postnate mouse a distinct cellular configuration is observed (figs. 31, 36, and 37): the nuclei are aligned parallel to each other (figs. 36 and 37) and this alignment is maintained and observed in the older mouse (fig. 32). These ligamental cells show a strong affinity for alcian blue. The incudomalleolar and incudostapedial joints continue to differentiate so that in the newborn mouse they are easily recognized (figs. 31 and 32). In a six-day-old mouse, the joint capsule of the diarthrodial incudostapedial joint is

present (fig. 32). The ossicles begin to ossify in the four-day-old mouse, but the tissues adjacent to the annular ligament and ossicular joints remain cartilaginous (fig. 32). The malleus is no longer continuous with Meckel's cartilage. The chain orientation previously observed is maintained, although growth, remodeling, and ossification of the ossicles slightly alter their overall appearance (figs. 32, 33, and 34). The stapedial artery persists in the differentiated middle ear, passing through the obturator foramen (fig. 33).

3. The Expansion of the Middle Ear Cavity and the Histogenesis of the Endodermal Epithelium

An extension of the first pharyngeal pouch, the tubotympanic sulcus, projects toward the developing ossicles by day 12 post-coitum, its proximal and distal portions represent the presumptive Eustachian tube and the middle ear cavity respectively. By day 15, the dorsal and ventral epithelium of the Eustachian tube are distinct from each other (figs. 28 and 38), the dorsal is a simple squamous epithelium, while the ventral is thicker, its basal cells are columnar and there are one to two layers of ovoid to flattened suprabasal cells. Lateral to the Eustachian tube, the presumptive middle ear cavity is intermediate between the two epithelial types. In the ventrolateral

portion, a basement membrane staining with alcian blue is observed. A basement membrane could not be identified in other regions based on staining characteristics. The epithelium of the dorsal aspect of the Eustachian tube and middle ear cavity is convoluted and folded into the regions of mesenchymal cells (figs. 39 and 40) and a basement membrane could not be identified associated with this convoluted simple squamous epithelium. Marovitz (pers. comm.), using EM, shows that the basement membrane is present. Occasional mitotic figures are observed in the epithelial cells.

Cellular heterogeneity within the endodermal epithelium is observed by two days after birth (fig. 41). Ciliated and non-ciliated mucous secreting cells (fig. 41), ciliated non-secreting cells, and cuboidal-squamosal cells are observed within the epithelium of the middle ear cavity (figs. 41, 42 and 43); their quantitative and qualitative differences depend on the region and stage observed. The epithelium of the Eustachian tube and medial tympanic cavity consists of many ciliated and mucous secreting cells (fig. 41), while the epithelium surrounding the ossicles and on the inner aspect of the tympanic membrane is a simple squamous epithelium (figs. 42, 43, and 44). The middle ear cavity continues to expand towards the

ossicles. Subdivisions of the endodermal pouch wrap anterior and posterior to the region of the ossicles, so that by day 6 after birth, the middle ear cavity is anterior to the ossicles but mesenchymal cells still surround them (fig. 32). In the 14-day-old mouse, mesenchymal cells are still adjacent to the stapes and dorsal aspect of the malleus and incus. The middle ear cavity is not completely devoid of mesenchymal cells until 16 days after birth when the ossicles are completely suspended within the middle ear cavity by ligaments (figs. 33 and 34). A simple squamous epithelium invests the ossicles, ligaments, and inner aspect of the tympanic membrane (figs. 33, 34, and 44). Acellular granular material is frequently associated with certain cell types within the lateral region of the cavity and the tympanic membrane (fig. 45). This material is not secreted by the secretory cell types identified earlier (fig. 41).

4. The Fate of the Mesenchyme During Middle Ear Cavity Expansion

The presumptive middle ear region is initially occupied with typical undifferentiated mesenchyme (figs. 27, 28, and 29). As development continues, the mesenchyme becomes more loosely packed (fig. 30). By day 18, two cell types, stellate and ovoid, can be identified within the mesenchyme. The stellate cells show a strong affinity

for alcian blue and their processes appear as fibrous bundels radiating from the cell body. The ovoid cells are frequently associated with small blood vessels within the mesenchyme.

The quantity of mesenchymal cells continues to decrease resulting in a web-like meshwork (figs. 31 and 32). Macrophages and pycnotic cells are observed within the mesenchyme. The number and size of blood vessels within this region do not show a substantial increase. By day 6 after birth, the mesenchyme is still adjacent to the stapes, incus, and the head of the malleus (fig. 32) and dorsal aspect of the tympanic membrane. Within this mesenchyme, pycnotic cells are identified. The quantity of mesenchymal cells within the region continues to decrease so that in the 16-day postnate, the ossicles are suspended within the middle ear cavity (figs. 33 and 34). The significance of the mesenchymal elimination and its relationship with the expanding endodermal epithelium will be discussed later.

5. The Histogenesis of the Tympanic Membrane and the External Auditory Meatus

The pars flaccida and pars tympani are two regions of the mammalian tympanic membrane (Lim, 1968a, 1968b); this description will deal with the membrane as a single

structure. The membrane is composed of an outer portion continuous with the ectodermal epithelium of the external auditory meatus, and an inner portion continuous with the endodermal epithelium of the middle ear cavity. These two epithelia are separated by the connective tissue of the lamina propria which is derived from mesenchymal cells. The inner epithelium has already been discussed in the section dealing with the middle ear cavity. Therefore, this description of the tympanic membrane will deal with the outer epithelium and the lamina propria.

The external auditory meatus, a depression in the surface epithelium projecting antero-ventro-medially is recognized by day 12 post-coitum. In a 15-day embryo (figs. 28-46), the length of the external auditory meatus has increased and its innermost aspect is the presumptive tympanic membrane. The epithelium of the external auditory meatus consists of columnar germinal cells overlain by rounded peridermal cells. The epithelium of the presumptive membrane is thinner, consisting of cuboidal germinal cells. The differentiating auricle can be identified by the thickened dermal cells adjacent to the external auditory meatus. A basement membrane can be identified adjacent to the tympanic membrane by its affinity for alcian blue, while it could not be identified associated with the external auditory meatus. In

the 15-day embryo, the ectodermal epithelium of the tympanic membrane is separated from the inner endodermal epithelium by mesenchymal cells which show no alcian blue affinity (fig. 46).

The external auditory meatus continues to grow anteroventro-medially as a meatal plate so that the two epithelia cannot be distinguished. The meatal plate is located ventral to the middle ear cavity (figs. 39, 40, 42, and 43). The tympanic membrane continues to narrow so that the two epithelia (inner and outer aspects) are now separated by one to three layers of alcian blue positive mesenchymal cells. In the three-day postnate and older, the meatal plate begins to break down and the epithelia of the tympanic membrane and the external auditory meatus are distinct (fg. 43). The ectodermal face of the tympanic membrane, composed of cuboidal basal cells with a single layer of peridermal cells remains relatively straight. The thick integument of the external auditory meatus is folded and sebaceous glands can be recognized (fig. 43).

The tympanic membrane will continue to narrow and differentiate. Although the inner aspect does not complete differentiation until 16 days after birth, the outer epithelium shows its differentiated configuration much earlier (fig. 40). The membrane becomes thinner due to

the differentiation of the mesenchymal cells of the lamina propria into compacted collagen fibers. A keratinizing squamous epithelium is first observed six days after birth on the external auditory meatus and continues onto the face of the tympanic membrane (figs. 43 and 44).

E. Discussion

1. General Introduction

The general sequence of histogenic events in the differentiation of the avian and mammalian middle ear agrees with that given by Jenkinson (1911), Romanoff (1960), and Stephens (1972). The pattern of differentiation of the avian paratympanic organ essentially agrees with Vitali's (1911, 1914) and Yntema's(1944) accounts; this specialized organ will not be discussed further in this thesis since its morphogenesis is important only as far as it represents a useful landmark for experimental analysis.

2. The Expansion of the Pharyngeal Pouch and the Fate of the Middle Ear Mesenchyme

The expansion of the endodermal pouch into a region previously occupied by mesenchyme is but one example of a major developmental problem--how do "large spaces" develop? Romanoff (1960 , p. 538) ascribes to the developing

epithelium of the pneumatized spaces the ability to "push the marrow ahead," and elsewhere quotes previous authors as speaking of "liquefaction" (p. 377) or "resorption" (p. 379) of mesenchymal cells during otic organogenesis: none of these suggestions are documented.

What then is the mechanism whereby the pouch itself expands? This question is most easily discussed with reference to the avian embryo. The histology of the differentiated avian middle ear epithelium is less complex than that of mammals, there being a simple squamous epithelium throughout. However, my results show that the epithelium is heterogeneous during development (figs. 4, 6, 10, 16, and 17). It appears that the epithelial structure is simplest, and a basement membrane is lacking, wherever "expansion" is occurring, e.g., in the diverticulum anterior to the columella at stage 35 (fig. 8). The presence of sporadic mitotic figures suggests that an increase in epithelial surface area could be achieved by simple cell multiplication. However, no epithelium, especially one consisting of such attenuated cells as those seen in the expanding regions, could possibly push mesenchyme aside (vide supra), so that the fate of the latter must therefore be considered.

Some of the differentiated middle ear mesenchyme must give rise to the submucosal connective tissue. This,

however, would not seem to account for the large number of mesenchymal cells originally occupying this region, nor the presence of macrophages and blood vessels during development. There is little, if any, evidence of cell death in this region. It is generally believed that some macrophages originate in embryonic mesenchyme, and from there they may migrate into blood vessels (Bloom and Fawcett, 1975; Ham, 1974). The sequential appearance of macrophages and sinus vascular spaces connecting to the jugular vein, suggest to me that the mesenchyme of the middle ear region may be eliminated as it performs this hemopoetic function. I interpret the celomic spaces (figs. 18 and 19) as the earliest stages of sinus differentiation. The events discussed above occur prior to the appearance of spaces with a continuous endodermal epithelium, and this supports my interpretation that the expansion of the pharyngeal pouch derivative occurs by simple epithelial growth following mesenchymal elimination. My observations pertaining to the mesenchyme of the avian middle ear, and the mechanism of subsequent growth of the endodermal pouch into that region, suggest that the middle ear cavity has no celomic component.

The developmental cytology of the mammalian middle ear is somewhat better known, but has been subject of a questionable interpretation, notably the possibility of

celomic contributions to the mature tympanic cavity. This interpretation was predicated on two factors, the heterogeneity of cell types in the adult epithelium and putative epithelial discontinuities during the process of pouch expansion. The presence and differential topographic distribution of heterogeneous cell types within the tympanic cavity has been interpreted as indicating a dual germ layer origin of the epithelium of the cavity (Buch and Jorgensen, 1964; Hussl and Lim, 1969; Schwartzbart, 1958). Recent investigations interpret the presence and distribution of heterogeneous cell types as indicating differentiation from a common stem cell (Hentzer, 1976). The differential differentiation of the cell types is probably due to epithelial mesenchymal interactions in the different regions of the tympanic cavity (Lim, 1974). Epithelial discontinuities within the tympanic cavity were described by Marovitz and Porubsky(1976) in the developing rat and I have reinvestigated this problem. The epithelium of the tympanic cavity was thrown into extensive and convoluted folds which infolded deep within the undifferentiated mesenchyme (figs. 39 and 40). It was frequently difficult to follow a single epithelium within an embryonic series. After careful examination of the development of the mouse middle ear, I could find no evidence of epithelial discontinuities. I conclude

that the entire tympanic cavity epithelium of both birds and mammals is derived from an endodermal epithelium. This conclusion, supported by recent cytological investigations on the mammalian middle ear (Hentzer, 1976; Lim, 1974) concur with current thought on the monophyletic origin of the anniote middle ear (Allin, 1975).

In mammals, the problem of the mechanism of pouch expansion into a region previously occupied by mesenchyme is as perplexing as in the chick. Otology textbooks usually discuss this problem with a few cursory sentences, e.g., "the loose mucoid connective tissue has given way to the expanding tympanic epithelium" (Anson and Donaldson, 1973). Even when the development of the middle ear has been investigated in mammals, the descriptions of the mesenchyme undergoing liquefaction, resorption, dissolution (Guggenheim et al., 1956; Guggenheim, 1971), or the pouch eroding the mesenchyme (Stephens, 1972) do not offer mechanical explanations. Guggenheim and his co-workers (1956, 1971) have investigated the fate of the mesenchyme within the middle ear cavity. Two cell types are observed within this mesenchyme: one disintegrating into a disorderly melee or modulating into macrophages and migrating via fluid filled cavities, the other retreating toward the lateral wall to line the cavity. According to their observations, mesenchyme contributes to the epithelium of

the middle ear cavity, notably the tympanic antrum region, suggesting that the epithelium has only a minor contribution from the endodermal pouch in this region. I agree with the description of mesenchyme within the middle ear region; two cell types are observed within this region. Cell death is observed within the mesenchyme, increasing as development continues and usually associated with an increase in the number of macrophages. I therefore agree that some mesenchymal cells differentiate into macrophages, as stated by Ham (1974). In the mouse embryo, these cells are usually associated with small blood vessels within the mesenchyme, but I could find no evidence of the extensive vascularization observed in the chick embryo. The macrophages eliminate the dead cell debris from the region and migrate via these small blood vessels. Yet, I could not account for the majority of the mesenchymal cells. A portion of the other cells probably differentiate into the submucosal connective tissue, the periosteum, and other components of the middle ear region. It appears unlikely that this convoluted simple squamous epithelium could push the mesenchyme in front of it. Instead, the mesenchymal cells are eliminated by a combination of cell death, differentiation, and/or migration. Only after the mesenchymal cells are absent from the region can the epithelium expand.

Is the mesenchyme within this region programmed to be eliminated? Foley et al. (1965) successfully cultured rat middle ear mesenchyme in vitro and reported that cells remained viable and underwent cell division. Therefore, it appears that the mesenchymal cells must be influenced by some environmental factor(s) during development that induce cell death, differentiation, and elimination. Marovitz et al. (1967, 1968) indicate that this process might be under endocrine control. They investigated dwarf Snell mice and hypophysectomized rats and observed that the mesenchyme persisted in the middle ear region and the otic capsule and ossicles retained a juvenile morphology.

What is the mechanism of pouch expansion? The presence of mitotic figures within the epithelium and the absence of an observable basement membrane indicate that the epithelium increases by simple growth. A folded epithelium is observed in certain regions of the cavity. This folding probably accommodates a rapid expansion of the pouch in a short period of time. Because the epithelium increases in size by simple growth, a rapid increase in surface area would be difficult to accommodate. Therefore, the epithelium increases its surface area very early in development at a constant rate, and this "excess" epithelium is available when needed.

The middle ear cavities of chick and mouse are dissimilar in certain respects. The chick middle ear is lined by an homogeneous simple squamous epithelium while the mouse epithelium shows cellular heterogeneity. This difference in cytology is probably important in understanding the secretions and/or clearance of material from the middle ear, a topic which will not be discussed in this thesis. Epithelial differences should have no effect on pouch expansion and mesenchymal elimination.

Are mesenchymal cells eliminated by a common mechanism in the chick and mouse? One basic mechanism is observed in both embryos: the differentiation and/or migration of mesenchymal cells. In the mouse embryo, there is the additional mechanism of cell death, which seems to be insignificant in the chick embryo. In the chick, the mesenchymal cells are predominantly eliminated by differentiation and cell migration via the extensive vascularization. In the mouse, these cells are mainly eliminated by cell death and differentiation, with a small number migrating within the vascular system. In both animals, once the region is devoid of cells, the pouch can expand by simple epithelial growth. The morphological differences in mouse and chick epithelia (see above) are probably an accommodation for the different rate of mesenchymal elimination from the middle ear cavity (see below).

3. The Differentiation of the Tympanic Membrane in the Chick and Mouse

The differentiated trilaminar tympanic membrane is composed of an outer ectodermal aspect, an inner endodermal aspect, and an intermediate lamina propria. The outer aspect is a simple keratinizing squamous epithelium continuous with the external auditory meatus; the inner aspect is a simple squamous epithelium continuous with the epithelium lining the middle ear cavity. The lamina propria is composed of compacted collagen fibers. The cytology of the tympanic membrane is similar in both the chick and the mouse (compare figs. 17 and 44), although the pars flaccida portion of the mammalian tympanic membrane has different histological characteristics. Since this structure is not important in the context of this thesis, I will not discuss it further. The relative stages of development of the middle ear and the tympanic membrane are significantly different in the chick and the mouse (Table 1), especially with respect to the rate of mesenchymal elimination from the tympanic cavity.

In the chick, the columella is suspended within the tympanic cavity by HH stages 37-38 (11-12 days). At this time, the inner aspect of the membrane is a simple squamous epithelium, while the intermediate mesoderm and the outer ectodermal epithelium must subsequently

TABLE 1

A COMPARISON OF THE DEVELOPMENT OF THE MIDDLE EAR IN THE CHICK AND MOUSE*

	Chick/ HH Stages	Mouse/Days Gestation
Ossicle(s) condensation	25	11
Ossicle chondrification	32-34	14
Footplate 'fusion' with otic capsule	32-34	15**
Footplate suspended by annular ligament cells	35	15-16
Annular ligament differentiation	38	18-22 (1 day postnate)
Ossicle(s) ossification	41	25 (4 days postnate)
Middle ear cavity surrounds ossicle(s)	38	16 days postnate
Tympanic Membrane differentiation		
a. inner aspect	37-38	14-16 (postnate)
b. outer aspect	40-41	2-16*** (postnate)
Keratinization	41	6 (postnate)

*The stage that a character is first observed will be given except in cases where it continues over a period of time.

**The footplate is never completely continuous with the otic capsule (see results).

***The appearance of the ectodermal epithelium changes little from days 2-16 postnate.

differentiate to culminate in the differentiated membrane. By HH stage 41 (15 days) the outer epithelium is keratinizing. The compacting of the intermediate lamina propria is observed and continues after hatching. In the mouse embryo, the ossicles are not completely suspended within a middle ear cavity until 16 days after birth (Table 1). Prior to this, the inner portion of the membrane is not completely differentiated and remains separated from the ectodermal epithelium by a large mass of mesenchyme. The ectodermal epithelium differentiates much earlier: by three days after birth the meatal plate begins to break down and epithelia begin to show their differentiated histological characteristics. Keratinization begins in the meatus on day 6 and this process continues until the ectodermal epithelia are keratinized. The late elimination of the mesenchyme from the middle ear region may also account for the relatively late appearance of a keratinizing epithelium relative to the differentiation of the other components of the middle ear. A keratinizing epithelium may be influenced by the densely compacted lamina propria which only appears after the mesenchyme condenses and the endodermal epithelium differentiates. Many workers have investigated the differentiation of skin appendages in the chick embryo (see review, Sengel, 1967). The

dermis controls the differentiation of the ectodermal epithelium. Therefore, the lamina propria could be inducing the differentiation of the ectodermal epithelium of the membrane. It appears that the differentiation of the tympanic membrane may be the result of epithelial-mesenchymal interactions, controlled by the differentiation of the endodermal epithelium.

In comparing the development of the chick and mouse tympanum, I observed that it is retarded in the mouse. Is the development of the mouse middle ear generally retarded relative to the chick? I examined a six-day-old mouse and compared the middle ear components with those of the chick embryo (Table 1). The middle ear cavity is still relatively filled with mesenchyme, compared to a chick HH stage 36 (11 days). The ossicles, on the other hand, are relatively well advanced; they are beginning to ossify. Columella ossification is observed in chick HH stages 41-42 (15-16 days) (Table 1). The development of the endodermal aspect of the tympanum is retarded in the mouse. Yet, it appears that the other components of the mouse middle ear are more advanced than the equivalent structure in the chick. The functional significance of this relatively late elimination of the mesenchyme from the middle ear region of the mouse, and

the accompanying late differentiation of the tympanum, is not clear.

4. The Histogenesis of the Ossicles with Special Emphasis on the Differentiation of the Fenestra Ovalis Region

The ossicles are derived from the visceral arches (Hansen et al., 1962; Smith, 1905) with the stapedial (columella) footplate receiving a contribution from the otic capsule (Reagan, 1917; Anson et al., 1960). Recent sophisticated techniques applied to the chick embryo have indicated a very widespread contribution of the neural crest to a variety of cranial tissues (Le Lievre and Le Douarin, 1975) including possibly the otic capsule as well as the ossicles (Le Lievre, pers. comm.). It would appear that ossicle differentiation, particularly with respect to the formation of the region of the fenestra ovalis, is a complex event and its embryogenesis must be considered in terms of modern theories of cell differentiation.

The relative simplicity of the chick middle ear with its single ossicle permits a presentation of the major steps in the process of differentiation of the fenestra ovalis region. The results of the present study have depended heavily on the staining characteristics of the otic tissues, with alcian blue as an indicator of

chondrogenesis and chlorantin fast red as an indicator of osteogenesis (Lison, 1954; Hall, 1972). Figure 47 is a schematic representation of the major steps in the process of differentiation. Figure 47 details the heterogeneity of the skeletal tissues within this region by stage 41. The problem is therefore to determine exactly which components of the "footplate" arise from which embryonic precursors.

The region of undifferentiated mesenchymal cells between the chondroblast populations of the proximal columella and the otic capsule (fig. 47A) appears at first sight to move towards the inner ear as indicated by its initial narrowing and subsequent translocation (figs. 47B adn C). The final result of this apparent "migration" is the delineation of the peripheral annular ligament and the proximal perichondrium, and the appearance of a uniform chondrocyte population throughout the columella (figs. 47D and E). Does this imply that the chondrogenic cells of the columella invade the more proximal region, so that the cells of the otic capsule are mechanically forced towards the more proximal and peripheral regions, or are we observing the effects of inductive interactions between adjacent cell populations? A mechanical action seems unlikely due to the fact that there exists a band

of nonmatrix-surrounded cells between the two cell populations during stages 28-34. However, it is apparent that the sequential patterns of histogenesis of the cells of the otic capsule surrounded by the rectangles in Figures 47B and C are quite different than those of adjacent cells. The perichondrium becomes distinct on the surface of the presumptive footplate well before it is differentiated elsewhere, but the chondrocyte maturation is relatively delayed. Although the entire thickness of the otic capsule consists of chondroblasts with alcian blue positive material around them by stages 28-30 (fig. 47A), as the band "migrates" across this region during stages 32-34 (figs. 47B and C), this matrix material disappears and the cells therein become elongated, oriented parallel to the proximal face of the footplate (fig. 14). Such dedifferentiated cells appear to eventually augment the precociously produced columella perichondrium most proximally, or differentiate peripherally as annular ligament. Thus, although all the cells enclosed by the rectangles in Figures 47A-E are potentially chondrogenic, patterns of specific interactions operating with elapsed time appear to modulate this potential. I interpret the developmental sequence as showing that the columella chondrocyte population influences the proximal capsular chondrocytes,

producing retardation and regression of their chondrogenic potential. On the basis of alcian blue staining intensity, it appears that columella chondrocyte differentiation is initially more advanced than in the capsule (figs. 12 and 14, and page 25). The cells of the "light band" might therefore be influenced initially by columella chondrocytes, but eventually, when the band has "retreated," they might then come under the retarding/regressing influence of capsule chondrocytes. Caught between the modulating influences exerted by the columella and capsular chondrocytes, the subsequent differentiation of the cells in the light region proceeds as follows. Those closest to the two differentiated chondrocyte populations are determined respectively as the persistent perichondrial tissues of the mature fenestra margin and the periphery of the mature footplate (fig. 47F), while the remainder never chondrify, they differentiate as ligamental tissues. The results of experiments using teratogens to influence middle ear development (see Section III) can be interpreted according to this model and will be discussed later.

The differentiation of the fenestra ovalis region in the mouse, appears at first glance, to be unlike that observed in the chick, but closer evaluation indicates that the major steps of this process agree with those

described above. The stapedial condensation is initially separated from the otic capsule by a region of undifferentiated mesenchymal cells. By day 13, the two cell populations are no longer separated by a "light band." The more densely packed cells of the stapes are distinguished from the adjacent capsule cells by their staining characteristics (fig. 27). The cells derived from the otic capsule, the lamina stapedialis, are not chondrogenic. Unlike the morphology in the chick embryo, the appearance of a uniform chondrocyte population in the footplate is observed early in the development (fig. 31). The cellular configuration of the lamina stapedialis and presumptive annular ligament are quite distinct from the adjacent chondroblasts of the footplate and capsule (figs. 29 and 30). This cellular heterogeneity within the fenestra ovalis region permits the interpretation of its development according to the model presented above for the chick embryo. The differentiation of the capsular component, the lamina stapedialis, is probably influenced initially be the stapedial chondrocytes, but eventually they come under the influence of the capsule chondrocytes. Caught between the modulating influence of the stapedial and capsular chondrocytes, differentiation proceeds as follow. Those cells closest to the vestibular (proximal) aspect differentiate into the perichondrium of the footplate and the fenestra

margin, while the remaining cells form the ligament. The annular ligament inserts only onto that aspect of the footplate derived from the lamina stapedialis (Marovitz and Shapiro, 1969), indicating an affinity in cellular origin (figs. 36 and 37).

Two obvious dissimilarities between the development of the fenestra ovalis region in the chick and mouse are the different morphology of the annular ligament and the absence in the mouse of an intermingling of capsule and hyoid arch cells. In the chick, all the cells within the region are chondrogenic so that the presumptive annular ligament cells are initially identical with the surrounding chondroblast cells. Only subsequence differentiation followed by the attainment of ligamental form, permits their identification. In the mouse, on the other hand, the presumptive annular ligamental cells are not chondrogenic and are always distinct from the adjacent stapedial and capsular chondroblasts. Early in development the ligamental cells show an alignment of their nuclei (figs. 36 and 37); this configuration is maintained in the differentiated structure but is absent from the chick. This morphological difference might be related to the second dissimilarity, the absence of intermingling of hyoid arch and capsular cells in the presumptive footplate. In both the chick and mouse, these two cell

populations are initially separated by a "light band" of undifferentiated mesenchymal cells. In the chick, it appears that the translocation of this band and its interaction with the stapedial and capsular chondrocytes result in the differentiation of the perichondrium and annular ligament. In the mouse, this "light band" is not observed at a very early stage, yet the cells of the lamina stapedialis are distinct from adjacent prechondroblasts in this region (fig. 20). The differences in staining characteristics of the different cell population indicate that these cells remain separated throughout the process of development. It is possible that cellular interactions similar to those observed in the chick do occur in the mouse, but the transitory "light band" is not observed.

