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dissertation entitled

OTOTOXICITY IN CHICK EMBRYOS

presented by

James D. Fikes

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pathology

Major professor

Date August 27, 1992

OTOTOXICITY IN CHICK EMBRYOS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pathology

1992

ABSTRACT

OTOTOXICITY IN CHICK EMBRYOS

By

James D. Fikes

The most commonly used research animal in testing for ototoxicity is the guinea pig. A chick could possibly serve as an alternative test animal, but it would be more advantageous to use a chick embryo. This research examined the possibility of using the chick embryo as a test animal for ototoxicity. Initially, chick embryos were exposed to a variety of ototoxic aminoglycoside antibiotics and a loop diuretic, ethacrynic acid, to determine if ototoxicity, as characterized by cochlear hair cell loss, was established. Next, the distribution of the aminoglycoside antibiotic, gentamicin, to the avian cochlea was evaluated. Finally, the uptake of gentamicin in relationship to phosphatidylinositol 4,5-bisphosphate (PIP_2) by cochlear hair cells was also evaluated. Administration of the aminoglycoside antibiotics alone and in combination with the loop diuretic failed to cause cochlear hair cell loss. Gentamicin was distributed to the avian cochlea early in the treatment period, but intracellular gentamicin was not detected in cochlear hair cells until later in the treatment period. Phosphatidylinositol 4,5-bisphosphate was present in the cochlear hair cells at the earliest time point evaluated. Chick embryos appear to be insensitive to the ototoxic effects of the aminoglycoside antibiotics and

ethacrynic acid and the insensitivity does not appear to be due to a lack of presence of PIP_2. The insensitivity may be related to insufficient or poorly developed cellular uptake mechanisms in the hair cells, therefore, the chick embryo would not be an appropriate alternative test animal to be used in screening for ototoxins.

Dedicated to my parents

Clinton H. and Paula M. Carroll.

ACKNOWLEDGEMENTS

I would like to extend a special thanks to Dr. Jim Render, my major professor, for his encouragement and dedication to assisting me in completing this research. I appreciate his insight and direction and the time expended on reviewing my work.

I would also like to thank the other members of my committee, Dr. Steve Bursian, Dr. Robert Poppenga, Dr. Willie Reed, and Dr. Stuart Sleight for their suggestions, support and contributions to this research.

I am indebted to Carol Ayala, Ralph Common, and Donna Craft of the Department of Pathology, Electon Microscopy Laboratory. Their attention to detail, accommodation of my work into their schedule, and technical advice have been tremendously appreciated. I am grateful to Jeff, Phil, Chris, and Angelo, of the Department of Animal Science, Poultry Science Research and Teaching Center for animal care and assistance in this project. I would also like to thank Drs. Mike Yaeger, Dev Paul, Nina DiPinto, Sue Stejskal, Gail Walter, Leslie Williams, Dave Dorman, John Sullivan, and Bill Valentine for their input and encouragement during my graduate program.

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ABR	auditory evoked brainstem response
AC	air cell
AS	allantoic space
BP	basilar papilla
BSA	bovine serum albumin
CAP	compound action potential
CM	cochlear microphonic
CP	cuticular plate
DAB	3,3 diaminobenzidine tetrahydrochloride
dB	decibel
DC	direct current
EA	ethacrynic acid
ED	embryonic day
EP	endocochlear potential
G	gentamicin
GFR	glomerular filtration rate
IOD	integrated optical density
IP_1	inositol 1-monophosphate
IP_2	inositol 1,4-bisphosphate
IP_3	inositol 1,4,5-triphosphate
K	kanamycin
kHz	kilohertz
L	lysosome
NSE	neuron-specific enolase
PIP_2	phosphatidylinositol 4,5-bisphosphate
RER	rough endoplasmic reticulum
RT	room temperature
S	streptomycin
Sub	submersion
TBS	tris buffered saline
TTBS	tween tris buffered saline
TV	tegmentum vasculosum
YS	yolk sac

INTRODUCTION

INTRODUCTION

The structure and function of the inner ear, cochlea and vestibular structures, of birds and mammals are comparatively similar (Lewis et al., 1985; Rebillard and Rubel, 1981). The auditory system is relatively mature at birth for human beings and at hatching for chickens, even though development of both structure and function still occurs (Rubel, 1978). The analogy between the avian basilar papilla and mammalian organ of Corti justifies the current routine use of the avian cochlea in studies of general problems of inner ear development and organization (Rubel and Parks, 1988). An understanding of the fundamental development of the inner ear is important in understanding lesions that affect hearing, balance, or posture control.