SECTION III

EXPERIMENTAL STUDIES

A. Chorioallantoic Membrane Grafting Experiments

1. General Introduction

Two types of experimental studies could be used to investigate the development of the avian middle ear, *in vivo* or *in vitro*. Because of the inaccessibility of this region in the embryo, *in vitro* studies seem to be the logical initial experiments. Other organ systems containing skeletal tissues do not show normal joint differentiation *in vitro* (Bradley, 1970) but may show good development on the chorioallantoic membrane. Since I am interested in the development of the fenestra ovalis region and the differentiation of the footplate and the annular ligament (the "joint" region of the avian middle ear), I decided to determine the developmental capacities of middle ear rudiments as chorioallantoic grafts to evaluate the merits of this technique for further studies.

2. Materials and Methods

Chick embryos HH stages 15-29 removed from the egg and dissected in balanced salt solution (Earle's Balanced

Salt Solution [IX], Grand Island Biological Co.) under sterile conditions. The embryo's head was severed from the pharyngeal region posterior to the aortic arches, and the anterior region including the eyes was removed from it. The remaining tissue was bissected so that right and left sides could be grafted. Each donor ear region contained a portion of the brain, an otocyst, and one-half of the pharyngeal tissue and aortic arches. Following removal of the ear region, a number of "donor" tissues and donor embryos were selected at random and prepared for histological examination. Three types of donor tissue were grafted. In most, the entire otic region, including a portion of the rhombenchephalon, the otic vesicle, visceral and aortic arches, and the associated endodermal and ectodermal epithalia were grafted. In ten of these, the heart and aortic arches were also included. In two other series, either the otic vesicle, the associated surrounding mesenchyme, and the brain together, or the otic region minus the otic vesicle and the brain were grafted. The donor tissue was grafted onto the seven- to nine-day chorioallantoic membrane (CAM) (Hamburger, 1960) and recovered five to ten days later, depending on the age of the donor tissue at extirpation. The grafts were allowed to develop to at least an equivalent of HH stage 34, the

minimum age at which middle ear differentiation can be reasonably assessed (see page 25).

The grafts were recovered, fixed in Bouin's fixative for twenty-four hours, and processed for histological examination as described previously. Serial sections of 133 grafts were examined and scored for completeness of differentiation.

3. Results

a. Evaluation Criteria

The recovered grafts were examined with reference to a variety of structures identifiable in an intact middle ear region at HH stage 36 (fig. 10; normal development). Various specific components were evaluated for presence or absence, and if present, for their degree of differentiation.

1. Inner ear region: The presence of an inner ear sensory epithelium, surrounded by a cartilaginous capsule facilitated the identification of other components.

2. Columella: The single middle ear ossicle is normally suspended proximally within the fenestra ovalis by the annular ligament, and inserts distally onto the tympanic membrane (fig. 10). A cartilage which had such a characteristic shape and insertion was scored "normal"

(fig. 48), one defective in any of these characteristics was scored "deformed" (figs. 49-56).

3. Middle Ear Cavity: The avian middle ear cavity is a space lined by a simple squamous epithelium of endodermal origin, surrounding the columella (fig. 10; see page 23) and its histological characteristics are therefore clearly distinguishable from other endodermal epithelia (Hodges, 1974) or from ectoderm (see page 32).

4. The External Auditory Meatus: The meatus is a depression on the surface integument whose deepest component is the ectodermal face of the tympanic membrane. The inner (endodermal) face of the membrane is separated from the ectoderm by a mesodermal derivative, the lamina propria. The positive evaluation of a meatus/tympanic membrane depended mainly on the presence of such distinctly recognizable ectodermal and endodermal epithelia in the vicinity of the extracolumella (fig. 56).

5. The Paratympanic Organ: This is an asymmetrical vesicular structure with a sensory epithelium containing characteristic alcian blue positive material within the lumen (fig. 57). It derives in part from an epibranchial placode (Yntema, 1944) and yet completes its development within the middle ear region, initially breaking off from the most distal extremity of the tubotympanic sulcus

(Yntema, 1944). It is a useful landmark for identifying the middle ear region.

6. Neural Tissue: Since the brain was grafted in most experiments, the presence or absence of recognizable neural tissue in a graft permits analysis of the possible role of such tissues in controlling development.

7. Jaw Components: In evaluating those experiments where both the brain and otic vesicle were removed from the explant, the otic capsule should be absent (Reagan, 1917; Simons, 1974, 1975). To assess the competence of the mesenchyme remaining in such explants, the bones and cartilages associated with the jaws were evaluated.

b. Graft Evaluation

In 96/110 grafts developed from the complete otic rudiment, the inner ear epithelium and otic capsule are recognizable (Table 2). In the remaining fourteen, although cartilage may be present, it is impossible to evaluate otic differentiation. The differentiation of the middle ear and tympanic tissues are not as consistent as that of the inner ear (Table 2).

A columella is present in seventy-five grafts. In nearly half of them, the ossicle shows the characteristic columella-extracolumella configuration. Although all three

TABLE 2

SUMMARY ANALYSIS OF AVIAN OTIC
RUDIMENTS GROWN ON THE CAM

Donor H-H Stage	#	IE	Columella	MEC	EAM	TM	PT	Neural Tissue
16	10*	6	5	5	0	1	1	9
17	9	8	6(2)***	4	3	0	1	6
18	12**	10	7	3	1	0	3	11
19	33	32	29 (4)	23	4	0	14	24
20	17	17	11	9	0	0	8	9
21	5	5	2 (1)	1	0	1	0	3
23	3	2	2	2	1	0	2	0
24	2	2	1	1	0	0	1	0
25	11	10	9 (2)	6	0	1	5	6
26	5	3	2	1	0	0	0	1
29	3	1	1	0	0	0	0	0
Total	110	96	75 (9)	55	9	3	35	68

NOTE: # = the number of grafts recovered; IE = inner ear epithelium; MEC = middle ear cavity; EAM = external auditory meatus; TM = tympanic membrane; PT = paratympanic organ.

*Two of these grafts were set with heart and aortic arches.

**Eight of these grafts were set with heart and aortic arches.

***In the columella scoring, those "normal" are shown in parentheses after the total number identified at any age.

processes of the extracolumella are rarely present, the extracolumella is usually observed. In nine grafts the differentiation is so complete that the columella footplate is suspended within the fenestra ovalis by the annular ligament (fig. 48). I have arbitrarily subdivided the columella abnormalities observed in the other grafts into three categories defined as follows.

Type I: the footplate is either completely (45/75) (figs. 49 and 50) or partially (3/75) (figs. 51 and 52) fused with the otic capsule. The chondrocytes of the columella are continuous with those of the otic capsule. The annular ligament does not differentiate (figs. 49-52). The perichondria of the columella shaft and otic capsule are contiguous.

Type II: the columella is not fused with the otic capsule and does not insert into a fenestra ovalis (16/75) (figs. 53 & 54). The columella is separated by a small (fig. 54) or large (fig. 53) distance from the otic capsule; the two skeletal components are separated by a perichondrium. There is no differentiation of an annular ligament suspending a footplate within a fenestra ovalis. Yet, in many cases, the characteristic shape of a footplate is often identifiable (fig. 53).

Type III: the morphology of the columella is abnormal. This category can include a wide range of

deformities, ranging from a slightly abnormal columella to the absence of the footplate, shaft, and/or extracolumella (fig. 59). In 35/110 grafts, a columella could not be identified, indeed in sixteen of these grafts, the absence of recognizable inner ear tissues precluded identification of a middle ear region.

The differentiation of the other components of the middle ear are not consistently associated with the columella nor with each other. A cavity associated with the middle ear structures was designated the tympanic cavity, regardless of germ layer origin of the epithelia (see discussion p. 83). This structure is observed in 55 grafts (figs. 48, 49, 50, 52): in many instances, the epithelium associated with the middle ear cavity is continuous with the surface eptihelium (figs. 54 & 55). It is not unusual to observe a keratinizing ectodermal epithelium continuous with a "digestive tract" endodermal epithelium in the grafts. Because of the presence of epithelia of both ectodermal and endodermal origin, and the extensive folding of the surface epithelia, it is quite difficult to identify a true depressed external auditory meatus. In the nine grafts where the external auditory meatus is identified, it is due to its association with otic components (fig. 50): other regions of epithelial infoldings are ignored. Perfect differentiation of the two epithelial faces of the tympanic

membrane is only observed in three grafts (fig. 56). This is due in part to the absence of the external auditory meatus. A well differentiated columella is not necessarily associated with a differentiated tympanic membrane, and in Figure 56, only the extracolumella is present. In the absence of a true tympanic membrane, the extracolumella inserts into the connective tissue adjacent to the endodermal epithelium (figs. 48 & 49) or in the mesodermal tissue surrounding the columella (fig. 53).

A possible mechanical effect is suggested by the abnormal differentiation frequently observed in the fenestra ovalis region. The otic region in vivo is usually associated with the development of the heart and aortic arches. In ten grafts, I included the heart and aortic arches with the otic rudiment. Differentiation in these grafts is identical to those lacking the heart and aortic arches.

Differentiation of the paratympanic organ is often indistinguishable from the normal intact embryo (compare figs. 23 and 57). The paratympanic organ differentiated in association with the seventh nerve (Yntema, 1944). Therefore, it is interesting to determine whether neural tissue is necessary for the differentiation of the paratympanic organ on the CAM. The sensory organ is frequently, but not always, associated with nervous tissues. Many grafts containing neural tissue lack the paratympanic organ.

Table 3 presents the summary of the differentiation of the grafts from the otic region minus the otic vesicle and brain. The otic vesicle and brain are present in 4/14 and 2/14 grafts suggesting experimental error. In five grafts, a columella is not fused with any structure and the footplate is absent (Type II abnormality). The extra-columella is the only portion of the ossicle present in the single graft with a tympanic membrane (Type III abnormality). The columellae are frequently associated with a middle ear cavity. The paratympanic organ is only present in one graft, possibly indicating a relationship between the nervous tissue and this sensory structure. In the absence of the otic vesicle (12/14) or a paratympanic organ (13/14), it is often difficult to identify otic tissue. It is possible that some jaw skeletal components might really be deformed columellae.

All nine grafts from the donor tissue including the otic vesicle, associated mesenchyme, and the brain, showed inner ear differentiation (Table 4). A columella-like structure is fused with the otic capsule in 2/9 (Type I abnormality). Apart from the single paratympanic organ, no other components of the middle ear are present.

c. Summary of Results

1. The otic rudiment differentiates on the CAM.

TABLE 3

SUMMARY ANALYSIS OF AVIAN OTIC RUDIMENTS WITHOUT THE OTIC VESICLE AND BRAIN GROWN ON THE CAM

Donor H-Stage	#	IE	Columella	MEC	EAM	TM	PT	NT	JAW
17	3	0	2	1	0	1	0	1	1
18	4	1	1	1	0	0	1	1	2
21	1	0	1	1	0	0	0	0	0
23	5	1	1	1	0	0	0	2	2
26	1	0	0	0	0	0	0	0	1
Total	14	2	5	4	0	1	1	4	6

NOTE: NT = neural tissue; JAW = identifiable jaw cartilages and bone; other abbreviations as in Table 2.

TABLE 4

SUMMARY ANALYSIS OF AVIAN OTIC VESICLE
AND BRAIN GROWN ON THE CAM

Donor State	#	IE	Columella	MEC	EAM	TM	PT	Neural Tissue
21	4	4	0	0	0	0	1	0
23	5	5	2	0	0	0	0	3
Total	9	9	2	0	0	0	1	3

NOTE: Abbreviations as in Table 2.

2. The columella differentiates in 75/110 grafts, over 50% show the characteristic columella-extracolumella configuration.

3. Three categories of columella abnormalities are observed:

TYPE I: the fusion of the footplate and the otic capsule;

TYPE II: the absence of the footplate and the separation of the columella shaft and the otic capsule; and

TYPE III: deformed morphogenesis of the columella.

4. The differentiation of the other components of the middle ear are not related to the differentiation of the columella, or each other.

5. A differentiated tympanic membrane is rarely present.

6. Abnormal epithelial associations are often observed in the grafts.

7. The paratympanic organ is well differentiated and provided a good landmark for locating the middle ear region.

8. Grafts of the otic rudiment minus the vesicle and brain are difficult to evaluate for middle ear structures. When the columella is observed, it shows a

Type II abnormality.

9. Grafts of the donor material including the otic vesicle, mesenchyme and brain, all have an inner ear. A columella-like structure appears as a rod fused with the otic capsule; the extracolumella is never identified.

4. Discussion

a. General Conclusions

At stage 23, the only morphologically recognizable primordia of middle ear structures are the tubotympanic sulcus and the paratympanic organ (p. 33). Not until stage 25 is the columella condensation first visible. The majority of the grafts show some degree of columella differentiation. There are no significant differences in the patterns of development from donor material stages 15-23. These observations clearly indicate that the determination of the columella occurs very early in development. Although the columella is present, the degree of differentiation ranges from normal to extremely deformed. It was rare that a completely normal columella is present. It is of interest that the fundamental shape is frequently manifested even when adjacent tissues are absent or defective, indicating a strong genetic component in skeletal differentiation (Hall, 1975).

Recent experiments by Simons (1974, 1975, 1976) confirm Reagan's (1917) work concerning the origin of the columella footplate. Unilateral extirpation of the otocyst results in the absence of the inner ear epithelium, the otic capsule, and the columella footplate; the other components of the columella are observed. These experiments clearly demonstrate that the footplate receives a contribution from both the otic capsule and the hyoid arch. Results from the CAM grafting experiments should provide further support for this conclusion. In the absence of the otic capsule and sensory epithelium, a footplate should be absent. Table 3 shows that whenever I observed the columella, a footplate is always absent (Type II abnormality). In the opposite experiments in which the otocyst and surrounding mesenchyme and brain are grafted, I would expect the presence of a footplate fused with the otic capsule, as has been reported by Friedmann et al. (1977) where the otocyst was grown in vitro. In two grafts (Table 4), a footplate fused with the otic capsule is observed (Type I abnormality). The columella shaft and extracolumella are absent. While these observations agree with the results of Reagan (1917) and Simons (1974, 1975, 1976), the majority of my grafts do not clearly support these conclusions.

In the grafts derived from the entire otic region,

a normal footplate is observed in nine grafts (Table 2). More commonly, one of the three categories of columella abnormalities is observed. Type II abnormality, similar to those observed in the grafts of the otic vesicle alone (Table 4) or in vitro studies (Friedmann et al., 1977) is frequently observed. The footplate is fused with the otic capsule, and it is difficult to distinguish where one chondroblast population ends and the other begins. It is very common that Type I abnormality--with the absence of the footplate and the fenestra ovalis region--is observed. This result is similar to those obtained by grafting the otic region without the vesicle (Table 3) or extirpation experiments (Reagan, 1917; Simons, 1974). Yet even when the columella shaft is separate from the capsule, the fundamental shape of the ossicle, including the shape of the footplate, is frequently observed. Therefore, the factors which influence the normal development of the footplate region are extremely complex and must be considered further.

b. Mechanical effects on middle ear development

The normal morphogenesis of skeletal structures and muscles in many embryonic systems has been shown to be dependent on mechanical factors (Bradley, 1970; Hall, 1971). Chick limbs grown in vitro have degenerated muscles and

abnormal joint differentiation (Bradley, 1970). When the limb was grown on the CAM, the muscles and joints frequently showed normal development (Bradley, 1970). Hall (1971) determined that the quadrate-quadratojugal joint would differentiate on the CAM graft, while in organ culture it would only differentiate in the presence of movement of the skeletal components. Therefore, it appears that any system containing skeletal components and joints might differentiate normally on the CAM if proper mechanical tension is provided. The chick middle ear region includes a single "joint," the insertion of the columella footplate into the fenestra ovalis. In many of the recovered grafts, the "joint" is fused--is this due to abnormal mechanical tension in the grafted otic region?

The posterior head region of the developing early embryo is normally under extreme mechanical stress, due to the constant dilation and contraction of the heart and aortic arches. The majority of the grafts did not include the heart and the complete aortic arches. To determine whether the addition of these structures would improve the degree of differentiation, the heart and aortic arches were included in ten grafts (see footnote, Table 2). There is no significant difference in the degree of differentiation in these grafts. However, I cannot exclude the role of all mechanical actions during development by this single

experiment, and the problem be discussed further in relation to other experiments (see Part III,

c. Cellular interactions in middle ear development

Transplantation, in vivo, and in vitro experiments on the differentiation of the otocyst and the otic capsule (Friedmann, 1969; Hoadley, 1924; Lewis, 1907; Simons, 1975; 1976) indicate the influence of the otocyst on otic capsule differentiation. A few of these works have included observations on the middle ear (Friedmann et al., 1977; Reagan, 1917; Simons, 1975, 1976), but otherwise little experimental work on the middle ear is available. Helff (1928, 1931, 1940) investigated the development of the anuran tympanic membrane. His work indicated the importance of the columella and other skeletal components in tympanic membrane formation. The tympanic membrane will only differentiate in the presence of skeletal components. Because so much of our information on development is based on amphibian experiments (Chibon, 1967; Holtfreter, 1968; Horstadius, 1950), it is logical to conclude from Helff's experiments (1928, 1931, 1940) that a well differentiated columella might be associated with a differentiated tympanic membrane. This is not observed in the CAM grafts. A differentiated tympanic membrane is observed in four grafts, and in each case, it is associated with only the extracolumella portion of the ossicle. The relative absence

of a differentiated tympanic membrane may be due to the absence of an external auditory meatus. Therefore, even when the endodermal epithelium of the tympanic membrane is observed, a definitive tympanic membrane could not be identified. It is difficult to determine the germ layer origin of the epithelia observed in the CAM grafts: I frequently observe endodermal and ectodermal epithelia continuous on the grafts' surface. This abnormal epithelial association is probably due to the artificial exposure of epithelia and mesenchyma which are normally never in contact. Since the extent to which the host chorioallantoic membrane might interact with the competent donor epithalia and mesenchyma has been inadequately studied (Sawyer, 1975; per. comm.), the melange of different differentiated epithelia might be of donor or host origin. The absence of correlation between differentiation of the other middle ear components and the columella warrants further investigations. The degree of differentiation of the middle ear components on the CAM cannot be predicted. Therefore, further experimental studies on the differentiation of the columella should be conducted in vivo. Experiments involving teratogenic intervention of normal development were conducted, and their results will be present later.

The paratympanic organ is associated with the middle ear during late embryonic, posthatching, and adult stages.

This sensory organ develops from an ectodermal placode in association with the seventh nerve (Yntema, 1944). I tried to correlate the differentiation of the paratympanic organ with the presence of nervous tissue. The presence of a single paratympanic organ associated with nervous tissue in Table 3 supports the theory that the paratympanic organ is associated with nervous tissue. However, nervous tissue is present in a graft, this organ can be absent. In the grafts of the otic vesicle (Table 4), the paratympanic organ should be absent since the surface epithelium was not included in the graft. The other graft results do not support nor disprove the possible relationship between the paratympanic organ and nervous tissue. This sensory organ is present in many grafts containing nervous tissue, but other grafts that contain nervous tissue are lacking the organ. These results could be due to the incorrect identification of a vesicular structure without a sensory epithelium as a paratympanic organ. Therefore, I could not relate the differentiation of the paratympanic organ with the presence of nervous tissue.

B. Teratogen Studies on the Chick Embryo

1. General Introduction

Environmental factors have been shown to influence developing cells frequently resulting in the production of

embryonic abnormalities. Based on this premise, experimental teratology has been a popular approach to investigating morphogenesis (Dagg, 1960; Hall, 1972; Kocher, 1977; Landauer, 1956). Teratology can be defined as "the science dealing with the causes, mechanism, and manifestation of developmental deviations of either structural or functional nature" (Wilson, 1973, p. 4), or in simpler terms: the cause → mechanism → manifestation of defect. In experimental teratology, the cause is known--the chemical teratogen--and the defect can be readily identified. It would be helpful and enlightening to determine the precise mechanism of teratogenic action, but the value of the teratological studies should not be diminished by the absence of this information. Since these teratologic studies are conducted in vivo, a teratogen could activate one or more mechanisms as well as a chain reaction of particular responses of the cell to particular initiating mechanism resulting in manifested abnormalities. The presence/absence of abnormalities can help to better understand the developmental processes that result in the differentiation of an organ system.

The avian middle ear has been shown to be a complex developmental system. To investigate the developmental events in this region, I chose three classes of teratogens: (1) Teratogens which inhibit the differentiation of neural

crest derivatives: Beta-2-thienylalanine (Kollar and Baird, 1968; Wilde, 1955) and Hadacidin (Kollar, 1976); (2) Anti-mitotic drugs that result in skeletal defects: 5 Fluorouracil (Dagg, 1960; Kury and Craig, 1966) and Triethylene melamine (Kocher, 1977; Scherschlicht, 1973, 1975); (3) Other teratogens that induce skeletal abnormalities: Pilocarpine (Landauer, 1953a, 1956); Beta-aminopropionitrile (Hall, 1972a, b; Levene and Gross, 1959).

These teratogens were administered to the chick embryo over a wide range of developmental stages and the middle ears of the recovered embryos were evaluated.

2. Materials and Methods

Eggs of the common fowl Gallus gallus were incubated at 37° C. Each teratogen was dissolved in volumes of 0.2-0.5ml 0.9% sterile saline which was injected through a hole in the shell onto the embryos at various stages and doses (Table 5). The hole was sealed with cellophane tape and incubation was continued without rotation. Control embryos were injected with 0.2-0.5 ml 0.9% saline.

Ear development cannot be assessed in normal embryos younger than HH stage 34 (8 days) (see page 25). The eggs were candled daily; embryos dying earlier than day 8 were not scored for deformities. Viable embryos were recovered at a minimum of eight days total incubation time. However, because the teratogens cause general embryonic

retardation, especially in cranial structures, it was desirable to permit embryos to develop for longer than eight days to achieve an equivalent of at least a stage 34. Surviving embryos were staged by adding the number of days since the injection to the HH stage at which each was injected. In those embryos demonstrating slight defects, limb development was used to substantiate this staging procedure. All embryos were evaluated for external malformations (Table 5). The embryos were fixed in 10% formalin for 24 hours. Heads, from normally appearing embryos and embryos showing external cranial abnormalities, and some abnormal right hindlimbs were processed for histology as described previously. The plane of section through the middle ear region was not uniform in all embryos as the cranial axis was difficult to determine in embryos with extreme deformities of the eyes and skull. Normal and malformed embryos were also cleared and stained with alizarin red and Alcian blue for bone and cartilage (Wasserzug, 1976). This technique permitted analysis of limb and cranial deformities, but because of the position of the columella relative to other skeletal components, only its presence or absence could be determined. In some of the whole stained embryos, the heads were removed, dehydrated for 24 hours in ethanol, and processed for histological examination as previously described. Despite the prior maceration

TABLE 5

TERATOGENIC EFFECTS OF SIX TERATOGENS
INJECTED INTO THE CHICK EMBRYO

Drug	Dose/mg	Stage/ HH	# Treated	SS	GA	#S	WM
BT Beta-2-thienyl-alanine (NBC)	1.0-56	5-25	350	109	5	18	4
BAPN Beta-aminopropri-onitrile (GIBCO)	0.156-0.312	8-16	250	26	21	4	2
	0.156-1.25	17-33	450	50	29	40	3
TEM Triethylene melamine	0.008-0.001	24&25	700	58	47	28	7
5FU 5 Fluorouracil (ICN)	0.1-0.15	13-16	150	14	7	2	0
	0.1-0.4	17-25	410	111	46	30	16
	0.4-0.5	26-28	60	20	8	6	2
Pilocarpine Pilocarpine Nitrate (ICN)	1.25-6.5	8-16	105	25	4	1	0
	5-15	17-25	285	119	95	40	14
Hadacidin N-Formylhydroxy-aminoacetic acid (Merck Sharp and Dohme)	0.75-4.0	5-9	480	62	50	35	0
	2-3	10-16	320	47	35	18	0
	1.5-4.5	17-25	600	127	93	39	15
	1.5-7.5	25+-30	185	44	9	1	3
0.9% saline		5-28	100	84	0	15	15

NOTE: SS = surviving embryos; GA = number gross abnormal; #S = number sectioned; WM = number cleared and whole stained.

with KOH, a relatively detailed evaluation of skeletal histogenesis was still possible.

3. Hadacidin

a. Rationale and mode of action

Hadacidin, N-Formyl hydroxyaminoacetic acid, has been shown to be teratogenic in mammalian embryos (Chaube and Murphy, 1963; Lejour-Jeanty, 1966; Shah, 1977). Hadacidin is a competitive inhibitor of the purine biosynthetic enzyme adenyl-succinate synthetase in prokaryotes (Demain, 1966), and in plants and animals (Shigeura and Gordon, 1962a, 1962b); the enzyme is essential in the conversion of iosinic acid to adenyl succinic acid so that the de novo biosynthesis of adenylic and deoxyadenylic acids are inhibited (Shigeura and Gordon, 1962b); this action is reversed by the addition of L-aspartate. At the cellular level, there is a cessation of mitosis (Kollar, 1976). In addition to this inhibition of purine biosynthesis, pyrimidine synthesis is partially suppressed when hadacidin is applied at a higher concentration (Shigeura and Gordon, 1962c). The formation of uridylic acid is inhibited presumably prior to orotic acid formation since the application of the drug after the formation of orotic acid does not result in the inhibitory affect. It has been shown that hadacidin is an apparent structural analog of aspartate

(Shigeura and Gordon, 1962c). At this higher concentration, cell death is frequently observed (Kollar, 1976). With this general metabolic affect, it is not surprising that hadacidin can cause a wide range of embryonic abnormalities.

Kollar's (1976) in vitro studies suggested a differential affect on cephalic neural crest derivatives since their differentiation was disrupted at doses that did not affect other cephalic tissues. Since the columella is derived from cephalic neural crest, I chose to investigate the teratogenic action of hadacidin on the ossicle and other embryonic structures.

b. Results

1. General comments

Hadacidin has teratogenic effects on chick embryos HH stages 5-30 when applied in a range of doses (Table 5). An analysis of external malformations in the 279 embryos recovered after teratogen treatment is given in Table 6. The various abnormalities observed in 187/279 embryos are related to dose and time of application. When compared to the control embryo (fig. 58), hadacidin treated embryos are retarded and show a variety of external malformations: microcephaly, beak abnormalities, unilateral and bilateral optic abnormalities, and limb abnormalities, especially in the hindlimb (figs. 59-65).

TABLE 6

INCIDENCE OF EXTERNAL MALFORMATIONS PRODUCED BY VARIOUS DOSES OF HADACIDIN INJECTED INTO CHICK EMBRYOS HAMBURGER HAMILTON STAGES 5-30

Stage	Dose/ mg	#	Normal	Eyes 1	Eyes 2	UB	FC	LB	Skull	FL	HL	Coel	Oedema
5-9	0.75	4	1	1	0	3	1	2	1	0	0	0	0
	1.0	6	3	0	1	4	0	1	1	0	0	0	0
	1.5	10	1	0	3	8	1	8	2	0	1	0	1
	2.0	22	4	3	8	16	1	9	4	0	0	2	1
	2.5	2	0	0	1	2	0	2	1	0	0	0	0
	3.0	14	2	2	5	8	3	6	2	0	0	1	1
	4.0	4	1	2	0	2	0	2	1	0	1	0	1
10-16	1.5	1	0	0	0	1	0	0	0	0	0	0	0
	2.0	35	8	9	7	16	8	10	4	2	2	7	5
	3.0	11	4	3	1	4	0	2	1	0	1	1	1
17-25	1.5	24	18	2	0	2	2	2	0	0	0	2	2
	2.0	56	13	16	0	14	21	38	1	15	23	0	3
	3.0	8	0	1	2	5	4	6	1	2	7	0	1
	3.5	20	1	0	12	8	11	18	0	14	17	1	1
	4.0	10	0	1	10	1	8	9	1	8	10	4	5
	4.5	8	1	1	3	3	1	2	0	0	6	1	1
25+ -	1.5	12	10	0	0	0	0	0	0	0	1	0	1
30	2.0	26	19	2	2	1	0	2	0	0	3	1	0
	7.5	6	6	0	0	0	0	0	0	0	0	0	0
Total		279	92	43	55	98	61	119	20	41	72	20	24

NOTE: # = number of embryos recovered; UB = upper back; LB = lower beak; FC = facial coloboma; Skull = deformed skull; FL = forelimb; HL = deformed hindlimb; Coel = coelosomia;
Eye abnormalities can be observed unilateral or bilateral.

2. A histological analysis of the effects of hadacidin on middle ear development

A summary of hadacidin induced defects is presented in Table 7. Thirty-six out of 93 embryos have a normal columella suspended in the fenestra ovalis and inserting onto the tympanic membrane (fig. 66). The footplate is suspended in the fenestra ovalis by an annular ligament of normal histological appearance (fig. 67).

I have subdivided the columella abnormalities into three categories (see page 71) and all three categories are observed after hadacidin application. The columella footplate is fused with the otic capsule (Type I). This abnormality varies from complete fusion (18/93) (figs. 68 and 69), in which the chondrocytes of the footplate are continuous with those of the capsule and the region where the annular ligament normally differentiates is indistinguishable from adjacent regions, to partial fusion (9/93) (fig. 70) in which the footplate is fused to the otic capsule (fig. 71) on one aspect and is separated from the capsule on the other aspect. In Figure 70, the dorsal aspect of the annular ligament is absent although the footplate appears normal: this abnormality is probably due to an abnormality in the development of the otic capsule. In 15/93 embryos, Type II abnormality is observed, in which the footplate is absent and the columella shaft is not

TABLE 7

HISTOLOGICAL ANALYSIS OF THE MIDDLE EAR IN CHICK EMBRYOS INJECTED WITH HADACIDIN AT HAMBURGER HAMILTON STAGES 5-30

Stage	Dose/ mg	#	NC	DC	EC	AbC	AbCS	FP F	AL	FP A	PT	EAM	TM	MEC
5-9	0.75	2	2	0	0	0	0	0	0	0	0	0	2	0
	1.0	5	4	0	1	0	0	1	3	0	0	1	5	2
	1.5	4	1	1	0	0	0	1	1	2	0	2	5	3
	2.0	12	4	6	2	2	0	3	0	1	2	6	8	5
	2.5	2	0	1	1	0	2	1	0	1	0	0	2	2
	3.0	9	1	4	2	2	0	1	0	5	3	2	8	6
	4.0	1	0	0	0	0	0	1	1	0	0	0	0	0
10-16	2.0	14	6	5	1	1	2	2	2	3	5	3	7	4
	3.0	4	2	2	0	0	1	1	1	1	1	2	3	0
17-25	2.0	18	9	5	1	0	1	7	2	1	0	1	5	4
	3.0	6	3	2	0	0	1	3	0	1	0	0	2	1
	3.5	11	2	0	1	0	0	5	4	0	0	0	3	1
	4.0	3	0	0	1	0	0	1	1	0	0	0	0	0
	4.5	1	1	0	0	0	0	0	0	0	0	0	0	0
25+-30	2.0	1	0	0	0	0	0	0	0	0	0	0	0	0
Total		93	36	26	10	5	7	27	15	15	11	17	50	28

NOTE: # = number embryos recovered; NC = normal columella; DC = deformed columella; EC = deformed extracolumella; AbC = absent columella; AbCS = absent columella shaft; FP F = footplate fused with otic capsule; AL = annular ligament retarded; FP A = footplate absent; PT = paratympanic organ deformed; EAM = abnormal external auditory meatus; TM = absent/abnormal tympanic membrane; MEC = abnormal middle ear cavity.

continuous with the otic capsule (figs. 72 and 73). The otic capsule remains uniform throughout (fig. 72). Each skeletal component shows an enclosing perichondrium (figs. 72 and 73). The third type of columella abnormality includes a wide variety of defects. In 26/93 embryos, the shape of the columella is deformed (fig. 74). In 7/93 embryos, the shaft and footplate are absent yet the distinctive shape of the extracolumella can be identified, frequently associated with a middle ear cavity. In 5/93 embryos, no structure identifiable as the columella is observed; in some of these embryos, the development of the otic capsule and inner ear is retarded and the identification of the middle ear region is difficult.

The configuration of the columella in Figure 75 is relatively normal yet the footplate is not suspended in a fenestra ovalis (compare figs. 66 and 75). The prootic and opisthotic, normally forming the margin of the fenestra ovalis, are absent in this region. The more dorsal and ventral portions of the capsule are observed. The footplate appears to be free standing within undifferentiated mesenchyme.

The other components of the middle ear show wide variation in their degree of differentiation; this variation could not be associated to the degree of differentiation of the columella or other middle ear components. The

external auditory meatus is absent in 17/93 embryos (figs. 69 and 75). In 28/93 embryos, the middle ear cavity does not surround the ossicle (figs. 69, 74, and 75) and the mesenchyme within this region is denser than normal. A double-faced tympanic membrane does not differentiate in 50/93 embryos; the extracolumella inserts into the connective tissue adjacent to the external auditory meatus (fig. 68) or into the mesenchyme surrounding the columella (fig. 69). Even when the columella and other middle ear components are deformed or absent, the paratympanic organ is usually well differentiated (fig. 75). The paratympanic organ is abnormal in 11/93 embryos (fig. 76).

3. A comparison of columella, beak, and limb abnormalities in selected externally abnormal embryos

The upper and lower beak are frequently abnormal after hadacidin treatment (Tables 8 and 9; figs. 59-63). The lower beak is usually more severely affected by the drug than the upper beak (Table 9). All embryos with abnormal columellae have abnormal beaks. Because many embryos with craniofacial abnormalities as in Figures 59 and 60 have normal columellae, I tried to correlate other skeletal abnormalities with columella abnormalities (Table 8). Hadacidin injected at HH stages 17-25 resulted in hindlimb abnormalities. The hindlimb showed extreme bending of the tibia close to the ankle joint (figs. 64 & 65),

TABLE 8

AN EVALUATION OF THE BEAK, COLUMELLA, AND LIMB IS SELECTED EXTERNALLY ABNORMAL EMBRYOS INJECTED WITH HADACIDIN

Animal	Stage	Dose/ mg	Beak	Columella	Limb
H 2	7	1	+++	+++	+++
H 35	11	2	+	++	+++
H 44	9	1.5	++	++	+
H 45	21	2	++	++++	++
H 64	18	2	+++	++	+
H 65	18	2	+++	+++	++
H 66	19	3	++	+	+
H 68	18	2	++	++++	+++
H 70	18	2	+	+	+
H 72	19	2	+	+	+
H 74	19	3	+	++	+
H 100*	19	2	+	+	+
H 161*	23	2	+++	++++	++
H 168*	23	2	++	++++	++
H 188*	24	3.5	++	++	++
H 190*	24	3.5	++	+++	+
H 191*	24	3.5	++	+++	+
H 199*	22	3	+	+	+
H 206*	24	3.5	+	+++	++
H 208*	24	3.5	+	+++	+
H 209*	24	3.5	+	++	+

NOTE: Hadacidin gives a wide range of abnormalities in each system. In this table an arbitrary score of normal (++++) to very abnormal (+) has been assigned in each case.

*Indicated whole stained embryos subsequently processed for histology.

TABLE 9

EVALUATION OF BEAK AND HINDLIMB IN SELECTED EXTERNALLY ABNORMAL EMBRYOS INJECTED WITH HADACIDIN

Animal	Stage	Dose/ mg	Hindlimb	LB	UB
H 74	19	3	+	+	++
H 100	19	2	+	+	++
H 146	29	2	+++	+++	+++
H 152	29	2	++	++++	++
H 157	29	1.5	++	++++	++++
H 158	23	2	+++	+++	++
H 161	23	2	++	++	+++
H 163	23	2	++	++	++
H 168	23	2	++	++	++
H 179	15	4.5	++	++	+++
H 186	25	4.5	++	++++	+++
H 187	24	3.5	+	++++	++++
H 188	24	3.5	+	++	+
H 190	24	3.5	+	+	++
H 191	24	3.5	+	+	++
H 199	22	3	+	+	++
H 206	24	3.5	+	+	++
H 208	24	3.5	+	+	+
H 209	24	3.5	+	++	+

NOTE: LB = lower beak; UB = upper beak; Hadacidin gives a wide range of abnormalities in each system. In this table an arbitrary score of normal (++++) to very abnormal (+) has been assigned in each case.

frequently associated with fusion and/or other joint abnormalities and metatarsal and/or phalanges bending, fusion, or absence (figs. 64 & 65). In experimental animals H66, H70, H72, H100, and H199 (Table 8), very deformed columellae are all associated with deformed beaks and limbs. Yet other embryos with deformed beaks and limbs have columellae that are slightly deformed or normal. Abnormal limbs are not preferentially associated with abnormal columellae, although in most cases the beaks are abnormal. Embryos with abnormal beaks and/or limbs could have normal columellae as did experimental embryos H45, H68, H161, H168. There appears to be no association between columella abnormalities and other skeletal abnormalities. The external malformations observed in the embryos could not permit the prediction that the columella is also abnormal except in experimental animals where the skull is extremely deformed. In these animals, the head and cranium are extremely malformed and therefore it would be impossible for the ear region to be normal. In all of these embryos, the middle ear region is abnormal.

4. Summary of Results

1. Hadacidin treatment results in all three categories of columella abnormalities.

2. The frequency of occurrence of each category depends on the stage of drug application.

3. The fenestra ovalis and the annular ligament may be abnormal in animals with otherwise normal columellae.

4. Other middle ear components are frequently abnormal.

5. All columella abnormalities are associated with craniofacial abnormalities.

6. There is no relationship between the extent and/or frequency of beak abnormalities to those of the columellae.

7. Limb abnormalities are stage specific; there appears to be no relationship between columella and limb abnormalities.

c. Discussion

1. General conclusions

Hadacidin was tested in view of its reported effects on neural crest derivatives (Kollar, 1976; Pourtois, 1972) and skeletal tissues (Lejour-Jeanty, 1966; Shah, 1977). I have determined its general effect on chick embryos, and conclude that it is a useful tool for understanding the dynamics of middle ear development.

2. The differentiation of the columella

All three categories of columella abnormality detailed previously have been observed after hadacidin

treatment; the relative frequency differs depending on the stage when the drug is administered. Thus the second and third type predominate when hadacidin was injected at HH stages 5-16, while the first predominates following injection during HH stages 17-25. Injections into stages older than HH stage 25 does not produce columella defects (see general discussion).

Complete absence of the columella is rare, but defects and/or absence of one or more component parts are common. It appears that if the embryo survives after drug administration, derivatives of the mesectoderm are rarely absent. Columella determination occurs, initial development proceeds, and such defects as do occur are due to disruptions of normal patterns of cell proliferation and/or orientation.

Footplate differentiation is the result of complex interactions between the chondrifying tissues of the columella and the otic capsule. Hadacidin injection prior to the proper orientation of the two cell populations could prevent their fusion. The columella shaft remains separate from the otic capsule and the footplate does not differentiate. If the drug is applied after the two cell populations become continuous, the interactions resulting in annular ligament differentiation could be disrupted. The footplate was therefore fused with the otic capsule and the

region which should be occupied by the annular ligament was not different than the adjacent regions.

4. 5-Fluorouracil

a. Rationale and mode of action

This fluorinated pyrimidine, 5-fluorouracil (5FU), is a mitotic inhibitor which produces skeletal and eye abnormalities when injected into embryos (Dagg, 1960; Karnofsky and Lacon, 1964; Kury and Craig, 1966). Since other teratogens, e.g., Hadacidin and TEM, which produce skeletal abnormalities also produce columella abnormalities (Scherschlicht, 1975, see page 83), therefore it was important to determine whether teratogens with different modes of actions, which result in skeletal abnormalities, produce similar different columella abnormalities.

The mode of action of 5FU has been extensively investigated due to its cancer therapeutic action (Dagg, 1960; Karnofsky and Lacon, 1964; Kury and Craig, 1966). Three possible mechanisms result in mitotic inhibition: inhibition of conversion of uracil and orotic acid into RNA uracil; fluorouracil metabolized to the ribosyl form to become incorporated into RNA to produce an abnormal RNA; or its metabolism into its deoxyribosyl form to form 5-fluorodeoxyurine (FUDR) which blocks a step involving the metabolism of deoxyuridylic acid (DUMP) to form thymydilic

acid (or thymidine) preventing DNA synthesis (Karnofsky and Lacon, 1964). Thus this teratogen can have its effects at three stages of mitosis-preventing RNA synthesis, producing an abnormal RNA, or preventing DNA synthesis, although there is no effect on total protein synthesis.

b. Results

1. General comments

Fluorouracil (5FU) is a mitotic inhibitor that produces external malformations when injected into chick embryos HH stages 11-28 (Table 5). Table 10 details the external malformations obtained in relation to the dose and stage of application. Sixty embryos show external malformations including retardation, abnormalities of the eyes, upper and lower beak, and limbs (figs. 78-80) when compared with a normal embryo (fig. 77).

2. A histological analysis of the effects of 5-fluorouracil on middle ear development

Table 11 presents a summary of the development of the middle ear region in 38 embryos. Eight of the examined embryos have normal columellae (fig. 84). The most frequently observed columella abnormality is the fusion of the footplate with the otic capsule (19/38); ranging from complete (14/38) (figs. 85-87) to partial (5/38) (figs. 88 and 89) fusion. The annular ligament is absent. Figure 87 demonstrates the differential affinity

TABLE 10

INCIDENCE OF EXTERNAL MALFORMATIONS PRODUCED BY VARIOUS DOSES OF 5FU INJECTED INTO CHICK EMBRYOS HAMBURGER HAMILTON STAGES 11-28

Stage	Dose	#	N	Eye	LB	FC	UB	FL	HL	Coel	Oed
11-16	0.05	8	5	2	2	0	1	0	0	0	0
	0.1	3	1	0	0	0	0	0	2	0	0
	0.15	3	2	1	1	1	1	1	1	0	0
17-25	0.05	4	4	0	0	0	0	0	0	0	0
	0.1	6	3	1	2	2	0	1	1	1	0
	0.15	30	25	1	13	4	0	5	8	1	0
	0.2	39	14	4	23	10	11	6	9	6	1
	0.25	7	1	1	6	0	3	2	3	1	0
	0.3	12	9	1	3	0	3	2	3	0	0
	0.4	13	9	2	3	2	2	2	2	0	2
26-28	0.4	19	12	0	7	3	2	2	7	1	4
	0.5	1	0	0	0	0	0	0	1	1	0
Total		145	85	13	60	23	23	21	37	11	7

NOTE: Abbreviations as previous; Oed = oedema.

TABLE 11

HISTOLOGICAL ANALYSIS OF THE MIDDLE EAR OF CHICK EMBRYOS
INJECTED WITH 5FU AT HAMBURGER HAMILTON STAGES 11-28

Age	Dose/ mg	#	NC	DC	EC	Abs C	FP F	AL	MEC	TM	EAM	PT
11-16	0.05-0.15	2	1	0	0	0	0	1	0	0	0	0
17-25	0.10	2	1	0	0	0	0	1	1	1	1	0
	0.15	11	3	2	1	0	7	2	3	5	2	0
	0.20	12	1	0	0	2	5	4	7	8	4	2
	0.25-0.30	1	0	0	0	0	1	0	1	1	0	0
	0.30	2	0	0	0	0	1	1	1	1	0	0
	0.40	2*	0	0	0	0	2	0	1	1	0	0
26-28	0.40	6	2	0	0	0	4	0	0	1	0	0
Total		38	8	2	1	2	20	9	14	18	7	2

NOTE: Abbreviations as previous.

*In one embryo, the otic capsule is absent so the annular ligament and fenestra ovalis are absent.

for alcian blue frequently observed in teratogen treated embryos. Therefore, just by observing the staining characteristics of skeletal tissue, one is often able to identify teratogen treated animals. In Figure 89, the annular ligament cells did not differentiate, and the cells in their position show staining characteristics similar to those of the otic capsule. In 1/38 embryos, the dorsal aspect of the annular ligament is absent while the ventral aspect has begun to differentiate (fig. 88). This abnormality is due to the absence of a portion of the otic capsule resulting in a deformed fenestra ovalis. A columella could not be identified in 2/38 embryos. In one embryo, the columella shaft and extracolumella are not continuous; the seventh nerve traverses the region where the chondrifying tissues should have fused.

A differentiated tympanic membrane is not identified in 18/38 embryos (figs. 85, 86, and 88). When this structure is not observed, the extracolumella inserts into the connective tissue adjacent to the external auditory meatus (fig. 85) or the mesenchyme surrounding the ossicle (fig. 86). In 14/38 embryos, the middle ear cavity is not yet surrounding the columella (fig. 86) and the mesenchyme is denser than normal (compare figs. 7 and 86). The external auditory meatus is absent in 7/38 embryos.

3. A comparison of columella, beak, and limb abnormalities in selected externally abnormal embryos

5FU produces beak abnormalities (Tables 12 and 13). The upper beak is frequently short and curved while the lower beak is usually more severely deformed (Tables 12 and 13) (figs. 78-83). Columella abnormalities are always associated with beak abnormalities (Table 12); experimental embryos F77 and F116 have very abnormal columellae. Embryos with craniofacial abnormalities, e.g., F65, F25, and F123 (Table 12) can have normal columellae. The hindlimb is often abnormal after teratogen application, conditions including micromelia, hemimelia (figs. 78-80), syndactyly, bending/fusion of the metatarsals (figs. 81 and 82). There appears to be no association between limb and columella abnormalities.

In Table 12 it appears that the effects of 5FU are dose dependent; all embryos treated with 0.15 mg 5FU have normal columellae while higher doses produce columella abnormalities. When more embryos are examined (Table 11), there does not appear to be a significant difference in normal versus abnormal columella at all teratogenic doses.

4. Summary of results

1. 5-fluorouracil produces columella deformities; the most frequent abnormality is Type I.

TABLE 12

AN EVALUATION OF THE BEAK, COLUMELLA, AND LIMB IN SELECTED EXTERNALLY ABNORMAL CHICK EMBRYOS INJECTED WITH 5FU

Animal	Stage	Dose/ mg	Beak	Columella	Limb
F 55	18	0.2	++	+++	+
F 65	18	0.15	+	++++	++
F 73	17	0.2	+++	+	+
F 76	16	0.15	+	+++	+
F 77	23	0.2	+	+	+
F 85	23	0.2	++	+++	+++
F 25*	17	0.15	+++	++++	+
F 84*	23	0.2	+++	++	+++
F 98*	23	0.15	+++	++	+++
F 112*	18	0.15	+++	++	++
F 116*	25	0.4	+	+	+
F 123*	26	0.4	+++	++++	++++

NOTE: 5FU gives a range of abnormalities in each system. In this table an arbitrary score of normal (++++) through very abnormal (+) has been assigned in each case.

*Cleared and whole stained embryos that were later processed for histology.

TABLE 13

EVALUATION OF BEAK AND HINDLIMB IN SELECTED
EXTERNALLY ABNORMAL EMBRYOS
INJECTED WITH 5FU

Animal	Stage	Dose/ mg	Hindlimb	LB	UB
F 25	17	0.15	+	++	++
F 28	17	0.15	++	+++	++++
F 37	18	0.2	+++	+++	++++
F 50	18	0.15	++++	+++	++++
F 59	24	0.15	++++	+++	+++
F 80	23	0.2	+++	++	++++
F 81	23	0.2	+++	+++	+++
F 84	23	0.2	+++	+++	+++
F 89	23	0.2	+++	+++	+++
F 94	23	0.15	+++	+++	++++
F 98	23	0.15	++++	++	+++
F 109	18	0.15	+++	++	++++
F 112	18	0.15	+	++	++++
F 113	18	0.15	++++	++	+++
F 116	18	0.15	+	+	++
F 123	26	0.4	++	++	++++
F 131	28	0.4	++++	+++	++++

NOTE: 5FU gives a wide range of abnormalities in each system. In this table an arbitrary score of normal (++++) to very abnormal (+) has been assigned in each case.

2. The columella could not be identified in 2/38 embryos.

3. The extracolumella is separate from the columella shaft in one embryo and this disruption is probably due to the presence of the seventh nerve between the two chondrifying tissues.

4. Columella abnormalities are always associated with craniofacial abnormalities.

5. There is no association between limb and columella abnormalities.

6. There are no significant differences in the frequency of columella abnormalities once the teratogenic dose is attained.

7. The relationship of the fusion of a portion of the annular ligament with the otic capsule could be important.

c. Discussion

1. General conclusions

The antimitotic drug, 5-fluorouracil (5FU), produces skeletal abnormalities in the chick embryo (Karnofsky et al, 1955). The action of this drug on nucleic acid synthesis is well known (Dagg, 1960; Kury and Craig, 1966); 5FU can inhibit RNA or DNA synthesis, or becomes incorporated into RNA to produce an abnormal RNA

form. Each mode of action could act upon different cell populations and/or different stages in the cell cycle. Because I am interested in the production of skeletal abnormalities in the embryo *in vivo*, the precise mode of action discovered *in vitro* is not important in my using this drug. It is important that teratogen application results in skeletal abnormalities.

2. The differentiation of the columella

The columella is rarely absent, but defects and/or absence of one or more component parts are common. It appears that the determination of the columella occurs, initial development proceeds, and such defects as do occur are due to the disruption of normal patterns of cell proliferation and/or orientation.

Type I abnormality is the most common one observed in the columella. This drug probably inhibits mitosis of the chondrifying cells, possibly preventing the proper number of cell generations necessary for normal differentiation (Holtzer et al., 1972). This results in an interruption of the interactions due to abnormal proliferation and/or positioning of the chondrifying tissues and the annular ligament does not differentiate.

The extracolumella is separated from the shaft by the intervention of the seventh nerve in the region of the

columella-extracolumella junction. This abnormality could be due to a differential rate of mitosis within the two cell populations, thereby permitting the nerve to push the two populations apart. Once the nerve physically separates the two chondrifying tissues, they are prevented from becoming continuous. The etiology of this abnormality is different from one appearing similar in TEM treated embryos (see p. 133), which have no physical barrier separating the two discontinuous cell populations.

5. Pilocarpine

a. Rationale and mode of action

Pilocarpine is teratogenic when injected into chick embryos, producing abnormalities of the beak and limbs (Landauer, 1953, 1956), and it has been extensively studied (Landauer, 1953a, b, 1956). Pilocarpine induces limb abnormalities; syndactylism, shortening or bending of the tarsometatarsus, and reduction in size or suppression of the first toe. At higher doses, there is a shortening of the mandible, frequently associated with facial coloboma. Abnormalities of feather pigmentation are observed after pilocarpine application in some embryos that otherwise appear normal; in chicks with skeletal abnormalities, changes in pigmentation are now common and extensive. The possible etiology of pigmentation abnormalities is that

melanoblast activity is disturbed in the effected region, or neural crest migration is disrupted preventing the formation of pigment cells that would normally occupy these distal regions.

The pharmacologic action of pilocarpine has been studied (Battersby and Openshaw, 1953) because of its cholinergic action in mammals, resulting in parasympathetic stimulation. Although its teratogenic action in chicks has been investigated, Landauer (1953a, b, 1956) concluded that in the chick embryo the action of pilocarpine cannot be the same as that in mammals. He arrived at this conclusion because of the absence of atropine action as an antagonist or as a teratogen, the absence of arecoline teratogenic action, and the differences in the degree of teratogenic activity of pilocarpine isomers in the chick and mouse.

Pilocarpine can be a useful tool in the investigation of middle ear development even though its exact mode of action in the chick is not known, since it produces beak abnormalities, which like the columella, is derived from cephalic neural crest cells. Because of the observations of pigment abnormalities, it is possible that pilocarpine preferentially affects some neural crest derivatives (Landauer, 1956).

b. Results

1. General comments

Pilocarpine is teratogenic when injected into chick embryos HH stages 8-25 (Table 5). External malformations were observed in 99 of the 147 embryos recovered (Table 14). The external malformations are related to stage of application so that drug application prior to stage 17 results predominantly in normal embryos. When the drug is administered to HH stages 17-25, the recovered embryos are retarded and have abnormalities of the upper and lower beak, eye, and limbs (figs. 91-96) when compared with the normal (fig. 90).