Ototoxicity, although infrequently encountered in clinical practice, may have devastating outcomes (Govaerts et al., 1990). Cochlear effects can range from temporary tinnitus (ringing in ears) to permanent deafness (Brummett, 1980). Vestibular effects include vertigo, nystagmus, and ataxia (Hawkins and Preston, 1975). These clinical symptoms and signs may be temporary (reversible) or permanent (Hawkins and Preston, 1975). Clinically, patients may appear to compensate for permanent vestibular deficits,

however, there may be little actual improvement of vestibular performance as measured by vestibular function testing methods (Hawkins and Preston, 1975).

A long and diverse list of compounds may be considered as ototoxicants. The vast majority of these are pharmaceutical agents such as the aminoglycoside antibiotics, antimalarials, and chemotherapeutic agents (Anniko, 1986; Brummett, 1980; Norris, 1988; Prosen and Stebbens, 1980; Rybak, 1986). Industrial compounds with potential for ototoxicity include solvents such as carbon disulfide (Arlien-Soborg et al., 1981; Miller, 1985; Wood, 1981) and xylene (Aschan et al., 1977; Miller, 1985). Arsenic (Anniko and Sarkady, 1977; Benko et al., 1977; Bencko and Symon, 1977; Miller, 1985), organic mercury (Amin-Zaki et al., 1978; Miller, 1985; Mizukoshi et al., 1975), and lead (Miller, 1985; Ursan and Suciu, 1965) are considered ototoxic metals and metalloids. In addition to ototoxicants, noise should also be considered as an environmental cause of serious harmful effects on hearing (Hamernik et al., 1982).

The guinea pig is the test animal of choice in ototoxicologic research because they have morphologic and functional features similar to those of human beings. Both have a dense petrous portion of the temporal bone which precludes the inner ear from being easily accessed for evaluation of otologic lesions. Because of this bone, histologic evaluation requires lengthy decalcification

procedures with delicate dissection if the morphology of the inner ear is to be preserved (Micheals, 1988). Alterations in the histologic appearances of some of the tissues occur as a result of the prolonged exposure to solutions used in the decalcification process. Many alterations ascribed to postmortem autolysis are in fact the result of damage by decalcification solutions (Michaels, 1988).

To overcome problems associated with the use of the guinea pig for ototoxicologic investigations, investigators have utilized other species of animals or in vitro methods. Animals utilized include mice (Henry et al., 1981), rats (Osaka et al., 1979), cats (Bernard, 1981), chickens (Lippe et al., 1991), frogs (Kroese and van der Berken, 1982), and parakeets (Hashino et al., 1992). For in vitro studies, the embryonic inner ear (otocyst) from the mouse (Richardson and Russell, 1991) or chicken (Friedmann, 1965) has been removed at various stages of development and exposed to ototoxic agents in an explant cell culture system. All of these alternatives have advantages and disadvantages.

Fertile chicken eggs have been used in alternative ocular irritation tests which have been loosely characterized as an in vitro test. They may provide an opportunity to combine the desirable aspects of using an animal and using an in vitro system. The advantages include the use of a whole live animal which allows for easy comparisons of results and the ability to quickly, cheaply and easily work with the structures of the inner ear.

To evaluate the usefulness of chick embryos in otologic research, one needs to have an understanding of the otologic anatomy of mammals and birds, otologic embryology, concepts of ototoxicology, and the use of birds in otologic research. This understanding will provide a basis of knowledge needed to evaluate the distribution of ototoxic drugs to the inner ear and the sensitivity of cochlear hair cells.

LITERATURE REVIEW

LITERATURE REVIEW

OTIC ANATOMY AND EMBRYOLOGY

Mammalian External and Middle Ear. The external ear consists of a pinna (auricle) and the external auditory canal (Breazile, 1976; Junqueira et al., 1977). The pinna has a central plate of thin elastic auricular cartilage covered on both sides with tightly attached skin. The external auditory canal is divided into a lateral cartilaginous and medial osseous part.