2. A histological analysis of the effects of pilocarpine on middle ear development

A detailed summary of the differentiation of the middle ear is presented in Table 15. The columella is present in all 41 embryos examined and is normal in 15/41 (fig. 97). The degree of columella abnormality observed after pilocarpine administration is minor. In the majority of the embryos, the columella is well differentiated and the annular ligament has begun to differentiate; the degree of ligament differentiation is retarded (18/41) when compared to the normal (figs. 98 and 99). Because of the dual technique of whole staining and subsequent histological

TABLE 14

INCIDENCE OF EXTERNAL MALFORMATIONS PRODUCED BY VARIOUS DOSES OF PILOCARPINE INJECTED INTO CHICK EMBRYOS HAMBURGER HAMILTON STAGES 8-25

Stage	Dose/ mg	#	N	Eye	LB	FC	UB	FL	HL	Coel	Oed
8-16	1.25	14	12	0	1	0	0	0	0	2	0
	3.25	9	8	1	0	0	0	0	0	1	
	6.5	2	1	0	0	0	0	0	0	0	1
17-25	5.0	8	3	0	5	1	0	2	4	1	1
	6.5	25	8	2	14	1	5	1	15	0	0
	7.2	2	0	2	0	0	0	1	2	0	2
	8.0	19	0	2	14	2	8	5	14	7	2
	9.3	17	2	5	14	8	1	6	15	1	2
	10.0	18	3	8	14	3	4	3	14	6	4
	12.5	3	1	0	2	0	2	1	2	0	1
	15.0	6	1	0	6	0	3	3	6	5	2
Total		144	45	22	85	10	38	26	87	24	20

NOTE: Abbreviations as previous.

TABLE 15

HISTOLOGICAL ANALYSIS OF THE MIDDLE EAR OF CHICK EMBRYOS INJECTED WITH PILOCARPINE AT HAMBURGER HAMILTON STAGES 11-25

Stage	Dose/ mg	#	NC	DC	FP F	AL	TM	MEC
11-16	3.25	1*	0	0	0	1	0	0
17-25	5.0	4	0	0	1	3	1	0
	6.5	8**	2	0	2	4	4	4
	8.0	3	1	1	2	0	1	1
	8.2	5	1	1	1	3	0	0
	9.3	6	3	0	2	1	0	0
	9.5	2	2	0	0	1	0	0
	10.0	6	5	0	0	1	0	0
	12.5	2	0	1	0	2	0	0
	15	4	1	0	0	3	0	0
Total		41	15	3	8	18	6	5

NOTE: Abbreviations as previous.

*Fenestra ovalis abnormal in 1 embryo.

**Fenestra ovalis abnormal in 2 embryos.

processing and examination, the annular ligament in Figure 99 stains differently than in other embryos. The ligament shows staining characteristics identical to those of the perichondrium of the footplate and otic capsule and different from those of the footplate and capsule. The footplate is completely (4/14) (fig. 100) or partially (4/14) fused with the otic capsule. Yet even when the footplate is classified as completely fused with the otic capsule, the cells within the "annular ligament region" can be identified by their alingment (fig. 101, arrow). In three embryos, the fenestra ovalis region is abnormal due to the absence of the otic capsule; the footplate is separated from the capsule but one aspect of the annular ligament is absent.

The external auditory meatus is observed in all embryos examined (figs. 97-99). A middle ear cavity is absent in 3/14 (figs. 99 and 100) and the mesenchyme is denser than in the normal middle ear region (compare figs. 7 and 100). A differentiated tympanic membrane is not observed in 6/41, in which case the extracolumella inserts into dense connective tissue adjacent to the external auditory meatus (fig. 100).

3. A comparison of columella, beak and limb abnormalities in selected externally abnormal embryos

The lower beak is usually shorter and bent when compared with a normal untreated embryo (figs. 91-96).

Meckel's cartilage shows symmetrical bending midway along its long axis (fig. 96). The upper beak is usually less affected by drug treatment; it is slightly bent (Tables 16 and 17) (figs. 94 and 95). The chick embryos are able to differentiate until HH stage 40 and older. In these older embryos, the entire beak appears shorter than normal, yet the beak does not appear bent. After whole staining, the skull was examined in detail (Table 17). Although the dermal bone of the lower jaw appears normal, a half loop is observed in Meckel's cartilage. The cartilaginous abnormality is not reflected in the dermal bone. It appears that bones are able to continue differentiation in the presence of cartilaginous abnormalities.

All columella abnormalities are associated with craniofacial abnormalities (Table 16); in experimental animal P30, the columella is abnormal. Yet more frequently abnormalities are associated with normal or slightly abnormal columellae; experimental animals P53, P54, P141, and P146 have deformed beaks, and relatively normal columellae.

Pilocarpine will induce various hindlimb abnormalities, usually syndactyly, hemimelia (figs. 91-94), and bending of the metatarsals (fig. 95). The columella, beak, and hindlimb were examined in twelve externally abnormal embryos (Table 16). There appears to be no relationship

TABLE 16

AN EVALUATION OF THE BEAK, COLUMELLA, AND LIMB IN SELECTED EXTERNALLY ABNORMAL CHICK EMBRYOS INJECTED WITH PILOCARPINE

Animal	Stage	Dose/ mg	Beak	Ear	Limb
P 30	19	6.5	+++	++	+++
P 33	19	6.5	+++	+++	+
P 34	19	6.5	+++	+++	+++
P 38	24	6.5	+++	++++	++
P 53	24	6.5	+	+++	+
P 54	19	5	++	+++	+
P 59	24	6.5	+++	+++	+++
P 61*	24	6.5	+++	++++	+++
P 83*	23	9.5	++	+++	+
P 135*	22	12.5	++	+++	++
P 141*	22	15	+	++++	++
P 146*	22	10	+	++++	++

NOTE: Pilocarpine gives a wide range of abnormalities in each system. In this table an arbitrary score of normal (++++) to very abnormal (+) has been assigned in each case.

*These embryos were whole stained and later processed for histology.

TABLE 17

EVALUATION OF BEAK AND HINDLIMB IN SELECTED EXTERNALLY ABNORMAL EMBRYOS INJECTED WITH PILOCARPINE

Animal	Stage	Dose/ mg	Hindlimb	LB	UB
P 61	24	6.5	+++	++	+++
P 66	21	8	+++	++	++
P 72	21	8	+++	++	++
P 75	21	8	++	++	+++
P 83	23	9.5	+	++	++
P 85	23	9.5	+	++	++
P 89	23	8.0	++	+++	+++
P 90	23	8	+	+	++
P 117	25	9.3	++	++	++
P 118	25	9.3	+	++	++
P 135	22	12.5	++	++	++
P 136	25	8.2	+	+	++
P 141	22	15	++	+	++
P 146	22	10	++	++	++

NOTE: Pilocarpine gives a range of abnormalities in each system. In this table an arbitrary score of normal (++++) to very abnormal (+) has been assigned in each case.

between limb and columella abnormalities. The degree of limb and beak abnormalities differ and there appears to be no direct relationship.

4. Summary of results

1. Pilocarpine never produces a very deformed columella although other skeletal tissues are abnormal.

2. The annular ligament region can be identified even in embryos with complete fusion of the footplate with the otic capsule.

3. The most common abnormality of the columella is the retardation of annular ligament differentiation.

4. The other components of the middle ear are usually well differentiated.

5. All abnormal columellae abnormalities are associated with craniofacial anomalies.

6. There is no relationship between columella and limb abnormalities.

c. Discussion

1. General conclusions

Pilocarpine produces skeletal abnormalities of the beak, usually associated with pigment abnormalities in these embryos (Landauer, 1956). Because of the common origin of the columella, beak, and pigment cells from

neural crest, I wanted to determine whether this drug would also affect columella differentiation. The columella is always present in pilocarpine treated embryos, and major defects of it are rarely observed. I conclude that pilocarpine is ineffective in interrupting the normal interactions that result in the development of the columella.

2. The differentiation of the columella

The annular ligament region can be identified in all embryos treated with pilocarpine. In many embryos, the annular ligament begins to differentiate, but the differentiation is retarded. This abnormality is difficult to interpret since I cannot determine whether the ligament would eventually "catch up" or remain retarded. This abnormality is probably due to a general retarding effect of the drug on the embryo rather than an interruption of columella-capsule interactions.

Pilocarpine causes cranial abnormalities; in three embryos the otic capsule is abnormal resulting in an abnormal fenestra ovalis and the footplate suspended within mesenchyme. In these embryos, the annular ligament does not differentiate. This abnormality is due to the action of the drug on the capsule cells, which because of the absence of the capsule cells, prevents the formation of the annular ligament by preventing interaction between the columella and otic capsule.

6. Triethylene melamine

a. Rationale and mode of action

The alkylating agent triethylene melamine (TEM) has been extensively used in experimental studies (Jurand, 1958; Kocher, 1977; Murphy, 1959) due to its cancer therapeutic action. It is a potent embryonic teratogen, producing skeletal abnormalities (Kocher, 1977; Murphy, 1959; Scherschlicht, 1973, 1975). Scherschlicht (1975) reported that the otic region is affected in TEM treated chick embryos, although the middle ear is less drastically affected than the inner ear and otic capsule. The columella, on the other hand, was described as having one type of abnormality, the fusion of the footplate with the otic capsule. The other components of the middle ear were normal except for a dimunition of the middle ear cavity. Because of this reported effect on the middle ear region, although it was described as minor (Scherschlicht, 1975), TEM would be an effective tool in investigating middle ear development.

Studies on the action of this alkylating agent on fibroblasts in vitro (Denoel, 1966; Plummer et al., 1952) indicate an antimitotic action. After an initial period of mitotic inhibition, the cells attempt to divide but disintegrate during a prolonged metaphase. The rate of

DNA synthesis is slower than that observed in control experiments (Denoel, 1966). At higher concentrations, TEM is cytotoxic (Plummer et al., 1952). The action of TEM is related to the presence of ethylenimine groups (Buckley et al., 1952). The mitotic changes are probably due to interference in nucleic acid metabolism involving the inter- or intrastrand cross linking of DNA, within the interphase nuclei (Connors, 1975). In vivo chick studies (Jurand, 1958) indicate that TEM is directed against mesodermal cells, especially those of the somites. Enlarged nuclei and nucleoli are observed in somites, the notochord, and the neural tube, as a consequence of mitotic inhibition. Yet, although TEM is a mitotic inhibitor, wound healing experiments (Friedenwald et al., 1948) have indicated that cellular growth and differentiation can continue in its presence.

b. Results

1. General comments

Triethylene melemine (TEM) was injected into chick embryos HH stages 20-26, but produced an extremely high mortality rate (Table 5). The embryos survived for 24 hours after teratogen interruption, and would subsequently die. When a narrow dose range was injected into HH stages 24-25 (Table 5), 58 embryos were recovered, of

which 47 were externally abnormal (Table 18). The external malformations were related to dose and time of application (Table 18). External malformations included retardation, cranial abnormalities--facial coloboma, beak abnormalities and less frequently eye abnormalities--and limb abnormalities (figs. 103-108) when compared with a normal embryo (fig. 102).

2. A histological analysis of the effects of TEM on middle ear developments

Table 19 summarizes the development of the middle ear in 28 embryos; 5/28 had a normal columella (fig. 109). The footplate was fused with the otic capsule in 14/28; this abnormality ranged from complete (11/14) (figs. 110-113) to partial fusion (3/14). The region where the annular ligament would normally differentiate (fig. 109), is indistinguishable from the adjacent footplate and otic capsule (figs. 110-113), all three cells populations consist of chondroblasts. In Figure 124, showing a partially fused footplate, the annular ligament has begun to differentiate in the ventral region while the dorsal aspect is completely fused with the otic capsule. In 5/28, the annular ligament has begun to differentiate but the differentiation is retarded. The fenestra ovalis is abnormal in 2/28 due to the absence of portions of the otic capsule (fig. 115). The footplate is separated from the otic

TABLE 18

INCIDENCE OF EXTERNAL MALFORMATIONS PRODUCED BY VARIOUS DOSES OF TEM INJECTED INTO CHICK EMBRYOS HAMBURGER HAMILTON STAGES 24-25

Stage	Dose/ µg	#	N	Eyes	LB	FC	UB	FL	HL	Coel	Oed
24	.85	27	8	1	17	4	5	5	8	2	1
	.88	2	0	0	1	0	1	2	2	0	0
	1.0	1	0	1	0	0	0	1	1	0	0
25	.81	28	3	1	19	1	17	19	22	17	2
Total		58	11	3	37	5	23	27	33	19	3

NOTE: Abbreviations as previous.

TABLE 19

HISTOLOGICAL ANALYSIS OF THE MIDDLE EAR IN CHICK EMBRYOS
INJECTED WITH TEM AT HAMBURGER HAMILTON STAGES 24-25

Stage	Dose/ µg	#	NC	DC	C Abs	FP F	AL	EC	PT	MEC	TM	EAM	OC
24	.85	14	5	3	0	6	3	3	0	2	5	0	0
	.88	2	0	0	2	0	0	0	2	2	2	2	2
	1.0	1	0	0	1	0	0	0	1	1	1	1	1
25	.81	11*	0	1	1	8	2	1	3	5	6	1	0
Total		28	5	4	4	14	5	4	6	10	14	4	3

NOTE: OC = otic capsule is deformed; other abbreviations as previous.

*Two of these embryos have abnormal fenestra ovalis.

capsule and the dorsal portion of the annular ligament is absent (fig. 115, arrow).

In 3/28, the extracolumella does not fuse with the columella shaft (fig. 113). A perichondrium separates the chondrifying cell populations which would normally be continuous. The development of the extracolumella is more advanced than that of the shaft, and it is completely surrounded by a perichondrium while the shaft and footplate have an intermittant perichondrium. In all of these embryos, the footplate is fused with the otic capsule. The columella could not be identified in 4/28 embryos, three of which showed extremely deformed otic capsules and the middle ear region could not be identified.

When the columella is absent or abnormal, a differentiated tympanic membrane is frequently absent (14/28) (fig. 113). The extracollumella inserts into the connective tissue adjacent to the external auditory meatus (fig. 113) or into the mesenchyme surrounding the ossicle. In most of the embryos observed, the middle ear cavity should be surrounding portions of the columella. In 10/28, the middle ear cavity had not reached the columella (figs. 112 and 113). The mesenchyme within this region is denser than in the normal embryo (compare figs. 113 and 116).

3. A comparison of columella, beak, and limb abnormalities in selected externally abnormal embryos

Triethylene melamine produces beak abnormalities (Tables 20 and 21). The lower beak is short and bent (figs. 103-107) and the upper beak is usually less affected by the drug. A deformed columella is always associated with craniofacial abnormalities (Table 20). In experimental animals T2, T12, T33, and T34, a very deformed columella is observed in each one although the external morphology of the beak is not very abnormal. Other embryos with craniofacial abnormalities (experimental animals T40, T50, T53, and T58) have relatively normal columellae (Table 20). To try to associate the affect of TEM on the columella with other skeletal components, I investigated the hind limbs (Table 20 and 21). The metatarsals are usually bent (figs. 106 and 108), although the degree of bending is very variable. Other hindlimb abnormalities, including long bone bending, fusion of bones, and the absence of digits, are frequently observed. The occurrence of hindlimb abnormalities are compared with that of beak and columella abnormalities (Table 20). There appears to be no relationship between abnormalities of the limb and columella. Beak abnormalities are usually associated with limb abnormalities.

TABLE 20

AN EVALUATION OF THE BEAK, COLUMELLA, AND LIMB IN SELECTED EXTERNALLY ABNORMAL CHICK EMBRYOS INJECTED WITH TEM

Animal	Stage	Dose/ µg	Beak	Columella	Limb
T 2	24	0.88	++	+	+++
T 12	25	0.88	++	+	+++
T 17	25	0.81	++	++	+
T 26	25	0.81	+	+	++
T 27	25	0.81	+	++	+++
T 28	25	0.81	+	++	+++
T 33	24	0.85	+++	+	+
T 34	24	0.85	++	+	+
T 44	24	0.85	++	++	+
T 40*	24	0.85	++	++++	+++
T 47*	24	0.85	+++	+++	++++
T 50*	24	0.85	+++	++++	+++
T 53*	24	0.85	+++	++++	+++
T 58*	24	0.85	+++	++++	+++

NOTE: TEM gives a wide range of abnormalities in each system. In this table an arbitrary score of normal (++++) to very abnormal (+) has been assigned in each case.

*whole stained and histologically treated embryos.

TABLE 21

EVALUATION OF BEAK AND HINDLIMB IN WHOLE STAINED SELECTED EXTERNALLY ABNORMAL EMBRYOS INJECTED WITH TEM

Animal	Stage	Dose/ µg	Hindlimb	LB	UB
T 36	24	0.85	+++	+++	+++
T 40	24	0.85	+++	++	++
T 41	24	0.85	++	++	++
T 47	24	0.85	++++	+++	++
T 50	24	0.85	+++	++	++++
T 53	24	0.85	+++	++	++
T 58	24	0.85	+++	++	+++

NOTE: TEM gives a wide range of abnormalities in each system. In this table an arbitrary score of normal (++++) to very abnormal (+) has been assigned in each case.

4. Summary of results

1. Mortality is very high: survival is only over a narrow dose and stage of application.

2. Abnormal columellae are observed; the most frequent type of abnormality is footplate fusion with the otic capsule.

3. The extracolumella does not fuse with the columella shaft in 3/28 embryos. There appear to be differences in the degree of chondrification and perichondrial differentiation.

4. The columella is absent in 4/28 embryos.

5. The degree of differentiation of the other middle ear components varies; a definitive tympanic membrane is frequently absent.

6. Abnormal columella are always associated with abnormal beaks.

7. There appears to be no association between limb and columella abnormalities.

c. Discussion

1. General conclusions

Scherschlict (1975) administered triethylene melamine (TEM) into the chick embryo at three stages of development -3-1/2, 4, and 4-1/2 days (equivalent to HH stages 20-25) and reported on the resulting skeletal

abnormalities. The inner ear and otic capsule are very deformed when TEM was administered at 3-1/2 days; the middle ear shows slight abnormalities when the drug was administered 4-4-1/2 days. The columella footplate is partially of completely fused with the otic capsule, and the size of the middle ear cavity is reduced. The mesenchyme surrounding the ossicle is thicker. This is the first report in the literature of teratogen induced avian middle ear abnormalities. Because Scherschlicht (1975) did not detail the histology of the middle ear, I attempted to repeat his experiments and reinvestigate the effect of TEM on the middle ear. I could get no embryonic survival at the dose range reported by Scherschlicht (1971). The embryos did survive longer than one day after teratogen treatment when I injected the drug in lower doses reported elsewhere (Kocher, 1977; Romanoff, 1972). The mortality was very high. I could only recover 58 embryos injected during HH stages 24 and 25, although a wider age range was investigated. TEM is a potent teratogen that is able to induce a high incidence of Type III columella abnormality.

2. The differentiation of the columella

Complete absence of the columella is rare, but defects and/or absence of one or more component parts--footplate, shaft, or extracolumella--are common.

In three embryos, the differentiation of the extracolumella is more advanced than that of the columella shaft or footplate. The extra-columella is surrounded by a perichondrium; this results in a separation. The differentiation of the columella components appear to proceed at different rates probably due to disruption in cell proliferation and/or orientation in the component cell populations.

3. Footplate differentiation

Footplate differentiation is one of the most complex aspects of columella formation. Primordial chondrogenic cell populations associated with the second visceral arch and otic capsule become continuous, and both contribute to the definitive footplate (Reagan, 1917; Simons, 1974, 1975). This interaction between the chondrifying tissues of the columella and the otic capsule results in the differentiation of the annular ligament (see p. 58). If this interaction is disrupted, the differences in the sequential patterns of chondrification of the capsule and columella are not seen. The footplate is fused with the capsule, and the position that should be occupied by the annular ligament is not significantly different than the adjacent regions. This type of abnormality, frequently observed after TEM treatment, agrees with the observations of Scherschlicht (1975). If this interaction between the

two chondrifying populations is allowed to commence, but is later interrupted, the footplate can differentiate and would be suspended within mesenchyme, but no annular ligament would be observed. This abnormality is observed in two embryos, where the fenestra ovalis is abnormal due to the absence of a portion of the otic capsule.

7. Beta-aminopropionitrile

a. Rationale and mode of action

Lathyrogens induce cleft palate in mammalian embryos (Waddell et al., 1971) and it is usually accompanied by micrognathia and hindlimb abnormalities. Hall (1972a, b) investigated the teratogenic action of beta-aminopropionitrile (BAPN) on the chick embryo and showed that the membrane bones associated with the upper and lower beak and the tibia are the only abnormal skeletal structures. The effect of this drug was investigated in the younger chick embryo by Rosenberg (1957) and Levene and Gross (1959), but detailed descriptions of skeletal abnormalities were not provided. I am therefore interested in the histological details of the skeletal abnormalities produced by BAPN injection and in determining the differentiation of the middle ear in externally abnormal embryos.

Investigations concerning the action of lathryogens have been conducted (Levene and Gross, 1959; Siegel and

Martin, 1970) and several mechanisms for BAPN's teratogenic action have been proposed. BAPN inhibits collagen crosslinking by inactivating the enzyme level oxidase which converts lysine residues to aldehydes which participate in collagen crosslinking (Pinnel and Martin, 1968). BAPN induces cleft palate in rat embryos; Pratt and King (1972) show that BAPN inhibits collagen crosslinking in the palatal shelves and suggest that this biochemical lesion could contribute to the formation of cleft palate. The drug does not only interfere with lysyl oxidase metabolism, but in low doses may also interfere in intracellular metabolic activity (Kenney, 1978).

BAPN stimulates protein synthesis (Kenney, 1978), collagen synthesis (Aleo et al., 1967; Kenney, 1978) and glycosaminoglycan synthesis in the developing tissues (Aleo et al., 1967; Kenney, 1978). Carlson (pers. comm.) concludes that at low doses BAPN may alter the cellular microenvironment and/or metabolism of the cell due to an alteration in cellular synthesis. The significance of their altered secretory products on overall development may be greater than the effect of the inhibition of collagen crosslinking. Only structural rigidity of the collagen molecule needs the striated form (Hall, 1972a, b). The present study is to determine whether the teratogenic action of BAPN on cellular metabolism and/or structural

rigidity results in abnormalities of the avian middle ear.

b. Results

1. General comments

The teratogenic action of BAPN was investigated in chick embryos HH stages 9-33 (Table 5). Approximately one third (26/76) show no external malformations (Table 22). BAPN is most effective in producing external malformations, beak abnormalities and tibial bending (figs. 116-118), when injected into HH stages 17-33. When BAPN is injected into younger embryos, the recovered embryos are mainly coelosomic and retarded, but show few skeletal abnormalities (Table 22). Only 4/26 embryos show beak and limb abnormalities.

2. A histological analysis of the effects of BAPN on middle ear development

Table 23 summarizes the development of the middle ear components. The columella is normal in 41/44 embryos. Type I abnormality, footplate fused with the otic capsule, is observed in 2/44 embryos. Type II abnormality, the footplate absent with the columella shaft discontinuous with the otic capsule, is observed in only 1/44 embryos (fig. 119). The two skeletal components are poorly differentiated and it appears unlikely that they would become continuous. The otic capsule is uniform throughout

TABLE 22

THE INCIDENCE OF EXTERNAL MALFORMATIONS PRODUCED BY VARIOUS DOSES OF BAPN INJECTED INTO CHICK EMBRYOS HAMBURGER HAMILTON STAGES 9-33

Stage	Dose/ mg	#	N	UB	LB	Skull	Eyes	HL	Coel
9-16	0.156-0.312	26	5	1	2	3	0	4	20
17-25	0.156-0.312	26	11	4	7	0	1	8	0
	0.420	5	1	0	1	0	0	4	0
	0.625-1.25	13	8	0	5	0	0	4	1
26-33	0.156-0.420	4	1	0	3	0	0	3	0
	0.625 1.25	2	0	0	2	0	0	2	0
Total		76	26	5	20	3	1	25	21

NOTE: Abbreviations as previous.

TABLE 23

A HISTOLOGICAL ANALYSIS OF THE MIDDLE EAR
OF CHICK EMBRYOS INJECTED WITH BAPN AT
HAMBURGER HAMILTON STAGES 9-33

Stage	Dose/ mg	#	NC	FP F	AL	FP A	OC
9-16	0.156-0.312	4	3	0	0	1	0
17-25	0.156-1.25	36	34	2	0	0	1
26-33	0.156 0.420	4	4	0	0	0	0
Total		44	41	2	0	1	1

NOTE: Abbreviations as previous.

and the region of the fenestra ovalis does not differentiate. In all embryos examined, all of the other middle ear components are normal.

3. A comparison of columella, beak, and limb abnormalities in selected embryos

BAPN induces lower beak abnormalities in the chick embryo (Table 24) (figs. 116-118). The lower beak bends midway along its long axis; this abnormality is shown in the bending of Meckel's cartilage (figs. 116 and 117). The upper beak is less frequently abnormal. All three embryos with abnormal columellae have abnormal beaks, while embryos with abnormal beaks have normal columella. BAPN treated embryos can show tibial bending (25/76 embryos) (figs. 117 and 118). Hindlimb abnormalities are stage dependent; abnormal hindlimbs are rarely observed when the drug is injected prior to stage 17. I could find no association between columella and limb abnormalities. In the single embryo that was cleared, whole stained, and subsequently examined histologically, the development of the middle ear is retarded but middle ear abnormalities are not associated with other skeletal abnormalities.

4. Summary of results

1. The effects of BAPN is stage dependent; embryos younger than HH stage 17 are less frequently

TABLE 24

EVALUATION OF BEAK AND HINDLIMB IN SELECTED EXTERNALLY ABNORMAL EMBRYOS INJECTED WITH BAPN

Animal	Stage	Dose	Hindlimb	LB	UB
B 72	8	0.312	++++	++++	++++
B 75	8	0.312	++++	++++	++++
B 90	25	1.25	++	++	++++
B 91**	25	1.25	++	++	++++
B 95	23	0.625	+	++	++++

NOTE: BAPN gives a wide range of abnormalities in each system. In this table an arbitrary score of normal (++++) to very abnormal (+) has been assigned in each case.