The thin, concave, semitransparent, oval tympanic membrane separates the external and middle ear. Centrally, the membrane is thinner than the periphery. The lateral surface is covered by a thin layer of epidermis and the medial mucosal surface is covered by simple cuboidal epithelium. Between the epidermal and mucosal layers is a tough connective tissue layer composed of collagenous and elastic fibers.

The middle ear consists of the tympanic cavity, which communicates with the pharynx by way of the auditory (pharyngotympanic or Eustachian) tube, and the three auditory ossicles with their associated ligaments and muscles (Junqueira et al., 1977). The auditory ossicles articulate by synovial joints (Junqueira et al., 1977). The malleus attaches to the medial tympanic membrane surface and then

articulates with the incus, which in turn articulates with the stapes. The footplate of the stapes attaches to the oval window. The tensor tympani muscle originates in the fossa tensor tympani and inserts on the muscular process of the malleus. Contraction of this muscle tenses the tympanic membrane. The stapedius muscle is the smallest skeletal muscle in the body and originates in the fossa musculae stapedius. It inserts on the muscular process of the stapes. Contraction of the stapedius muscle results in caudolateral movement of the anterior end of the base of the stapes.

The main function of the middle ear is to reduce the impedance mismatch between air, the medium of environmental sound wave delivery, and the cochlear fluids (Johnstone, 1988). Energy from sound waves impacting on the tympanic membrane is converted to hydraulic pressure waves in the cochlea by a pumping action of the ossicles at the round window. Contraction of the tensor tympani and stapedius muscles decreases the transfer of sound waves to the inner ear in an attempt to protect the cochlea during prolonged or repetitive loud sounds.

Avian External and Middle Ear. The external ear of birds lacks an auricle, but usually possesses specialized feathers, ear (skin) flaps, and an opercula (Meyer, 1986). The external auditory meatus of birds is a short, curved tube with a circular or oval opening. As in mammals, the tympanic membrane separates the middle and external ears of birds.

The middle ear consists of the tympanic cavity, columellar apparatus (avian equivalent to ossicles), and a single columellar muscle (Hodges, 1974). The tympanic cavity is directly continuous with the pharynx via the auditory tube. The internal surface of the tympanic membrane is in contact with the columella, which extends across the tympanic cavity and ends at the oval window. The function of the columella is equivalent to the ossicles and the hydraulic transformation quotients of adult bird columellae correspond with those of several mammals (Meyer, 1986). The columellar muscle, arising from the occipital bone outside the tympanic cavity, passes through a foramen to insert at the apex of the infracolumellar process and the posterior margin of the tympanic membrane.

Embryology of the External and Middle Ear. The embryological development of the external and middle ear of mammals and birds is similar. The pinna is formed from the first and second branchial arches (Crary, 1964; Presley, 1984; Wood-Jones and Wen, 1934). The external auditory meatus begins as an invagination of ectodermal tissue within the first branchial groove. A medial enlargement of the meatus provides the epithelial cover of the tympanic membrane. As other cephalic structures expand, the primary external auditory meatus is forced closed with the epithelial layers left in apposition. Later, secondary extension of the external meatus occurs, and the epithelial layers separate with the lumen once again appearing. Separation of these layers is highly variable among different species of mammals.

Species with more mature hearing capabilities at birth may have a fully formed external auditory meatus in late gestation, while in other species (i.e. many rodents), the meatus may not open fully until many days postpartum. The primary external auditory meatus closely correlates with the portion of the meatus surrounded by cartilage in the adult mammal, while the secondary extension relates to that portion surrounded by bone.

The middle ear is formed from the first pharyngeal pouch and its associated endodermal tissue (Altmann, 1950). This pouch extends, creating the tubotympanic cavity. This cavity continues to expand by moving in apposition to the external auditory meatus at the point of the future tympanic membrane. The primary extension of the pharyngeal pouch remains constricted and forms the auditory tube (Eustachian tube). The more distal extensions expand to form the tympanic cavity, tympanic recess, and air cells. With continued differentiation and maturation, specific outpouching of the tympanic cavity occurs along with air cell formation (pneumatization) in adjacent bone. The mucosa of the middle ear consists of different types of epithelial cells including nonciliated, nonsecretory cells; nonciliated, secretory cells (including goblet cells); ciliated cells; intermediate cells; and basal cells. The mucosa of the auditory tube contains ciliated cells and glands and closely resembles that of the respiratory tract.