**Indicates that B 91 has been examined histologically and the columella is abnormal.

affected than those stage 17 and older.

2. The lower beak and hindlimb are frequently abnormal.

3. The columella is abnormal in 3/44 embryos: Type I and Type II columella abnormalities are observed.

4. Columella abnormalities are associated with beak abnormalities.

5. Limb abnormalities are stage dependent.

c. Discussion

1. General conclusions

Beta-aminopropionitrile induces beak and hindlimb abnormalities when injected into chick embryos (Hall, 1972a; Levene and Gross, 1959; Rosenberg, 1957). Hall (1972a) injected BAPN into 7 day old chick embryos (HH stages 30-32) and observed that only the tibia and the membranous bones of the beak were abnormal. Because the important stages of middle ear development occur prior to stage 30 (see page 78), I am interested in the effect of this lathyrogen on the younger embryo. Previous investigations indicate that BAPN is teratogenic only after the formation of an extensive vascular system (Romanoff, 1972). Lathyrogen application prior to day 2 (HH stages 1-6) does not inhibit primary differentiation nor is it lethal. I injected this drug after this initial period of development:

BAPN was injected HH stages 9-16 and the majority of the embryos are slightly abnormal with only 4/26 showing skeletal abnormalities. Concentrations higher than 0.312mg result in 100% death. When BAPN is injected at the same doses (0.156-0.312mg) into HH stages 17-33, a higher frequency of skeletal abnormalities are observed. Higher concentrations result in a lower survival rate (Table 5). Within the teratogenic dose range of BAPN, the frequency of skeletal abnormalities are not dose dependent. The teratogenic action of this lathyrogen is stage dependent, with the younger embryos being less affected by the drug. This observation is relatively perplexing as the chick embryo is a 'closed' system.

The skeletal abnormalities observed must be interpreted relative to the different action of the drug. BAPN inhibits collagen crosslinking (Pinnel and Martin, 1968); this structural development is not necessary for developmental activity of collagen fibers (Carlson, pers. comm.). Collagen crosslinking is important for the structural rigidity of connective tissue (Hall, 1972a). When crosslinking is inhibited, connective tissue loses its rigidity and becomes more soluble, making these tissues less likely to resist stresses during development (Hall, 1972a). This results in bending of skeletal tissues (Hall, 1972a).

The action of BAPN has been investigated in vitro (Kenney, 1978) and its action is probably different from that observed in vivo. Therefore, although BAPN disrupts tooth (Kollar, 1972b) and feather (Goetinck and Sekellick, 1970) differentiation in vitro by disrupting the collagen matrix, the action of the lathyrogen in vivo could be very different (Carlson, pers. comm.). The reason that abnormalities are less often observed in the chick embryo could be related to drug action, metabolism, and elimination.

2. The metabolism and elimination of BAPN from embryonic systems

The lathyrogen, BAPN, produces a high incidence of cleft palate when injected into rodent embryos (Stivers et al., 1971); the production of this abnormality is very time dependent (Pratt and King, 1972). Investigation of radioactive BAPN in maternal and fetal tissues after lathyrogen injection into pregnant rats and mice indicate that one third of the drug is metabolized into cyanoacetic acid (CAA), in inactive form, within 20 minutes and is eliminated soon after (Waddell et al., 1974). BAPN is quickly localized in fetal neural and cartilaginous tissues, especially the fetal palatal shelves and paraoral tissues. This localization within the palatal shelves could account for the high frequency of cleft palate, and its absence from the middle ear region could account for normal ossicular

differentiation (Stivers et al., 1971). It is possible that the stage of development that is susceptible to cleft palate formation does not coincide with the susceptible stage, if any, of the middle ear region. Therefore, the observation of normal ossicles in mice with cleft palate and other craniofacial abnormalities (Stivers et al., 1971) could be due to missing the 'developmental window' of the middle ear. To eliminate this problem of maternal metabolism and elimination of the drug, the effect of BAPN and other teratogens should be investigated in the chick embryo.

The chick embryo is a closed system. During the primitive streak stage of development, the embryo has no organ systems for the elimination of teratogens. Therefore the observations of the lack of effect of BAPN when applied to early chick embryos are perplexing. Romanoff (1972) reports that application of BAPN during the earliest stages of chick development has no effect on development. Only after a vascular system is formed does BAPN have a teratogenic effect. Why doesn't the active drug wait within the egg for the development of the vascular system? Why is the drug inactive in later stages of development? Because radioactive tracing experiments have not been conducted in the chick embryo, the metabolism and elimination of BAPN in this species are as yet unknown.

7. Beta-2-thienylalanine

a. Rationale and mode of action

Beta-2-thienylalanine, an analog of phenylalanine, inhibits cellular growth and activity (Caviness, 1966a, b; Kollar and Baird, 1968). Although the precise action of this teratogen is not known, numerous investigations of it have been conducted (Hruban et al., 1965; Rabinowitz et al.,1954; Savchuck et al., 1964). Beta-2-thienylalanine is not cytotoxic since histological integrity is maintained and mitotic activity is observed (Kollar, 1968; Kollar and Baird, 1968). Caviness' (1966a, b) study on adult macrophages indicates that this drug does not inhibit RNA or protein synthesis; it is rapidly incorporated into cellular proteins. The data suggests that the synthesis of specific structural or enzymatic proteins, of as yet unknown nature, may be disrupted and results in the subsequent blockage of further development of the cells (Kollar, 1976).

Wilde (1955) investigated the effect of this drug on amphibian cephalic neural crest differentiation. Most mesenchymal derivatives are inhibited, but pigment cell differentiation is observed, demonstrating that two cell populations derived from a single source may be differentially affected. Tooth germ differentiation *in vitro* is inhibited in the presence of this drug (Kollar and Baird,

1968), by disrupting epithelial-mesenchymal interactions. Based on Wilde's (1955) in vitro studies, beta-2-thienylalanine has been classified a neural crest inhibitor (Kollar, 1976). Kollar and Baird (1968) conclude that tooth germ differentiation is disrupted by its action of the dental papilla which is derived from neural crest.

I am interested in determining whether this drug is teratogenic to chick neural crest derivatives in vivo; if it were, it would be a useful tool in the investigation of the dynamics on avian middle ear development.

b. Results

1. General comments

Beta-2-thienylalanine was injected into chick embryos HH stages 6-26 (Table 5). At low doses, this drug is relatively harmless to the embryos. Because of the low solubility of the drug, it was difficult to increase the concentration of the drug above 30mg/0.5ml. I was able to inject some embryos with as much as 56mg/0.5ml but because of the short period of time that the drug remained in solution, I could not be certain of the concentration once the drug was injected into the egg. In the dose range over which I was able to conduct this series of experiments, this drug was relatively ineffective in producing external malformations; 82/109 embryos were externally normal

although some embryos were slightly retarded (fig. 120).

Table 25 details the external malformations observed after beta-2-thienylalanine treatment. When the drug is injected into HH stages 6-16, the embryos are retarded and show coelosomia (20/109). The beak and skull are abnormal in 2/109 and 2/109 embryos respectively. The limbs are abnormal in only 3/109 embryos. Embryos treated at HH stages 17-26 are normal.

2. Histological analysis of the effects of beta-2-thienylalanine on middle ear development

The development of the middle ear components is summarized in Table 26. The columella is normal in 15/18. The footplate is completely fused with the otic capsule in 2/18 (figure 121) (Type I abnormality). The chondroblasts of the footplate are continuous with those of the otic capsule, and the annular ligament does not differentiate. In 1/18, the annular ligament begins to differentiate, but the degree of differentiation is retarded when compared to the middle ear region of a normal embryo of a comparable age. The other components of the middle ear region are all normal.

3. A comparison of the columella, beak, and limb abnormalities in selected externally abnormal embryos

The three embryos with abnormal columellae

TABLE 25

THE INCIDENCE OF EXTERNAL MALFORMATIONS PRODUCED BY VARIOUS DOSES OF BETA-2-THIENYLALANINE INJECTED INTO CHICK EMBRYOS HAMBURGER HAMILTON STAGES 6-26

Stage	Dose/ mg	#	N	Eye	LB	UB	HL	Skull	Coel
6-16	1-50	94	67	4	2	2	3	3	20
17-26	2-50	15	15	0	0	0	0	0	0
Total		109	82	4	2	2	3	3	20

NOTE: Abbreviations as previous.

TABLE 26

A HISTOLOGICAL ANALYSIS OF THE MIDDLE EAR OF CHICK EMBRYOS INJECTED WITH BETA-2-THIENYLALANINE AT HAMBURGER HAMILTON STAGES 6-16

Stage	Dose/ mg	#	NC	FP F	AL	OC
6-16	2-50	18	15	2	1	1

NOTE: Abbreviations as previous.

show extremely deformed beaks. Other embryos with equally deformed beaks have normal columellae. Because of the relative absence of external malformations, the skeletal differentiation of treated embryos is evaluated in four whole stained embryos. The beak and limbs of these embryos are normal; therefore these embryos were not sectioned for histological examination. I could find no relationship between the abnormal limbs and columella abnormalities.

4. Summary of results

1. Beta-2-thienylalanine is relatively harmless to the chick embryo in concentrations of 1-56mg/embryo.

2. Only 3/18 columella are abnormal; 2/18 show Type I abnormality and 1/18 have a retarded annular ligament.

3. The absence of teratogenic effect could be due to the low solubility of the drug.

c. Discussion

1. General conclusions

Beta-2-thienylalanine has been classified as a neural crest inhibitor (Kollar, 1976) based on two in vitro studies (Kollar and Baird, 1968; Wilde, 1955). The relative absence of teratogenic activity of the drug is initially surprising, since many structures are derived from neural crest (LeLievre and Le Douarin, 1975). The results could be explained in either of two ways. First,

the classification of beta-2-thienylalanine as a neural crest inhibitor may be too general. Beta-2-thienylalanine also disrupts the development of other systems, i.e., mouse vibrissae, derived from neural crest cells (Caviness, 1966a,b; Kollar, 1968). Therefore, the drug's action is better described as disrupting epithelial mesenchymal interactions. It appears that in many systems, one cell population is more sensitive to this teratogen than another. There seems to be no good reason to conclude that this teratogen acts selectively on cephalic neural crest derivatives over other cell populations. My in vivo chick experiments do not show a selective sensitivity of any cell population to beta-2-thienylalanine. The majority of the embryos are externally normal, although some are slightly retarded. The absence of almost all teratogenic effects requires an alternative explanation.

All previous investigations with this drug were conducted in vitro (Caviness, 1966a, b; Kollar, 1968; Kollar and Baird, 1968; Wilde, 1955). Beta-2-thienylalanine has a low solubility, preventing an increase in the concentration in a given volume. Because the chick embryo is a closed system and any large volume injected into the egg results in embryonic malformations (Romanoff, 1972), the volume of injection could not be greatly increased. It is likely that the drug concentration injected into the embryo

is below the minimum teratogenic concentration so that for this reason, the embryos are relatively unaffected. I observed external malformations in some embryos; but these skull, beak and eye abnormalities are not dose-related. Columella abnormalities are observed in three of these externally abnormal embryos. The abnormalities observed could be due to a minor teratogenic action of the drug combined with the effects of relatively large volumes of fluid being injected into the embryos.

9. Discussion

a. Introduction

Histological studies reveal the morphological complexity of columella development. Recent experimental studies have indicated a much wider range of neural crest contributions to cranial elements than previously known (Le Lievre and Le Douarin, 1975). Information on the otic region is still incomplete, but if indeed the otic capsule as well as the columella receives a contribution from cephalic neural crest, this might explain the complex pattern of interactions between the adjacent primordia observed during the development of the tissues of the fenestra ovalis region. Teratological interventions provide one approach to understanding morphogenesis in complex systems.

b. General summary of the effects of teratogens on columella development

The development of the columella was evaluated in 263 teratogen treated embryos (Table 27); 122/263 have normal columellae. The columella is rarely completely absent in the viable embryo. Where a columella could not be identified, the embryos are extremely retarded and the otic region is very deformed. Yet, components of the columella are sometimes absent. In six embryos, only the extracolumella could be identified, the other components are absent (see Table 27, *cs). Of the remaining embryos evaluated, the fenestra ovalis region is most frequently affected by teratogen treatment. All three types of columella abnormality previously described (see pg. 71) are observed after teratogen administration over a wide range of developmental stages.

In 16/268 abnormal embryos, the columella shaft never becomes continuous with the otic capsule, and since both footplate and fenestra ovalis are always absent, there is no annular ligament. This Type II abnormality was seen in 15 hadacidin-treated embryos and one BAPN-treated embryo, but not as a result of any other teratogen.

In 73/263 embryos, the columella footplate is fused with the otic capsule, and the annular ligament does not differentiate. This Type I abnormality resulted from all teratogens except beta-2-thienylalanine. A 'minor'

TABLE 27

A SUMMARY OF COLUMELLA DEVELOPMENT AFTER TERATOGEN TREATMENT ON CHICK EMBRYOS

Drug	Stage	#	NC	DC	EC	AbsC	FPF	AL	FPAbs	OC	FO
H	5-9	35	12	12	6	6*2cs	8	5	10	0	0
BT	6-16	18	15	0	0	0	2	1	0	1	0
BAPN	9-16	4	3	0	0	0	0	0	1	0	0
P	11-16	1	0	0	0	0	0	1	0	0	1
F	11-16	2	1	0	0	0	0	1	0	0	0
H	10-16	18	10	7	1	4*2cs	3	3	3	0	0
BAPN	17-25	36	34	0	0	0	2	0	0	1	0
P	17-25	40	15	3	0	0	8	18	0	0	2
F	17-25	30	4	2	1	2	16	9	0	0	1
H	17-25	40	16	7	3	2*2cs	16	7	2	0	2
TEM	24-25	28	5	4	4	4	14	5	0	3	2
BAPN	26-33	4	4	0	0	0	0	0	0	0	0
F	26-28	6	2	0	0	0	4	0	0	0	0
H	25+-30	1	1	0	0	0	0	0	0	0	0
Total		263	122	35	15	18	73	50	16	5	8

NOTE: FO = fenestra ovalis abnormal. FPAbs = footplate absent.

*cs indicates that the columella shaft is absent; other abbreviations as previous.

abnormality is frequently observed; the degree of differentiation of the annular ligament is retarded (50/263). Because the development of the embryos must be terminated before I can evaluate the middle ear, I cannot determine whether a) this retardation is an intermediate step between normal fenestra ovalis formation and a fused footplate or b) the development would eventually 'catch up' and form a normal fenestra ovalis. This abnormality is most frequently observed after pilocarpine treatment (19/41).

The fenestra ovalis region is frequently abnormal due to malformations of the footplate as described above. It can also be deformed due to abnormalities of the otic capsule (see fig. 75, Table 27); this abnormality was not classified in the categories of columella abnormalities. In 8/263 embryos, a portion of the capsule adjacent to the footplate is absent. This results in the absence of a portion of the annular ligament with the footplate appearing free-standing. This abnormality is observed after hadacidin, TEM, 5FU, and pilocarpine treatment.

In 35/263 embryos, the size and shape of the columella is frequently deformed; the degree of malformation ranges from slight to very deformed. This Type III abnormality resulted from hadacidin, TEM, 5FU, and pilocarpine administration. In 15/35 embryos, the extracolumella is abnormal. In four of these, a discontinuity

is observed between the columella and extracolumella; these two components are separated by a perichondrium. This abnormality, observed after TEM and 5FU treatment, may be the result of different rates of chondrogenesis in the columella shaft and extracolumella. Once a chondrogenic tissue is surrounded by a limiting perichondrium, it is prevented from fusing with other chondrogenic tissues.

c. The effect of teratogens on different stages of columella development

Whether or not the otic skeletal tissues are wholly of neural crest origin (see pg. 152), data from studies on avian neural crest cell migration (Johnston, 1966; LeLievre, 1974; Noden, 1975), combined with the data from the descriptive and experimental studies of avian ear development previously discussed (see section IIA and IIIA), permit the division of columella development into four phases:

1. The period prior to neural crest cell migration, HH stages 5-9 (Johnston, 1966; Noden, 1975).

2. The period of neural crest migration and proliferation, HH stages 10-16 (Johnston, 1966; Noden, 1975).

3. A period of "covert" differentiation, HH stages 17-25. The chorioallantoic membrane grafting experiments have shown that initiation of columella differentiation

must occur prior to HH stage 17, but no morphological indications are apparent until HH stage 25+ (Romanoff, 1960).

4. A period of "overt" organogenesis and the beginning of cytodifferentiation, HH stages 25+ (Romanoff, 1960; see pg. 22).

Teratogens were administered during all phases of columella development, although some were only administered during one or two of these phases. The three categories of columella abnormalities were observed when teratogens were administered during the first three phases of development and relative differences in the frequency of abnormalities seem to occur. These differences could be due to the different actions of the drugs as well as the different phases of development at which the drugs were applied (see Table 27).

Hadacidin was injected over a wide range of developmental stages (HH stages 5-30) thus permitting a complete evaluation of the effect of the teratogen on columella differentiation. All three categories of columella abnormality are observed after hadacidin administration during phases 1-3, but relative differences in the frequencies of abnormality are seen. Types II and III predominate when the drug is administered during phases (1) and (2), while Type I predominates when the injection is

effected during phase (3). Injections during phase (4) do not produce columella abnormalities.

The other teratogens were administered over a narrower range of developmental stages (Table 27), but the abnormalities can be evaluated by considering at what phase of development the drug was applied. 5-fluorouracil (5FU) injected during phase (3) predominantly produces Type I columella abnormality. Pilocarpine administered during the same time period produces primarily a retardation of annular ligament differentiation, although some Type I abnormalities are also seen. Triethylene melamine (TEM) is extremely toxic to the early chick embryo. Viable embryos were only recovered after TEM administered during HH stages 24-25; a fused footplate is most frequently observed (Type I) although a deformed columella (Type III) is also observed. Beta-aminopropionitrile (BAPN), injected over a wide range of developmental stages (HH stages 9-33), results in a relatively normal columella. Only 3/44 columella are abnormal. Beta-2-thienylalanine is relatively ineffective in inducing columella abnormalities when injected during phase (2).

Teratogen administration during the period of overt differentiation (phase 4-HH stages 25+) produced variable results. Hadacidin and BAPN treatment results in a normal columella while 5FU can, in addition to a normal ossicle, result in a fused footplate.

d. The possible etiology of columella abnormalities

Footplate differentiation is one of the most complex aspects of columella formation. Primordial chondrogenic cell populations associated with the second visceral arch and otic capsule become continuous and both contribute to the definitive footplate (Reagan, 1917; Simons, 1974). This interaction between the chondrifying tissues of the columella and the otic capsule results in the differentiation of the annular ligament. Teratogens injected during the period of neural crest cell migration and proliferation [phases (1) and (2)] (pg.156) could prevent the proper orientation and/or fusion of the two cell populations. The columella shaft remains separated from the otic capsule. Once each chondrifying tissue becomes surrounded by a limiting perichondrium, establishment of continuity between them is prevented. If the drug is applied during the third phase, the cell populations can become continuous, but the interactions resulting in the annular ligament are disrupted. In normal differentiation, the presumptive annular ligament cells dedifferentiate after an initial period of cartilage formation. This sequential pattern of chondrification of the capsule and columella shaft characterizing the normal is not seen, and the position which should be occupied by a differentiating annular ligament is not significantly different than the adjacent regions.

Complete absence of the columella is rare, but defects and/or absence of one or more component parts--the footplate, shaft, or extracolumella--are common. It appears that whenever a drug is applied relative to neural crest migration and proliferation, if the embryo survives, derivatives of mesectoderm are rarely absent. Thus initiated columella development proceeds, and such defects as do occur are due to disruptions of normal patterns of cell proliferation and/or orientation.

e. Factors which influence neural crest cell migration

The cephalic neural crest cells migrate ventrally from the vicinity of the neural folds during its closure to form the neural tube (HH stages 9-16, Noden, 1975) to the region of the visceral arches (Johnston & Listengarten, 1972). The majority of the cells migrate beneath the surface ectoderm through a relatively cell free space surrounded by an amorphous matrix (Johnston and Listengarten, 1972). By studying labeled precursor incorporation, Pratt et al. (1975) demonstrated that hyaluronate is the predominate glycosaminoglycan within this space. Neural crest cells synthesize and secrete hyaluronate into the extracellular space (Greenberg and Pratt, 1977). Toole et al. (1972) proposed that hyaluronate may act at the cell surface during embryogenesis to regulate cell interactions.

Hyaluronate would be a suitable matrix for cell migration, and regulated timed removal by hyaluronidase could immobilize the cells, thus permitting cell aggregation, interaction, and differentiation (Toole et al., 1972). Pratt et al. (1975) propose that hyaluronate could provide a substrate suitable for neural crest cell migration, and the removal of the matrix by hyaluronidase at the completion of migration would ensue in cell aggregation.

Is there any experimental evidence concerning the effect of teratogens on neural crest cell migration? Vitamin A is a potent teratogen producing craniofacial anomalies in the mammalian embryo similar to the first arch syndrome or Treacher Collins Syndrome (Johnston and Pratt, 1975). Experiments have been conducted attempting to determine the etiology of this abnormality. Morriss (1975) injected excess Vitamin A into pregnant rats and observed facial, otic, and brain abnormalities. The otocyst was positioned at the level of the mandibular arch rather than the hyoid arch. In vitro experiments of mouse neural crest cells (Morriss, 1975) indicate that cell migration is abnormal in the presence of Vitamin A. Hassell et al. (1977) have conducted similar in vitro experiments on the chick embryo. Vitamin A prevented the appearance of cranial neural crest migration in the first visceral arch. They observed an increase in cell density at the

arch's base, suggesting that Vitamin A may alter cell migration. Kwasigrich and Kochhar (1975) investigated the effect of Vitamin A on limb mesenchymal cells and concluded that cell migration is altered. The exact teratogenic action of Vitamin A is not known, but experimental evidence indicates that it may alter the synthesis of glycoproteins and glycosaminoglycans (Deluca and Yuspa, 1974; Yuspa and Harris, 1974). Neural crest cells synthesize glycoproteins and glycosaminoglycans into the extracellular space. Vitamin A may alter the surface glycoprotein or the secretion and distribution of matrix during neural crest migration (Hassell et al., 1977). However, Vitamin A might also inhibit cell proliferation or cause cell death, therefore accounting for the reduction in the number of neural crest cells (Hassell et al., 1977).

I have not conducted in vitro studies on the teratogens in the experiments. Therefore, I cannot definitively state their actions on the chick cells. It is possible that, in part, the teratogens (TEM, 5FU, hadacidin) might interfere with neural crest cell migration. Yet it seems more logical that the antimitotic drugs would interfere during the phase of neural crest cell proliferation, and/or at higher concentrations, produce cell death. The end result would be the same--a decreased number of crest cells within the visceral arch leading to malformation of the ossicle.

f. The importance of cell number during development

In the above discussion of columella abnormalities I have concluded that teratogens rarely affect the initial determinative events, but probably influence subsequent patterns of cellular orientation and proliferation. The antimitotic action of hadacidin, TEM, and 5FU, and the resulting cell death when these drugs are administered at higher concentrations would result in a decreased number of mesectodermal cells, insufficient to become continuous with the otic capsule and form a normal columella (Type II and some Type III abnormalities). The importance of cell number has been investigated in other developmental systems.

Holtzer et al. (1972, 1975) proposed that the steps in cell differentiation are based on quantal mitosis. Every cell has the potential to divide and produce daughter cells with identical or different synthetic pathways from the mother cell. It is only after the completion of a specific number of cell division, quantal cell cycles, that the chromosomal structure can undergo the rearrangement required to reprogram the daughter cells. These cells could then produce the specific proteins characteristic of differentiated cells, i.e., muscle, cartilage, blood cells. While the experimental evidence underlying Holtzer's "quantal mitosis theory" has been subject to considerable debate, the basic premise of a relationship between cellular kinetics

and the onset of differentiation has been recently emphasized in limb development. According to the most recent theory of chick limb development, different limb segments are determined at sequential stages of time (Wolpert, Lewis, and Summerbell, 1975). The positional value may control the selection of a course of cytodifferentiation as muscle or cartilage. The mesenchymal cells pass through a 'progress zone' where they are responsive to positional signals. The cells migrate from the zone and begin to differentiate; this change in cell character within the zone is related to cell division. The last cells to leave this region will differentiate into more distal limb structures. Therefore, subsequent determinative events associated with the formation of limb segments appear to be a function of proliferation and morphogenesis (Summerbell and Lewis, 1975). Caplan and Koutroupas (1973) emphasized the role of cell position within the developing cartilage of the limb. They suggested that the proximity of any cell to the vasculature could affect its differentiation, and therefore its susceptibility to teratogens. Since cell position is the result of cell proliferation and aggregation, it therefore seems that cell number is extremely important in limb development.

Since the columella and beak are derived from cephalic neural crest, it is surprising that while embryos

with deformed columellae always have other craniofacial abnormalities, such anomalies may be seen in embryos with quite normal columellae. To try to understand the different effect of teratogens in two chondrogenic tissues of the same embryonic origin, I investigated a completely different type of chondrogenic tissue, that of the hindlimb which derives from somatic mesoderm.

Limb abnormalities are very time dependent, so that they are rarely observed when teratogens are administered prior to HH stage 17. Many teratological studies show that different times of drug application result in more distal deformities (Kocher, 1977). This might be explained according to the theory that limb segments are determined at sequential stages of development (Wolpert, Lewis, and Summerbell, 1975). The drugs used in the reported experiments (Table 27) did not produce such predictable results. I therefore conclude that the drugs might influence patterns of cellular orientation and proliferation in any and all parts of the determined organ. However, subsequent determinative events associated with the formation of the limb segments which appear to be a function of proliferation and morphogenesis (Summerbell and Lewis, 1975) may be affected indirectly so that distal deficiencies of varying forms may be observed on a random basis. This argument can provide an understanding of the

nature of craniofacial and columella defects I have observed in teratogen treated embryos.

The period of time associated with the overall growth of the rostral craniofacial components involves anterior extension of the trabeculii, pterygoquadrate bars, and Meckelian cartilages, and their interactions with dermal ossifications: it is as long as that occupied by limb growth. Thus we would describe all craniofacial defects as arising from distrubances of patterns of cell proliferation and orientation association with growth. One may not speak of the determination of mesectodermal derivatives being inhibited, if such an event occurs, the embryo dies. The morphogenesis of the columella itself does not involve "growth," rather it concerns specific modeling of constituent parts. Thus the columella is rarely absent; it is merely deformed, or resists any teratogen effect, and thus appears normal. Because the differentiation of the fenestra ovalis region is the result of complex interactions, this region is the most susceptible to teratogen effects. This is due to the fact that its determination occurs relatively late in time, and subsequent differentiation occupies a considerable length of time.

g. Other factors effecting columella development

I have been discussing the effect of cell

migration, proliferation, and orientation on the development of the avian columella, but the effect of the teratogens might be more general. All embryos with abnormal columellae are retarded and show craniofacial abnormalities. The columella might be abnormal as the result of malformation of the cranium rather than the direct action of the teratogens on the constituent cells. Abnormal positionings of the inner ear, otic capsule, beak, vasculature, and other cranial tissues could exert abnormal stresses on the developing columella resulting in malformations. Leibel (1976) investigated the effect of experimental manipulation of skull formation in Ambystoma and observed that in the absence of the otic capsule, skeletal components are frequently abnormal in size and shape. Benoit (1960) and Corsin (1972) observed similar results in the chick and another amphibian (Pleurodeles) respectively. Leibel (1976) described bending of the parasphenoid bone and parachordal cartilages which he attributed to epigenetic effects resulting from unequal mechanical stresses within the developing skull. Studies on the eye (Corsin, 1972; Coulombre and Crelin, 1958) provide additional evidence concerning mechanical effects during craniogenesis. The absence of the eye results in abnormal orbital and extraorbital bones, and trabecular cartilages. Since mechanical (epigenetic) factors might exert a major effect during

cranial development, I cannot ignore the fact that the skull is abnormal in all embryos with abnormal columellae when trying to interpret my experimental results.

The above interpretation suggesting that mechanical factors could produce abnormal columella development facilitates an explanation of the similarities in abnormalities observed in the CAM grafts and the teratogen experiments. In both types of experimental investigations, all three categories of columella abnormalities are observed. This could be due to the fact that three dimensional morphological integrity must be maintained in order for the columella to differentiate. Abnormalities could be produced by a disruption of normal cranial morphological relationships, as the result of CAM grafting or teratogenic interruption of development.

h. The effect of teratogens on other components of the middle ear region

Other components of the middle ear region can be affected by teratogen treatment. Table 28 summarizes the effects on the paratympanic organ, the middle ear cavity, the external auditory meatus, and the tympanic membrane.

The paratympanic organ is observed in all experimental animals; this structure frequently permitted the identification of the middle ear region in very deformed animals. In 19/263, the histodifferentiation of the

TABLE 28

A SUMMARY OF THE DEVELOPMENT OF THE MIDDLE EAR COMPONENTS* AFTER TERATOGEN TREATMENT ON CHICK EMBRYOS

Drug	Stage	#	PT	EAM	TM	MEC
H	5-9	35	5	11	30	18
BT	6-16	18	0	0	0	0
BAPN	9-33	44	0	0	0	0
P	11-16	1	0	0	0	0
5FU	11-16	2	0	0	0	0
H	10-16	18	6	5	10	4
P	17-25	40	0	0	6	5
5FU	17-25	30	2	7	17	14
H	17-25	40	0	1	10	6
TEM	24-25	28	6	4	14	10
5FU	26-28	6	0	0	1	0
H	25+-30	1	0	0	0	0
		263	19	28	88	57

NOTE: Abbreviations as previous;

*Excluding the columella.

organ is abnormal and it might be continuous with the middle ear epithelium.

A deformed columella is often associated with a dimunition of the middle ear cavity (57/263). The degree of pouch expansion and the corresponding mesenchymal elimination is retarded. The mesenchyme is usually more densely packed within the middle ear region than in the normal embryo. In 28/263 embryos, the invagination of the surface ectoderm to form the external auditory meatus is decreased or absent. Since the epithelia of the tympanic membrane are continuous with those of the middle ear cavity and the external auditory meatus, abnormalities in the latter two structures would result in an abnormal tympanic membrane. The tympanic membrane is also abnormal in three embryos each with a relatively normal middle ear cavity and external auditory meatus.

All teratogens that produce columella defects can induce abnormalities of the other middle ear components. I could not determine a relationship between the frequency of columella abnormalities of the other components or with each other. Therefore, a more detailed discussion of these components will not be presented in this thesis.

SECTION IV

OVERALL CONCLUSIONS

The development of the tetrapod middle ear is extremely complex. The morphological studies indicate that the development of the fenestra ovalis region in the chick and mouse is the result of a series of interactions between the chondrifying tissues. In the CAM grafting experiments and teratogen studies, the fenestra ovalis region is frequently disrupted while other middle ear components are normal. These experimental studies provide further evidence that the development of this region is the result of cellular interactions.

Middle ear abnormalities observed in humans (Altmann, 1957; Hough, 1963; Warkany, 1971) can be better explained by understanding the development of the middle ear components. The high frequency of occurrence of stapedial abnormalities, and the low frequency of malleal and incal abnormalities are probably associated with differences in the complexity of interactions between the proximal and distal ossicular elements. This conclusion supports that of previous investigators (Altmann, 1957).

The experiments described in this thesis do not permit any definitive statements regarding putative epithelial mesenchymal interactions in middle ear development such as have been demonstrated in amphibia (Helff, 1940; Holtfreter, 1968). A possible approach for further investigation of this problem is the in vitro cultivation of the middle ear region, permitting a separation of the individual components and the determination of possible interactions.

APPENDIX I

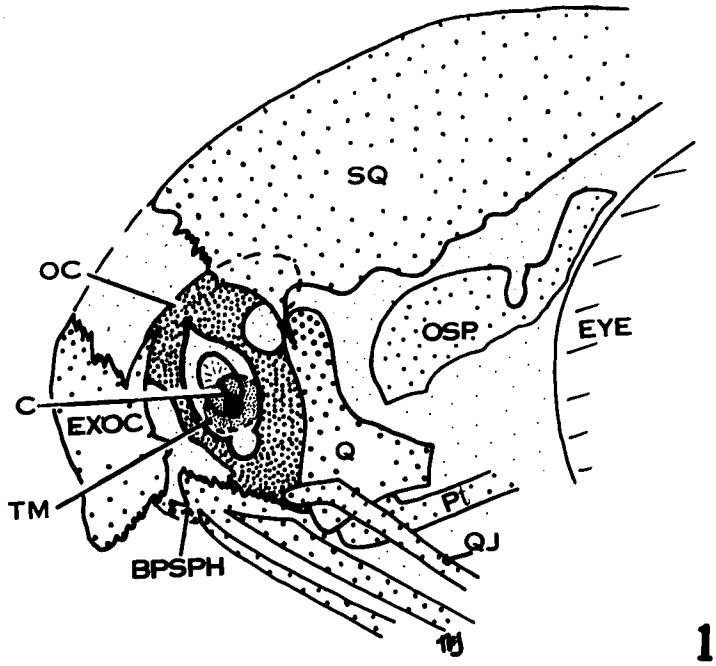
FIGURES

ABBREVIATIONS FOR FIGURES

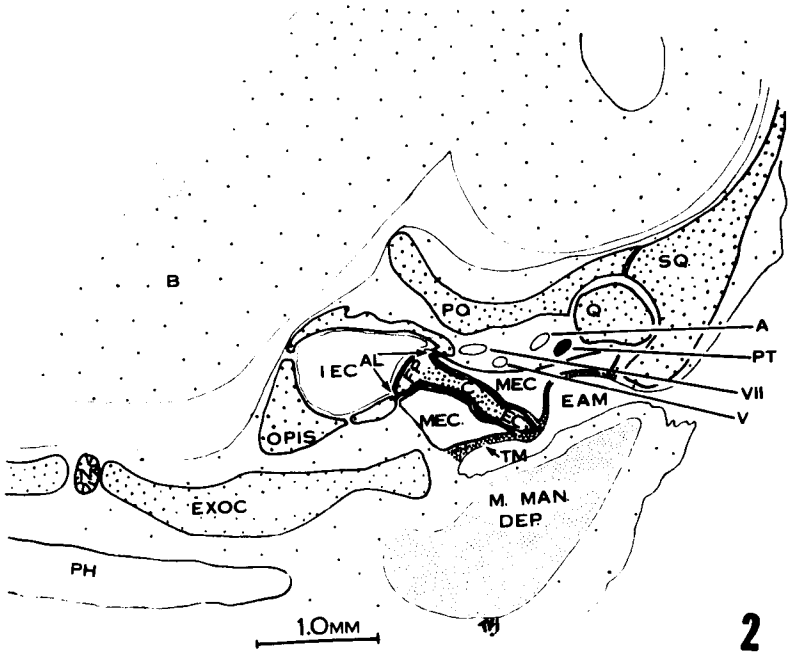
A	external ophthalmic artery	N	notochord
AL	annular ligament	OC	otic capsule
B	brain	OF	obturator foraman
BPSPH	basiparasphenoid	OPIS	opisthotic
BV	blood vessel	OSP	orbitosphenoid
C	columella	P	perichondrium
CS	columella shaft	PE	periderm
DET	dorsal eustachian tube	PH	pharynx
E	endodermal epithelium	PO	prootic
EAM	external auditory meatus	PT	paratympanic organ
EC	extracolumella	Pt	pterygoid
ET	eustachian tube	Q	quadrate
EXOC	exoccipital	QJ	quadratojugal
FP	footplate	S	stapes
I	incus	SA	stapedial artery
IE	inner ear	SC	sensory cells
IEC	inner ear cavity	SM	stapedial muscle
ISJ	incudostapedial joint	SQ	squamosal
K	keratinizing epithelium	SG	stratum germinumtivum
L	lagena	TM	tympanic membrane
M	mesenchyme	TTM	tensor tympani muscle
MA	malleus	TTS	tubotympanic sulcus
MEC	middle ear cavity	V	jugular vein
MM	malleus manubrium	VET	ventral eustachian tube
M. Man. Dep.	mandibular depressor muscle	VII	seventh nerve

1. A line drawing based on examinations of alizarin preparations of late embryos, stages 40 - 46, and newly hatched chicks of the posterior lateral region of the skull to demonstrate the avian ear region. The specimens were tilted dorsally, and viewed along the axis of the antero-ventro-laterally directed columella (C). The columella therefore lies approximately perpendicular to the plane of the figure.

2. An idealized cross section through the middle ear of a stage 39 - 40 embryo. The footplate is suspended in the fenestra ovalis by the annular ligament (AL). The middle ear cavity (MEC) communicates with the pharynx (PH) via the antero-medio-ventrally directed tube, the Eustachian tube, which therefore lies considerably anterior to this idealized cross section. The middle ear cavity is continuous anteriorly, posteriorly, and dorsally with the extensive pneumatized spaces which characterize the avian skull (Stork, 1972). The ossicle is tensed by a single muscle, the columella muscle, inserting on the extracolumella and the tympanic membrane. The muscle is innervated by the facial nerve (Pohlman, 1921). This structure will not be discussed in this thesis. For further explanation of the diagram, see text page 21.

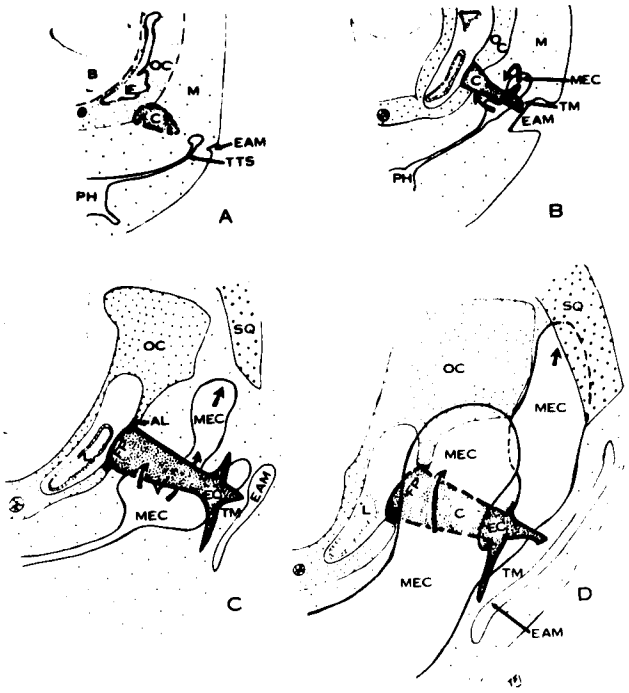

SQ
OC
OSP
EYE
C
EXOC
Q
TM
Pt
QJ
BPSPH

1


B
SQ
PQ
Q
A
PT
IEC
AL
MEC
VII
EAM
V
MEC
OPIS
TM
EXOC
M. MAN.
DEP.
PH
1.0MM

2

3. Schematized drawings of four phases in the development of the avian middle ear. A. Stages 29 - 30 (6 - 7 days). B. Stages 31 - 34 (7 - 8 days). C. Stages 35 - 36 (8 1/2 - 10 days). At this time, the external auditory meatus (EAM) has become oriented so that in a section showing the columella (C), it is seen as an enclosed space. D. Stages 37 - 38 (11 - 12 days). Arrows in B - D indicate the direction of expansion of the pouch diverticula.


B
OC
E
M
C
EAM
TTS
PH
A
OC
M
C
MEC
TM
EAM
PH
B
OC
SQ
AL
MEC
TM
EAM
MEC
C
SQ
OC
MEC
MEC
L
C
TM
MEC
EAM
D

3

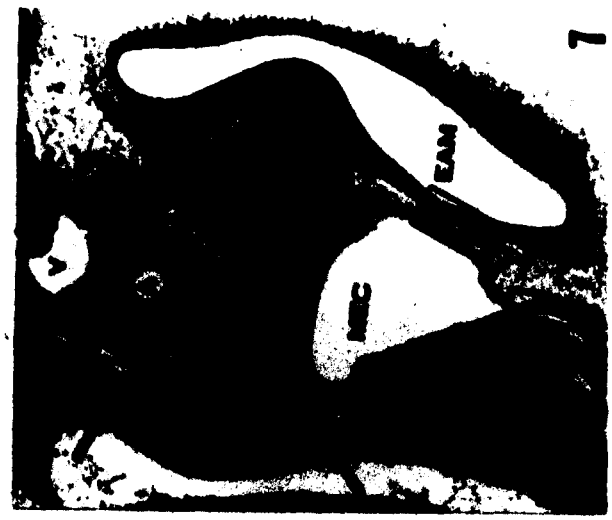
4. A cross section of stage 29 through the presumptive middle ear, showing the relationship of the tubotympanic sulcus (TTS) to the columella (C) condensation and the external auditory meatus (EAM). The columella is separate from the otic capsule (OC). The lateral extremity of the tubotympanic sulcus in this section is the paratympanic organ (PT) which is still continuous with the first pharyngeal pouch.

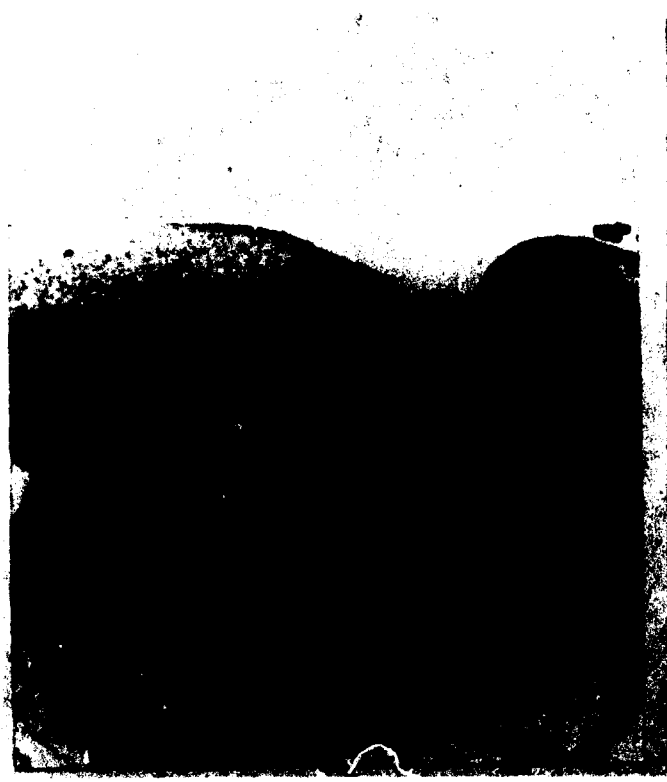
5. A cross section of stage 32 showing the further development of the columella (C), spanning the middle ear region which still contains mesenchyme (M). The mesenchyme adjacent to the ectodermal aspect of the tympanic membrane (TM) is denser than that of adjacent regions of the external auditory meatus (EAM). Numbered rectangles in this and subsequent figures refer to subsequent photomicrographs.

6. A cross section of stage 34 in which only the mid region of the columella (C) is present. The distal portion of the tubotympanic sulcus expands to form the middle ear cavity (MEC), while the proximal region becomes the Eustachian tube (ET). Note the differential density of the mesenchyme adjacent to the epithelium of the external auditory meatus (EAM) and the tympanic membrane (TM).

7. A cross section of stage 35 showing the extracolumella (EC) inserting on the tympanic membrane (TM). The footplate (FP) is separated from the otic capsule (OC) by the presumptive annular ligament (arrows). The ventral tympanic membrane (TM) is formed by the expansion of the middle ear cavity (MEC) ventral to the columella (C).


EAM
5


EAM
MC
7



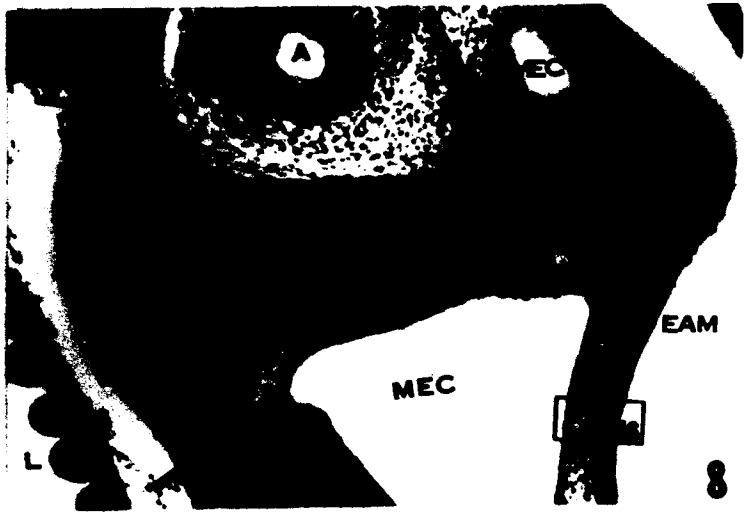

EAM
MC

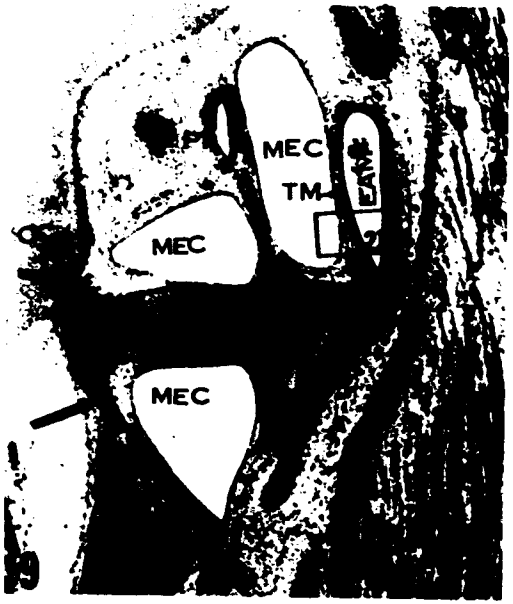
8. A section anterior to Figure 7 indicating a small diverticulum from the middle ear cavity (MEC) wrapping around the extracolumella (EC) to form a small middle ear cavity positioned dorsally. Arrows indicate the presumptive annular ligament which suspends the footplate (FP) within the fenestra ovalis.

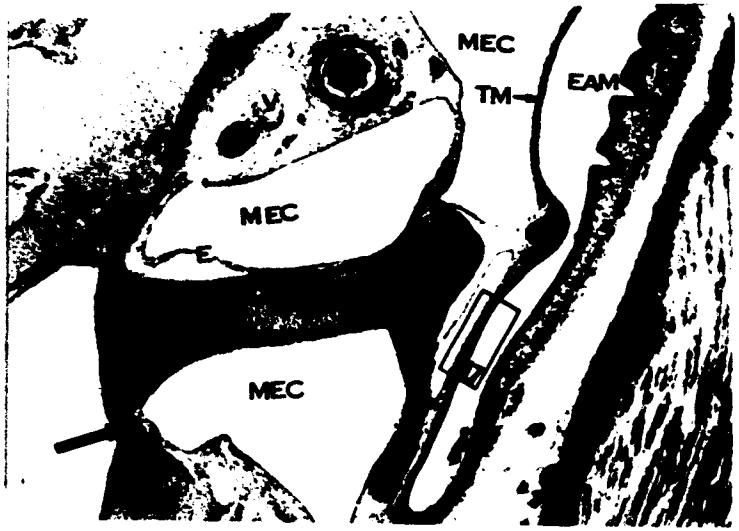
9. A cross section of stage 36 showing the continued expansion of the middle ear cavity (MEC) around the anterior and dorsal aspect of the columella-extracolumella. The extracolumella (EC) inserts by three processes: 1. supracolumella. 2. extracolumella 3. infracolumella, onto the tympanic membrane (TM). Arrows indicate the presumptive annular ligament.

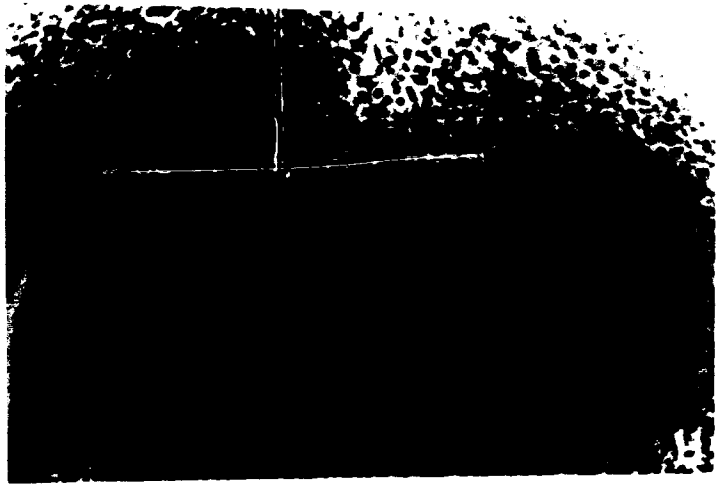
10. A cross section of stage 38 showing the differentiated middle ear. The columella (C) is now suspended within the middle ear cavity (MEC) so that a simple squamous epithelium surrounds the ossicle. The footplate (FP) inserts into the fenestra ovalis by the annular ligament (arrows). The tympanic membrane (TM) is thinner due to the differentiation of the mesodermal component. Note the folded wall of the external auditory meatus (EAM).

11. A high power view of the juxtaposition of the otic capsule (OC) and columella (C) at stage 30. A lighter staining region (arrows) of less dense cells separates the columella from the otic capsule.


A
EC
EAM
MEC
L
8


MEC
TM
EAM
MEC
MEC
9


MEC
TM
EAM
MEC
E
MEC


11

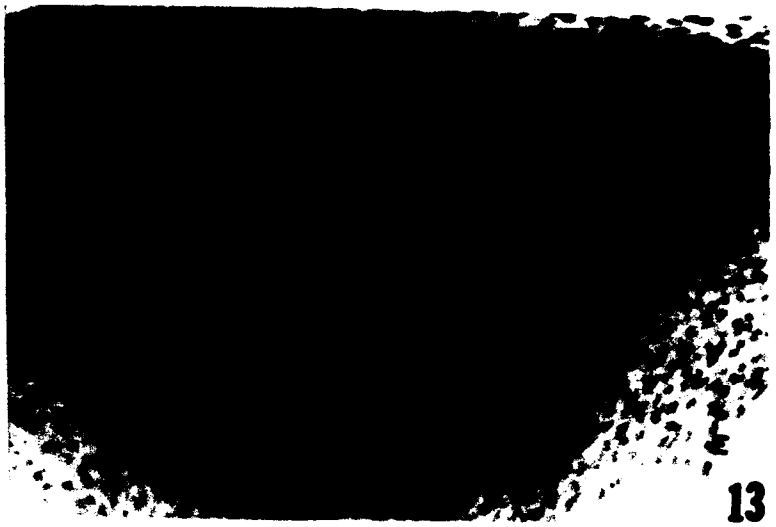
12. A cross section of stage 32 through the presumptive footplate region of the columella (C). The columella has fused with the otic capsule (OC), in which there are regionalizations. The arrows indicate a cell population separating one region of the capsule from the other. Note the difference in density of staining between the columella and the otic capsule.

13. A higher power view of the presumptive footplate region in Figure 12. At this magnification, the cells in the band (arrows) between the columella (C) and the otic capsule (OC) may be seen to be more elongate than the chondroblasts of the adjacent tissues.

14. A cross section of the footplate region at stage 34. This stage contrasts with stage 32 (fig. 12) by virtue of the differential staining densities throughout the chondrogenic tissues, especially in the band of lighter cells most proximal to the columella (heavy arrows), and the two lateral regions (light arrows) where the cells represent the presumptive annular ligament. Note also the differentiation of perichondrial tissues (P).

15. The Eustachian tube (ET) showing the characteristic histological differences between the dorsal (DET) and ventral (VET) epithelia. The mesenchyme adjacent to the ventral epithelium is denser and contains more alcian blue staining fibers than those adjacent to the dorsal region (cf. figs. 4 - 7).

12



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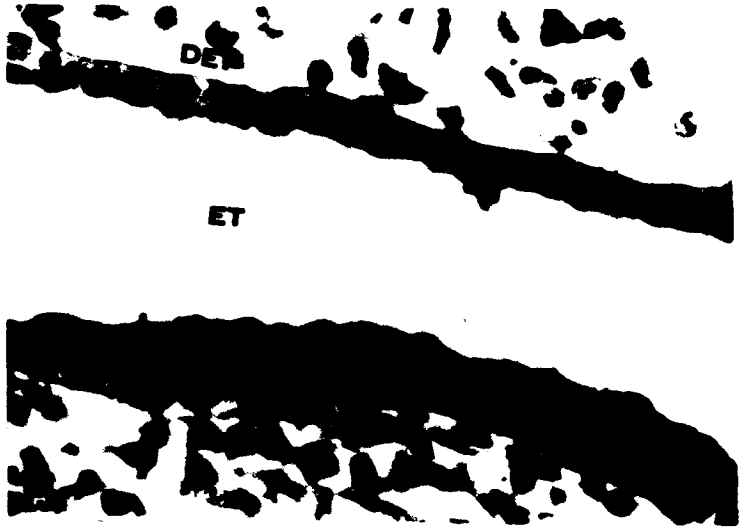


16. The tympanic membrane at stage 35 separating the middle ear cavity (MEC) from the external auditory meatus (EAM). The endodermal epithelium (E) against the middle ear cavity consists of fairly flattened cells with rounded nuclei. The ectodermal epithelium facing the external auditory meatus has a germinal layer (SG) overlain by flattened cells representing a periderm (PE). Note the differential staining of the mesenchymal cells next to the two epithelia (cf. figs. 7 - 8).

17. A differentiated tympanic membrane at stage 43. While the endoderm (E) is a simple squamous epithelium, the ectoderm has a keratinizing squamous epithelium (K). The intermediate mesodermal region is composed of collagen fibers. Note the acellular material (arrows) within the middle ear cavity (MEC) (cf. fig. 10).

18. A region of the presumptive middle ear cavity dorso-anterior to the columella and surrounded by the otic capsule (OC). Within the mesenchyme (M) are numerous spaces: 1. middle ear cavity, 2. coelomic spaces, 3. blood vessels. Note the distinct epithelial separation between the space types (arrow).

19. A high power view of the spaces in Figure 18. The spaces are separated by epithelia (arrow) and are distinct from each other.

20. The ectodermal aspect of the tympanic membrane (TM) of a stage 26, indicating the unspecialized epithelium. The mesenchyme (M) beneath the ectoderm is already dense, but the cells are randomly oriented and rounded unlike the situation seen in Figure 16 (cf. figs. 5 - 6).

21. The histology of the ectodermal epithelium at stage 36. Note the difference in thickness between the ectoderm over the tympanic membrane (TM) and that of the rest of the external auditory meatus (EAM) (cf. fig. 9).

18
C
M
1
2
3
EAM
17
EAM
TM
EAM
20
EAM
FE
MEC
16
1
2
3
19

22. The epithelium of the external auditory meatus (EAM) of stage 42 has been thrown into extensive folds. The cleft shows a typical keratinizing squamous epithelium (K). The cleft is filled with acellular material (arrow) (compare this material to that in the middle ear cavity in figure 17).

23. The paratympanic organ of stage 42 showing the sensory cells (SC) on its medial aspect. The lumen contains alcian blue staining material.

24. The paratympanic organ (PT) is discontinuous with the tubotympanic sulcus (TTS) in stage 32. Note the shape and assymetry of the organ.

25. The paratympanic organ (PT) is embedded in connective tissue lateral to the external ophthalmic artery (A) and a branch of the facial nerve (VII).

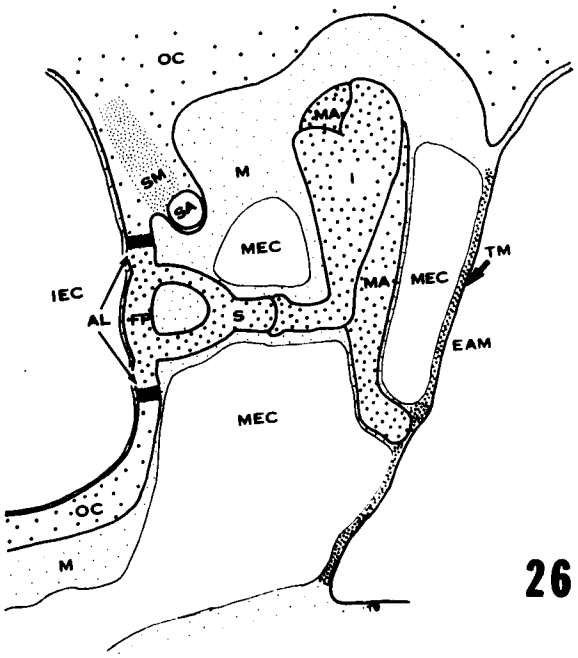
22

23

24

25

26. An idealized cross section through the middle ear of a 9 - 14-day-old mouse. The stapedial footplate is suspended in the fenestra ovalis by the annular ligament (arrows). The ossicles are not completely suspended within the middle ear cavity (MEC) and the body of the incus is still surrounded by undifferentiated mesenchyme (M). The malleus manubrium (MM) inserts onto the tympanic membrane (TM). The stapes is supplied with the stapedius muscle, innervated by the facial nerve. This structure will not be discussed further in this thesis.



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27. A cross section through the middle ear of a 13-day-mouse fetus showing the ossicle condensations. The prechondroblasts of the stapes (S), incus (I), and malleus (MA) are more densely packed than those of the otic capsule (OC). The external auditory meatus (EAM) is a distance from the ossicles.

28. A cross section through the middle ear of a 15-day-mouse fetus. The cells of the stapedial footplate (FP) is more densely packed than those of the otic capsule (OC). The presumptive annular ligament (arrows) can be identified. The malleus manubrium (MM) is adjacent to the endodermal epithelium (E) of the middle ear cavity, an expansion of the lateral portion of the tubotympanic sulcus (TTS).

29. A high power view of Figure 28, showing the fenestra ovalis region. The stapedial footplate (FP) can be divided into two regions: the portion derived from the second visceral arch (light arrows) can be distinguished from the medial lamina stapedialis derived from the otic capsule by different staining characteristics. The lamina stapedialis stains lighter than the other region of the footplate. The presumptive annular ligament is demarcated by the dark arrows.

30. A cross section through the middle ear of an 18-day-mouse fetus. The stapedial footplate (FP) is suspended in the fenestra ovalis by the annular ligament (arrows). The stapes (S) is pierced by the stapedial artery (SA). The chondroblasts of the stapes and incus (I) are separated by undifferentiated mesenchyme, the presumptive incudostapedial joint.


IE
EM

31. A cross section through the middle ear of a 2-day-old mouse showing the further development of the ossicles. The footplate (FP) is suspended by a differentiated annular ligament (arrows) in the fenestra ovalis. The incudomalleolar joint (light arrow) is beginning to differentiate. The malleus manubrium (MM) inserts into the endodermal epithelium of the middle ear cavity (MEC). The three ossicles, the malleus, incus, and stapes, are surrounded by a perichondrium (P). The external auditory meatus (EAM) is ventral to the middle ear cavity.

32. A cross section through the middle ear of a 6-day-old mouse. The ossifying ossicles articulate by the incudostapedial (ISJ) and incudomalleolar (light arrow) joints; the tissue adjacent to the joints remains cartilaginous. The annular ligament (heavy arrows) suspends the footplate (FP) in the fenestra ovalis. The middle ear cavity (MEC) is expanding toward the ossicles, but at this stage the stapes, incus, and head of the malleus (MA) are still surrounded by undifferentiated mesenchyme (M).

33. A cross section through the middle ear of a 16-day-old mouse. The ossicles are now surrounded by the middle ear cavity and invested with a squamous endodermal epithelium continuous with the epithelium of the middle ear cavity (MEC). The incus (I) is suspended by a ligament (light arrow) of endodermal epithelium. The obturator foraman (OF) is boundared by the anterior and posterior crura of the stapes.

34. A cross section of a 16-day-old mouse anterior to Figure 33. The tympanic membrane (TM) appears thinner due to the differentiation of the mesodermal component. The incus articulates with the malleus (MA) by the incudomalleolar joint (light arrow). The tensor tympani muscle (TTM), innervated by a branch of the trigeminal nerve, is attached to the malleus (MA). This structure will not be discussed further.

31 33 32 34

35. A high power view of the fenestra ovalis region of a 13 day old mouse fetus. The presumptive chondoblasts of the stapes (S) are more densely packed than those of the otic capsule (OC) (cf. fig. 27).

36. The fenestra ovalis region of a 2-day-old mouse showing the development of the stapedial footplate (FP) and adjacent tissue. The footplate appears homogenous throughout (compare with the chick footplate, figure 14). The annular ligament (arrows) suspends the footplate in the fenestra ovalis. The skeletal tissues are surrounded by perichondria (P) (cf. fig. 31).

37. A high power view of the annular ligament in Figure 36. Note the alignment of the nuclei of the ligament (arrow).

38. A cross section through the presumptive Eustachian tube of a 15-day-mouse fetus. The epithelium of the dorsal Eustachian tube (DET) is thinner than that on the ventral Eustachian tube (VET). Note the differential density of the mesenchyme adjacent to these epithelia (cf. fig. 28).


M
37

39. A cross section through the middle ear cavity (MEC) of a 7-day-old mouse. Its endodermal epithelium (E) shows regions of different cell types. The external auditory meatus (EAM) is ventral to the middle ear cavity.

40. A cross section through the middle ear cavity (MEC) and presumptive tympanic membrane of an 8 day old mouse. The malleus manubrium (MM) inserts into the endordermal epithelium (E) of the middle ear cavity. The epithelium of the middle ear cavity is a simple squamous epithelium; it is attenuated and infolded into the mesenchyme (M), and the mesenchyme appears devoid of an epithelium. The external auditory meatus (EAM) is still ventral to the middle ear cavity.

41. A high power view of the epithelium on the medial portion of the middle ear cavity. Nonsecretory celiated (single arrow) and secretory (double arrows) cells are identified within this epithelium (cf. fig. 39).

42. A cross section through the middle ear cavity of a 2-day-old mouse. The malleus manubrium (MM) inserts into the simple squamous epithelium (E) of the cavity. The external auditory meatus (EAM) is a meatal plate that has not yet begun to break down. The presumptive tympanic membrane (arrow) cannot be distinguished from the other epithelia of the external auditory meatus.

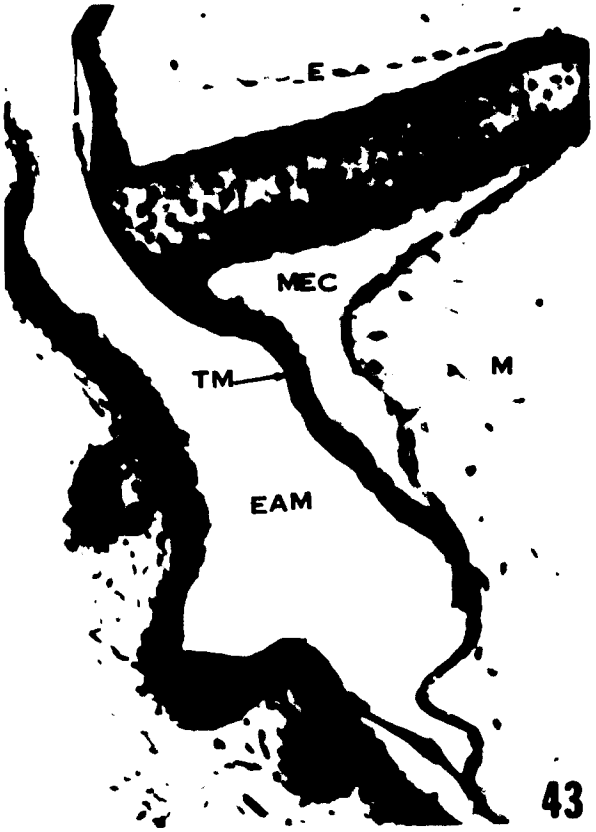

1
2
MEC
MEC

43. A cross section through the tympanic membrane (TM) of a 9 day old mouse. The malleus manubrium (MM) is invested with the endodermal epithelium (E) of the middle ear cavity. The ectodermal face of the tympanic membrane and the epithelium of the external auditory meatus are keratinizing.

44. The tympanic membrane of a 16-day-old mouse is extremely thin and delicate. The tympanic membrane (TM) consists of an inner simple squamous epithelium (C) continuous onto the malleus (MA), a dense intermediate region, and an outer keratinizing epithelium (K) (cf. fig. 34).

45. A cross section through a middle ear cavity of a 7-day-old mouse showing extracellular material (arrows). Compare this extracellular material with that associated with secretory cells in the medial tympanic cavity in Figure 41.

46. The presumptive tympanic membrane of a 15 day mouse fetus is relatively wide due to the region of undifferentiated mesenchyme (M). The malleus manubrium (MM) is adjacent to the epithelium of the middle ear cavity.



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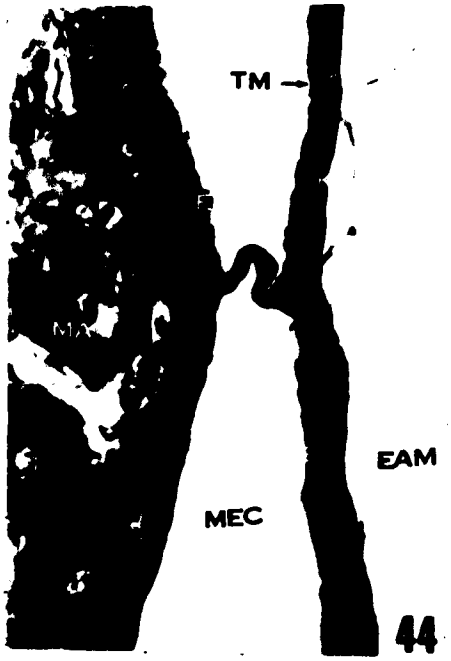

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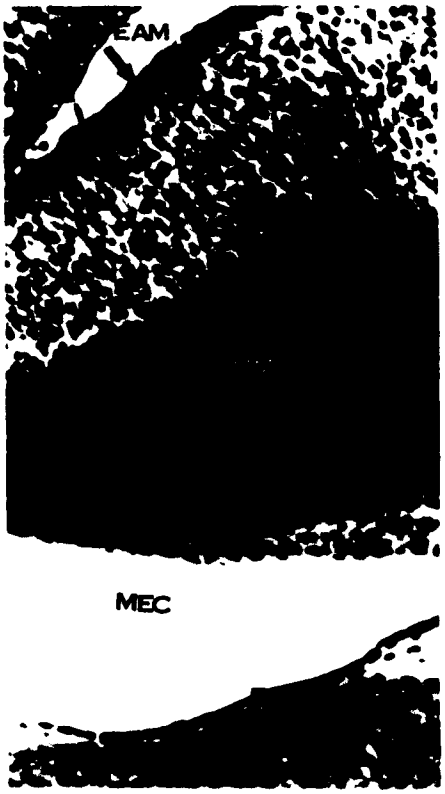
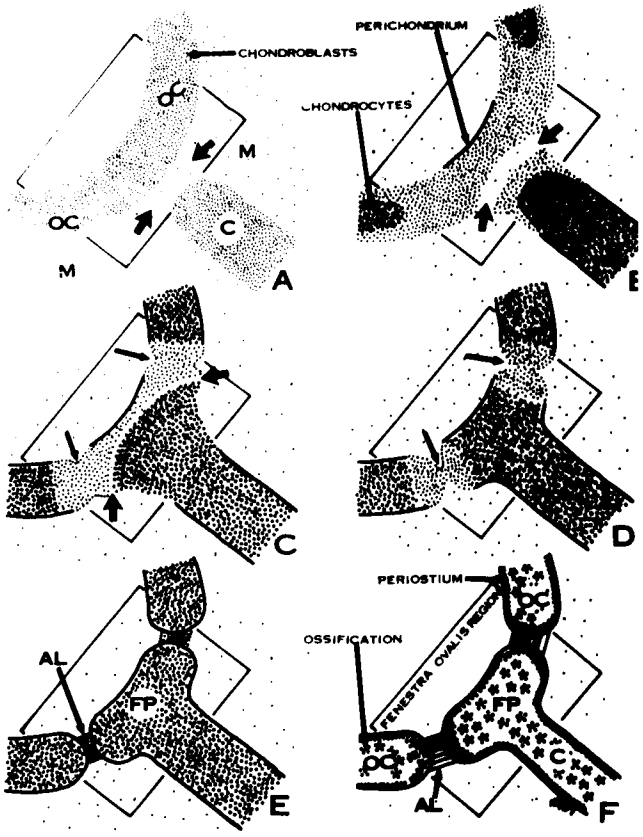


47. Schematic drawings depicting the differentiation of the tissues of the region of the fenestra ovalis in the chick embryo. The drawings are not to scale. A. Stages 28 - 30: the chondroblasts of the columella (C) are separated from those of the otic capsule (OC) by a band of less dense cells (heavy arrows). B. Stage 32: the cell populations of the otic capsule and the columella are nearly confluent, but there is a band separating them (heavy arrows). Perichondral and chondrocyte differentiation has occurred in certain regions. C. Stage 34: the cells representing the presumptive annular ligament (light arrows) are distinguishable from the adjacent skeletal elements. The band of less dense cells (heavy arrows) still narrowly separates the chondrocytes from the proximal chondroblasts. D. Stage 35: the capsule and columella consist almost entirely of chondrocytes, and are nearly completely surrounded by a perichondrium. At the margin of the presumptive fenestra ovalis, chondroblast populations are still visible, and the cells at the isthmus represent the presumptive annular ligament (light arrow). E. Stages 36 - 38: differentiation of the chondrocytes and a perichondrium is seen throughout the otic capsule and columella. The footplate (FP) is suspended within the fenestra ovalis by the annular ligament (AL). F. Stages 41+: endochondrial ossification has begun in the capsule and columella, except around the margin of the fenestra ovalis, which tissues are persistently chondrogenic. The differentiation of the annular ligament (AL) is now complete, and collagen fibers are visible therein.


CHONDROBLASTS
M
OC
C
M
A
PERICHONDRIUM
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B
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D
AL
FP
E
PERIOSTIUM
OSSIFICATION
FENESTRA OVALIS REGION
FP
OC
C
AL
F

47

48. A section of a graft from a donor stage 17 after 10 days on the CAM. The columella (C) shows the characteristic shape and the footplate (FP) is suspended in the fenestra ovalis by the annular ligament (arrows). A middle ear cavity (MEC) surrounds the columella. The extracolumella (EC) inserts into the mesenchyme adjacent to the endodermal epithelium (E), but note the absence of an ectodermal epithelium on the "tympanic membrane." The structure X has all the histological characteristics of a normal tongue (Hodges, '74).

49. A section of a graft from a donor stage 17 after 10 days on the CAM. The columella (C) is fused with the otic capsule (OC) and there is no differentiation of the annular ligament.

50. A section of a graft from a donor stage 18 after 6 days on the CAM. The columella footplate (FP) is partially fused with the otic capsule (OC).

51. A section of a graft from a donor stage 19 after 7 days on the CAM. The footplate (FP) is partially fused with the otic capsule (OC). The annular ligament (arrow) differentiates at the ventral aspect of the columella, while at the dorsal aspect, the chondrocytes of the footplate are continuous with those of the otic capsule.

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52. A section of a graft from a donor stage 21 after 9 days on the CAM. The annular ligament (arrow) suspends the footplate (FP) in the fenestra ovalis in the dorsal region. The footplate is fused with the otic capsule (OC) in the ventral region (double arrows). The columella (C) is suspended in the middle ear cavity (MEC) by "ligaments" of endodermal epithelia (E).

53. A section of graft from a donor stage 16 after 17 days on a CAM. Although an otic capsule (OC) is not adjacent to the columella (C), the shape of the footplate (FP) is identifiable. A middle ear cavity (MEC) lies above and below the ossicle. Note the paratympanic organ (PT) which is located more medial relative to the columella than is normal (compare with fig. 9).

54. A section of a graft from a donor stage 25 after 6 days on the CAM. The shape of the columella (C) appears relatively normal, with the extracolumella (EC) inserting onto the tympanic membrane. The footplate is adjacent to but not continuous with the otic capsule (OC) (light arrow) and each skeletal component is surrounded by a perichondrium (P). A middle ear cavity (MEC) lies ventral to the columella and the continuity of its epithelium with the surface epithelium (double arrows) is abnormal. However, ectodermal differentiation on the surface (heavy arrow) is normal.

55. A high power view of Figure 54 showing the continuity of the middle ear cavity epithelium with the surface epithelium (double arrows). The ectodermal differentiation on the surface is normal (heavy arrow).

56. A tympanic membrane of a graft from a donor stage 25 after 6 days on a CAM. The columella is represented by a single process of the extracolumella (EC). The tympanic membrane consists of an inner endodermal simple squamous epithelium (E), an intermediate mesodermal region (M), and an outer ectodermal epithelium of normal appearance (arrow, see fig. 21).

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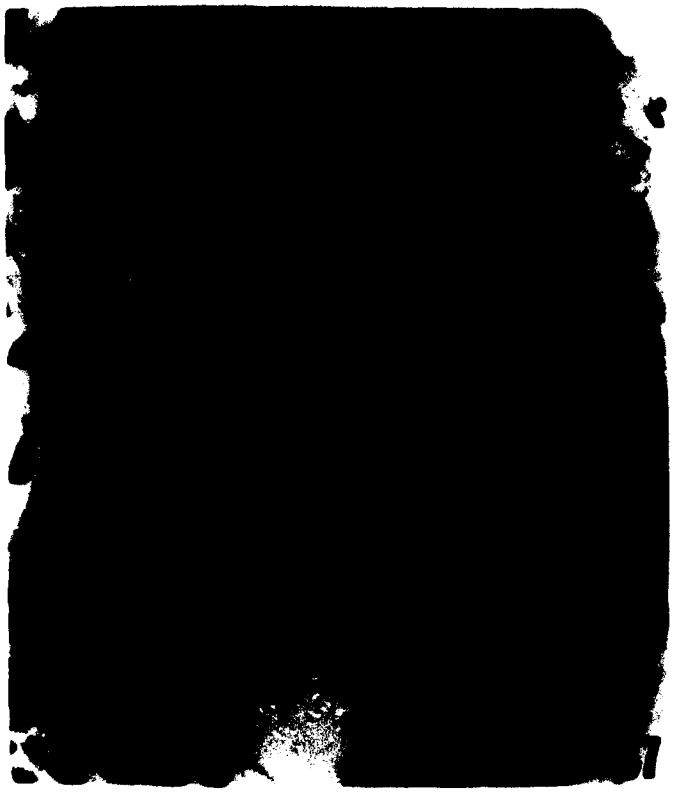
57. A high power view of a paratympanic organ from a donor stage 23 after 7 days on a CAM. Note the typical assymetrical orientation with the sensory cells (SC) on its medial aspect. The typical alcian blue positive material is present (compare with figure 23).



58. Control chick embryo of stage 35.

59. Chick embryo (H208) injected with 3.5 mg Hadacidin at HH st 24 and recovered 6 days later (HH st 35-36). Note the narrow upper beak, buphthalmia, and phocomelia.

60. Chick embryo (H207) injected with 3.5 mg Hadacidin at HH st 24 and recovered 6 days later (HH st 35-36). Note the micromelia, very short lower beak, facial coloboma (a gap between the maxilla and premaxilla), and buphthalmia of the left eye.

61. Chick embryo (H199) injected with 3.0 mg Hadacidin at HH st 22 and recovered 7 days later (HH st 36). Note the retardation, phocomelia, extremely small upper and lower beak, and facial coloboma.

62. The head of a chick embryo (H188) treated with 3.5 mg Hadacidin at HH st 24 and recovered 6 days later (HH st 35-36). Note the bilateral buphthalmia, facial coloboma, and very short lower beak.

63. The head of a chick embryo (H175) treated with 1.5 mg Hadacidin at HH st 24 and recovered 6 days later (HH st 35-36). Note the micropthalmia, and that the upper beak curves to the right. The development of this embryo is further advanced than previous injected embryos of equivalent ages.

64. A cleared and whole stained chick embryo (H163) injected with 2.0 mg Hadacidin at HH st 23 and recovered 6 days later (HH st 35). The skull is relatively normal. The hindlimb is abnormal, with a slight bending of the tibia and metatarsal bending (arrow).

65. A cleared and whole stained chick embryo (H185) injected with 3.5 mg Hadacidin at HH st 24 and recovered 7 days later (HH st 37). The head appears normal. Note the bending of the tibia (arrow) which is characteristic of most abnormal hindlimbs, and slight metatarsal bending.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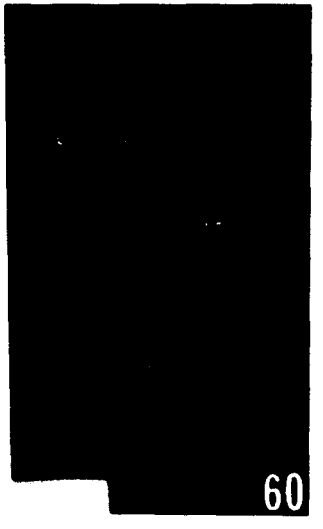
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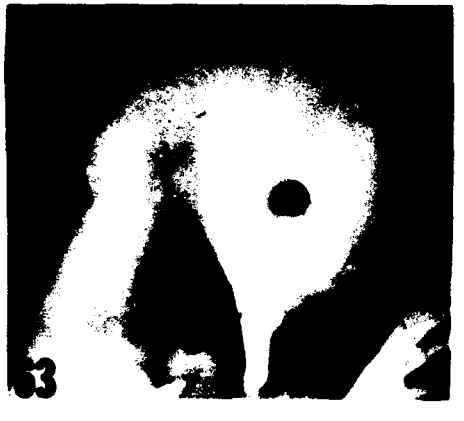
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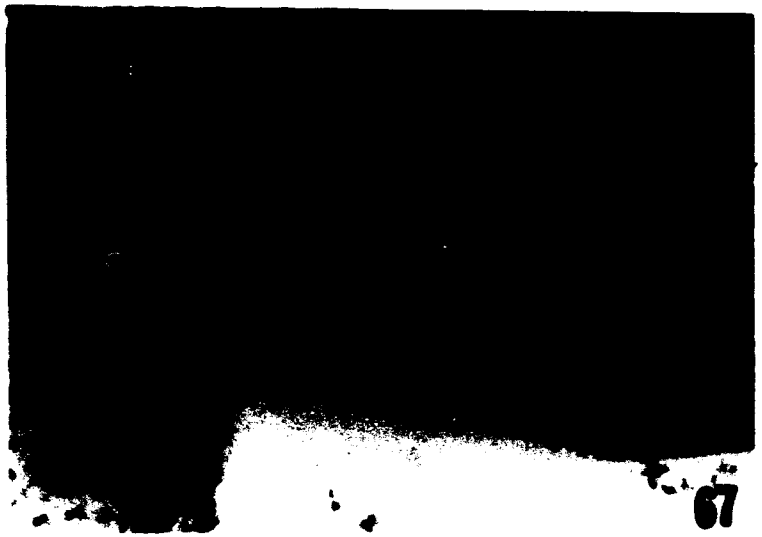
66. A cross section through the left middle ear of a chick embryo injected with 2.0 mg Hadacidin at HH st 10 and recovered 9 days later (HH st 38) (the degree of differentiation appears like a normal columella HH st 36). The columella (C) is normal, surrounded by a middle ear cavity (MEC) and suspended between the tympanic membrane (TM) and the fenestra ovalis (arrows). The footplate (FP) is suspended in the fenestra ovalis by the annular ligament.

67. A high power view of the fenestra ovalis region in Figure 66. The footplate (FP) is suspended in the fenestra ovalis by the annular ligament (arrows).

68. A cross section through the middle ear of chick embryo(H 100), injected with 2.0 mg Hadacidin at HH st 19 and recovered 8 days later (HH st 37). The morphology of the columella (C) is relatively normal. The footplate (FP) is completely fused with the otic capsule (OC).

69. A cross section through the middle ear of chick embryo (H 31), injected with 3 mg Hadacidin at HH st 7 and recovered 7 days later (HH st 35). The middle ear region is extremely deformed with the columella (C) projecting ventrolaterally; to faciliate identification of the middle ear structures, this region is rotated 45 degrees. The footplate (FP) is continuous with the otic capsule (OC). Note the absence of the middle ear cavity and the external auditory meatus.

66



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70. A cross section through the left middle ear of chick embryo (H 70) injected with 2.0 mg Hadacidin at HH st 18 and recovered 7 days later (HH st 36). The columella (C) inserts into the tympanic membrane (TM). The footplate (FP) is partially fused with the otic capsule (OC). Note the absence of the dorsal portion of the annular ligament (arrow) although the footplate is separated from the capsule.

71. A high power view of the fusion of the footplate (FP) with the otic capsule (OC) in Figure 70. Note the cartilaginous cells (arrows) in the region normally occupied by the annular ligament (compare figures 67 and 71).

72. A cross section through the left middle ear of chick embryo (H 76) injected with 2.0 mg Hadacidin at HH st 18 and recovered 8 days later (HH st 37). The columella shaft (CS) is separated from the otic capsule (OC), and the two populations are not continuous (compare figures 68, 70, and 72). Note the perichondrium (P) around each skeletal component.

73. A high power view of Figure 72 showing the separation between the columella shaft (CS) and the otic capsule (OC).

74. A cross section through the middle ear of chick embryo (H13)injected with 1.5 mg Hadacidin at HH st 9 and recovered 7 days later (HH st 35). The columella (C) is very deformed with poorly differentiated chondroblasts.

75. A cross section through the middle ear of chick embryo (H32)injected with 2.5 mg Hadacidin at HH st 8 and recovered 7 days later (HH st 35). The footplate (FP), extracolumella (EC), and shaft can be identified in the columella (C). An otic capsule is absent at the margin of the footplate although the capsule is observed more dorsal and ventral. The absence of the otic capsule results in the absence of the fenestra ovalis and the annular ligament.

76. A section through an abnormal paratympanic organ of chick embryo (H23) injected with 2.5 Hadacidin at HH st 8 and recovered 7 days later (HH st 35). The arrow indicates the abnormal extracellular material with the organ. Note the absence of sensory cell differentiation on its medial aspect which is characteristic of the paratympanic organ (compare with fig. 23).

15

77. A control chick embryo of stage 35.

78. Chick embryo (F 118) injected with 0.3 mg 5FU at HH st 25 and recovered 4 days later (HH st 35). Note the micromelia, and short beak.

79. Chick embryo (F 111) injected with 0.15 mg 5FU at HH st 18 and recovered 4 days later (HH st 34). Note the general retardation, short beak, and micromelia.

80. Chick embryo (F 110) injected with 0.15 mg 5FU at HH st 18 and recovered 4 days later (HH st 34). Note the general retardation, short beak, and micromelia.

81. A cleared and whole stained chick embryo (F 84) injected with 0.2 mg 5FU at HH st 23 and recovered 4 days later (HH st 34-35). Note the short lower beak.

82. A cleared and whole stained chick embryo (F 25) injected with 0.2 mg 5FU at HH st 17 and recovered 6 days later (HH st 35). Note the bending of the metatarsals (arrow).

83. The head of the chick embryo in Figure 82. Meckel's cartilage bends to the right in the anterior part of the lower beak (arrow).

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84. A cross section through the middle ear of chick embryo (F32)injected with 0.2 mg 5FU at HH st 21 and recovered 8 days later (HH st 37). The middle ear region appears relatively normal although the columella (C) appears bulkier than in normal embryos (compare with figure 10). The columella footplate is suspended in the fenestra ovalis by the annular ligament (arrows).

85. A cross section through the middle ear of chick embryo (F41)injected with 0.15 mg 5FU at HH st 18 and recovered 7 days later (HH st 36). The footplate (FP) is fused with the otic capsule (OC). The skeletal tissue is surrounded by a continuous perichondrium (P). The mesenchyme (M) around the ossicle is denser than normal (compare with fig. 9).

86. A cross section through the middle ear of chick embryo (F49)injected with 0.15 mg 5FU at HH st 18 and recovered 5 days later (HH st 34-35). The footplate (FP) is continuous with the otic capsule (OC). The mesenchyme (M) around the ossicle is denser than normal (compare with fig. 9).

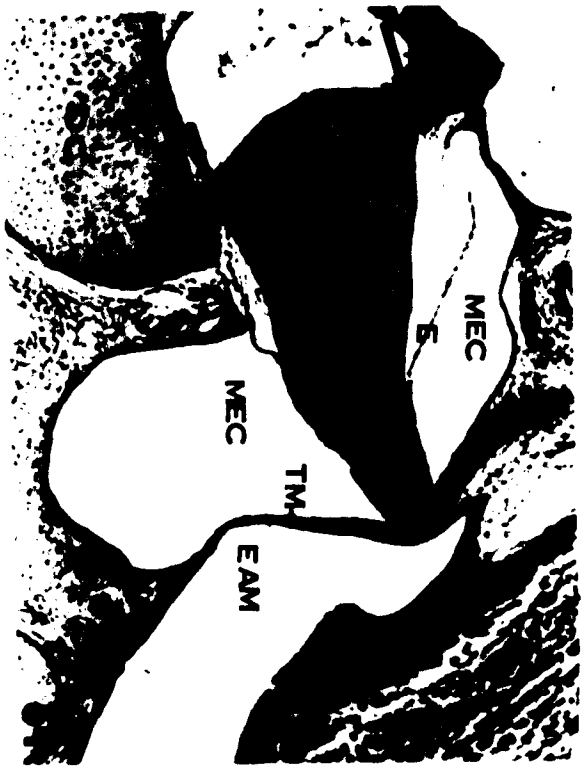
87. A cross section through the middle ear of a cleared and whole stained chick embryo (F130) injected with 0.4 mg 5FU at HH st 28 and recovered 4 days later (HH 36). The otic capsule (OC) stains similar to the cells within the region which normally differentiates into the annular ligament while the footplate (FP) stains darker. This difference in staining characteristics is frequently observed after clearing and whole staining and subsequent processing for histological examination.

MEC

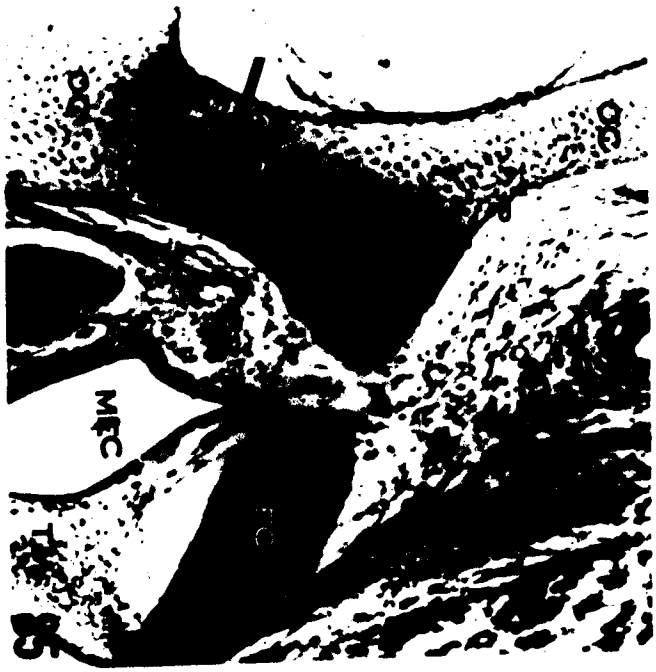
88. A cross section through the middle ear of chick embryo (F 116) injected with 0.4 mg 5FU at HH st 25 and recovered 4 days later (HH st 35). The footplate (FP) and otic capsule (OC) are continuous at the ventral aspect while the capsule and ligament are absent at the dorsal aspect (light arrow).

89. A high power view of the fenestra ovalis region in Figure 88. The cells which normally differentiate into the annular ligament are not different than the cells of the footplate (FP) and otic capsule (OC).


OC
V


MEC
MEC
TM
EAM


FP
OC


MEC
OC

90. Control embryo of stage 36.

91. Chick embryo (P 87) injected with 8.0 mg pilocarpine at HH st 23 and recovered 6 days later (HH st 36). Note a short lower beak and bending of the hindlimb.

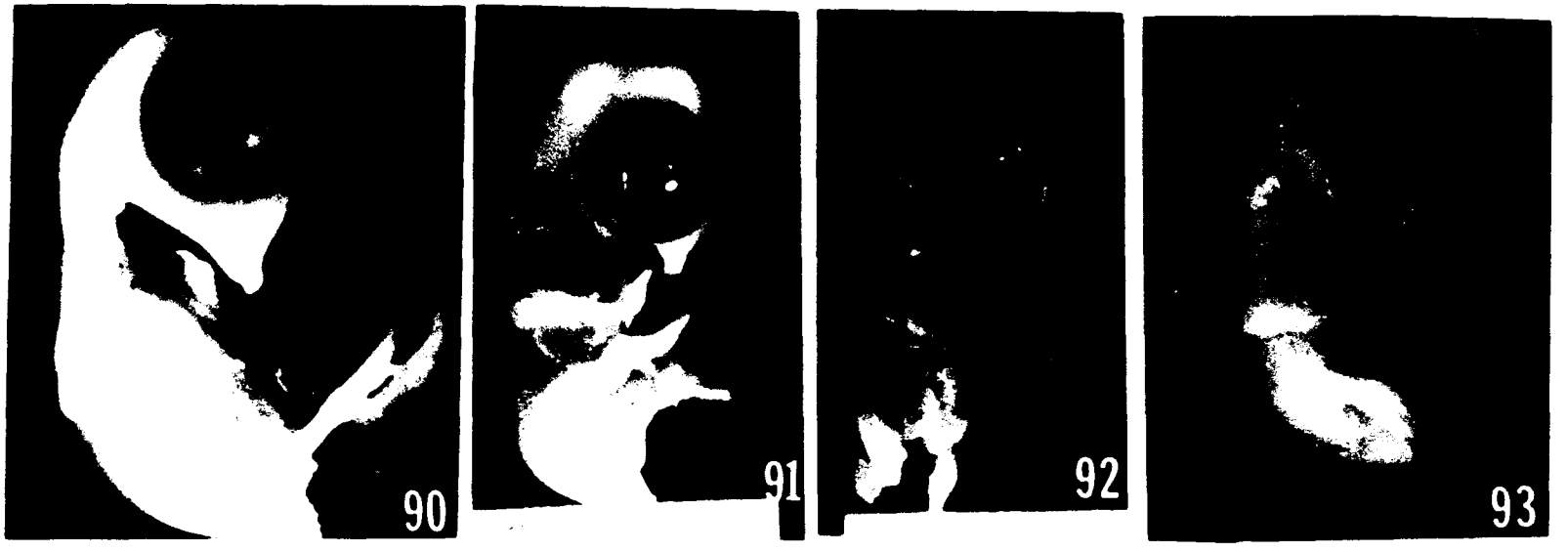
92. Chick embryo (P 96) injected with 9.5 mg pilocarpine at HH st 23 and recovered 7 days later (HH st 37). Note the general retardation, parrot beak appearance, and short lower beak.

93. Chick embryo (P 114) injected with 9.3 mg pilocarpine at HH st 25 and recovered 5 days later (HH st 36). Note the short lower beak, phocomelia.

94. A cleared and whole stained chick embryo (P 66) injected with 8.0 mg pilocarpine at HH st 25 and recovered 5 days later (HH st 36). Meckel's cartilage is bent.

95. A cleared and whole stained chick embryo (P 61) injected with 6.5 mg pilocarpine at HH st 24 and recovered 5 days later (HH st 36). Note the bending of Meckel's cartilage, the short lower beak, and the bending of the metatarsals (arrow).

96. The head of a cleared and whole stained chick embryo (P 83) injected with 9.5 mg pilocarpine at HH st 23 and recovered 6 days later (HH st 36). Note the symmetrical bending of Meckel's cartilage.



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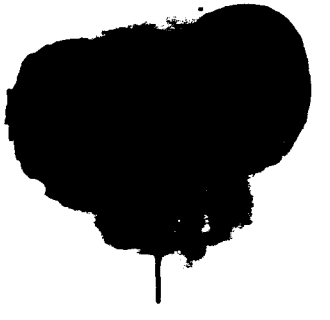

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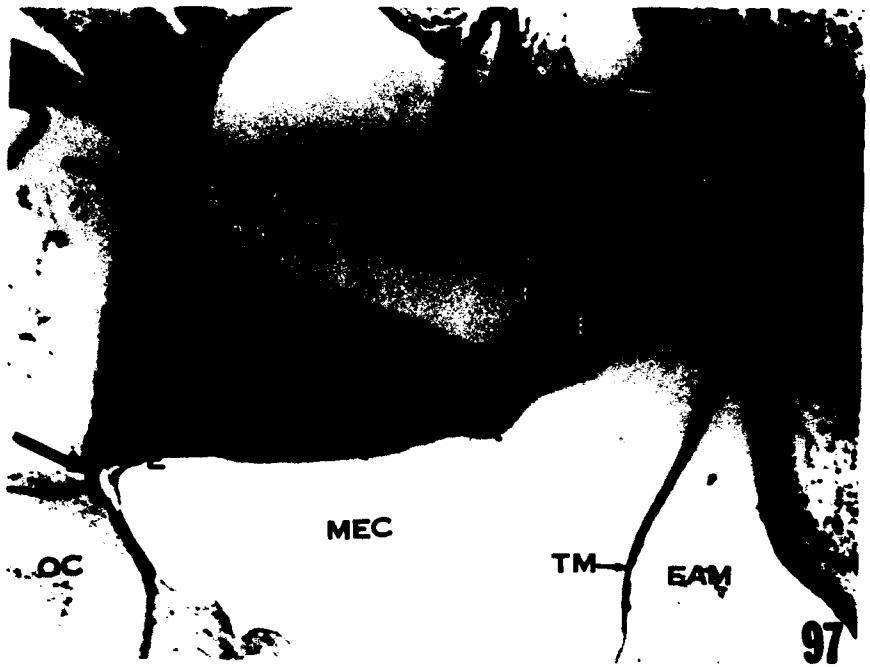
97. A cross section through the middle ear of chick embryo (P141) injected with 15 mg pilocarpine at HH st 22 and recovered 7 days later (HH st 37). The middle ear region appears normal. The annular ligament (arrows) suspend the footplate in the fenestra ovalis.

98. A cross section through the middle ear of chick embryo (P 137) injected with 15 mg pilocarpine at HH st 22 and recovered 7 days later (HH st 37). The columella (C) is bulkier than normal (compare with fig. 10). The annular ligament has begun to differentiate but its differentiation is retarded when compared with normal (see figs. 10 and 97).

99. A cross section through the middle ear of chick embryo seen in Figure 96. The staining characteristics of the middle ear structure are slightly altered, but analysis of them is not disturbed. The footplate (FP) is suspended by the annular ligament (arrows) in the fenestra ovalis. The middle ear cavity has not yet reached the ossicle. The tympanic membrane (TM) consists of an ectodermal epithelium (double arrows) and dense mesodermal cells.



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100. A cross section through the middle ear of chick embryo (P 30) injected with 6.25 mg pilocarpine at HH st 19 and recovered 6 days later (HH st 35). The footplate is continuous with the otic capsule (OC) and the annular ligament is absent. The mesenchyme surrounding the ossicle is denser than normal (compare with fig. 9). The double arrows indicate an artefact; the single arrow indicates the ectodermal epithelium of the tympanic membrane.

101. High power view of the fenestra ovalis region in Figure 100. The annular ligament is absent, yet the cells which normally differentiate into the ligament (arrows) are different than those of the footplate (FP) and the otic capsule (OC) (compare with fig. 89).

101
OC
E
a

100
EVE
E
a
OC
OC

102. Control chick embryo stage 36.

103. Chick embryo (T 45) injected with 0.85 µg TEM at HH st 24 and recovered 5 days later (HH st 36). Note the general retardation and short lower beak.

104. Chick embryo (T 56) injected with 0.85 µg TEM at HH st 24 and recovered 7 days later (HH st 38). This embryo is smaller than a normal HH st 36 (see fig. 102). The lower beak is extremely short and the upper beak is narrow.

105. The head of a chick embryo (T 57) injected with 0.85 µg TEM at HH st 24 and recovered 7 days later (HH st 38). The left eye appears normal while the right eye is microthalmic. Note the facial coloboma (arrow).

106. A cleared and whole stained chick embryo (T 50) injected with 0.85 µg TEM at HH st 24 and recovered 6 days later (HH st 37). Note the general retardation, the very short lower beak, and the bending of the metatarsals (arrow).

107. A cleared and whole stained chick embryo (T 40) injected with 0.85 µg TEM at HH st 24 and recovered 5 days later (HH st 36). Meckel's cartilage is bent in the anterior portion of the lower beak. Note the general retardation.

108. A high power view of the hindlimb in Figure 106. Note the bending of the metatarsals (arrow).

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109. A cross section through the middle ear of chick embryo (T 53) injected with 0.85 µg TEM at HH st 24 and recovered 6 days later (HH st 37). The middle ear region appears relatively normal. The footplate (FP) is suspended by the annular ligament (arrows) in the fenestra ovalis. The middle ear cavity (MEC) is beginning to surround the columella (C).

110. A cross section through the middle ear of chick embryo (T 18) injected with 0.81 µg TEM at HH st 25 and recovered 5 days later (HH st 36). The footplate (FP) is continuous with the otic capsule (OC) and the entire skeletal tissue is surrounded by a continuous perichondrium (P). The mesenchyme is very dense around the ossicle (compare with fig. 9).

111. A high power view of the fenestra ovalis region in Figure 110. The cells which would normally differentiate into the annular ligament (arrow) are identical with those of the footplate (FP) and the otic capsule (OC).

112. A cross section through the middle ear of chick embryo (T 8) injected with 0.81 µg TEM at HH st 25 and recovered 4 days later (HH st 35). The footplate (FP) is continuous with the otic capsule (OC). The skeletal tissue is surrounded by a continuous perichondrium (P).

III
EAM
III
III
IE

113. A cross section through the middle ear of a chick embryo (T 34) injected with 0.85 µg TEM at HH st 24 and recovered 4 days later (HH st 35). The footplate is fused with the otic capsule (OC). The columella shaft (CS) is separated from the extracolumella (EC) by a perichondrium (P, heavy arrow). The extracolumella is completely surrounded by a perichondrium while the columella shaft has a perichondrium on the dorsal aspect. The light arrow indicates the ectodermal epithelium of the tympanic membrane.

114. A cross section through the middle ear of a chick embryo (T 10) injected with 0.81 µg TEM at HH st 25 and recovered 4 days later (HH st 35). The annular ligament (double arrows) differentiates on the dorsal aspect of the footplate (FP) while the footplate is continuous with the otic capsule (OC) on the ventral aspect (arrow).

115. A cross section through the middle ear of a chick embryo (T 17) injected with 0.81 µg at HH st 25 and recovered 4 days later (HH st 35). The annular ligament differentiates on the ventral aspect of the footplate (FP) (arrow) while it is absent on the dorsal aspect (double arrows). Note the absence of the otic capsule at the dorsal margin of the footplate.


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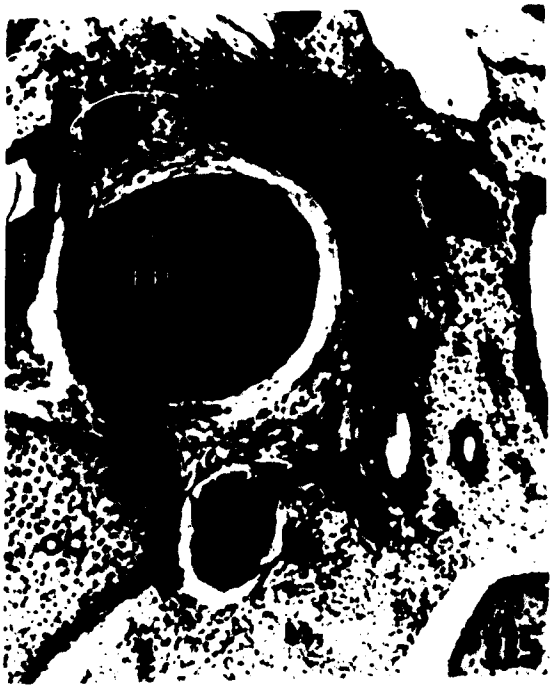
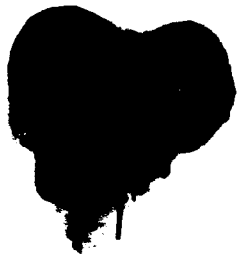


116. The head of a cleared and whole stained chick embryo (B 95) injected with 0.625 mg BAPN at HH st 23 and recovered 5 days later (HH st 38). Note the bending in Meckel's cartilage (arrow).

117. The complete emrbyo described in Figure 116. The lower beak is abnormal. Both hindlimbs are twisted and bent. Note the tibial bending (arrow) in the left hindlimb.

118. A cleared and whole stained hindlimb of chick embryo (B 91) injected with 1.25 mg BAPN at HH st 25 and recovered 3 days later (HH st 34). The tibia is curved (arrow).

119. A cross section through the middle ear of a chick embryo (B 68) injected with 0.312 mg BAPN at HH st 9 and recovered 8 days later (HH st 36). The columella shaft (CS) and the otic capsule (OC) are separated by a distance (arrow) and a footplate is absent. Cartilaginous differentiation is poor relative to untreated embryos (compare with fig. 9).



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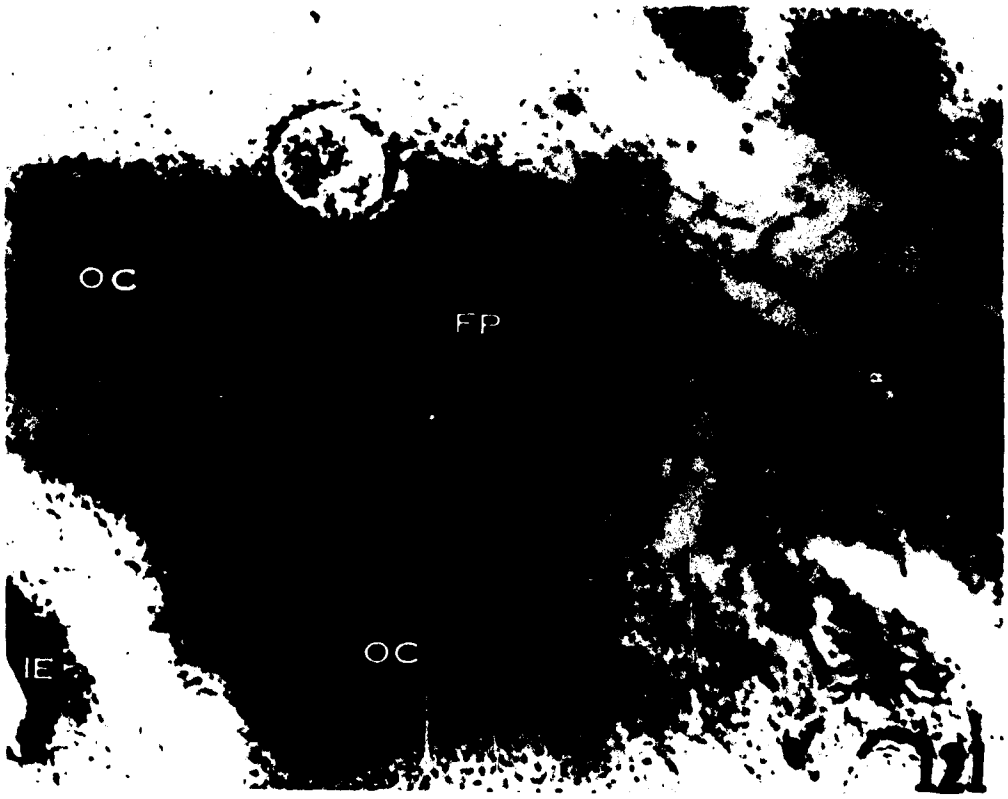
120. A cleared and whole stained chick embryo (BT 93)injected with 35 mg Beta-2-thienylalanine at HH st 9 and recovered 7 days later (HH st 35). The embryo is slightly smaller than normal (compare with fig. 77), but is otherwise normal.

121. A cross section through the middle ear of a chick embryo(BT 75)injected with 20 mg Beta-2-thienylalanine at HH st 5 and recovered 7 days later (HH st 35). The columella footplate (FP) is fused with the otic capsule (OC). Because of the orientation of the sectioning, the other components of the middle ear cannot be identified in this section.


OC
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