The ossicles of the middle ear are derived from the mesenchymal tissue of the first and second branchial arches (Hanson et al., 1962). The mammalian malleus and incus form from the first arch and the mammalian stapes forms from the second arch. The avian columella, a single ossicle corresponding to the mammalian stapes (Hodges, 1974), is derived from both neural crest and mesoderm (Noden, 1978; Maderson et al., 1982). Ossicles are suspended in mesenchyme until late in development, when the surrounding mesenchyme begins to disappear leaving the auditory ossicles suspended by ligaments. The tensor tympani, formed from the first pharyngeal arch, is associated with the malleus, and the stapedius, formed from the second arch, is associated with the stapes. The columellar muscle is considered to be homologous to the stapedius muscle on the basis of its branchial origin (second pharyngeal arch). The columellar muscle and the stapedius muscle are innervated by the facial nerve (Meyer, 1986). The tensor tympani is innervated by the mandibular branch of the trigeminal nerve (Vth cranial nerve).

Several types of tissue contribute to the embryologic development of the tympanic membrane. The same tissue which lines the external auditory meatus comprises the outer epithelial layer of the tympanic membrane. This tissue develops into the juxtastromal stratum basal, outer stratum spinosum, stratum granulosum, and stratum corneum. Cephalic mesenchymal tissue trapped between the expanding external auditory meatus and the expanding tympanic cavity forms the

fibrous lamina propria. The lamina propria contains capillaries and myelinated and unmyelinated nerves. Ciliated, columnar cells lining the tympanic cavity also cover the medial surface of the tympanic membrane. This particular mucosa is derived from endoderm of the first pharyngeal pouch (Jaskoll and Maderson, 1978).

Mammalian Inner Ear. The inner ear is a system of canals and cavities in the petrous part of the temporal bone (Breazile, 1976) and is referred to as the osseous labyrinth. Within the osseous labyrinth is the membranous labyrinth.

The osseous labyrinth consists of the vestibule, semicircular canals, and the spiral cochlea. The vestibule lies adjacent to the medial wall of the tympanic cavity. The semicircular canals open into the vestibule on one end and combine to form the crus commune at the other extremity. The canals are uniform in diameter except at one extremity where they dilate to form the ampulla. The osseous cochlea is a coiled structure. The core of the spiral is called the modiolus and the apex is referred to as the helicotrema. An osseous shelf, the spiral lamina, projects into the lumen of the osseous cochlea and spirals around the inner surface of the modiolus.

The membranous labyrinth lies within the osseous labyrinth and is separated from it by the perilymphatic space (Breazile, 1976; Junqueira et al., 1977). The utricle and saccule are located within the osseous vestibule. These structures communicate via a narrow, Y-shaped duct whose stalk

ends as a blind sac (endolymphatic duct and sac) in the subdural space. The membranous semicircular canals open into the utricle. The membranous labyrinth is lined with simple squamous epithelium, except in regions that differentiate to form specialized receptor organs. These are named the cristae ampullares (within the membranous ampulla), macula utricularis and macula saccularis (within the utricle and saccule, respectively), and the organ of Corti (within the cochlea).

The cristae ampullaris project into the lumen of the membranous ampulla. The epithelium is composed of Type I and Type II hair cells and supporting cells. The epithelium of the cristae ampullaris is covered by a gelatinous cap (cupula) that completely occludes the lumen of the ampulla. The function of the cristae ampullaris is rotational acceleration detection.

The macula of the utricle and saccule are rounded areas of sensory epithelium histologically similar to the cristae ampullaris. The surface of the macula is covered with a gelatinous mass containing small calcium carbonate concretions, otoconia. The utricle and saccule are involved in the detection of gravity, linear acceleration, and vibration.

The membranous cochlea is arranged into three chambers, the dorsally located scala vestibuli, the centrally located scala media, and the ventrally located scala tympani. The scala vestibuli and scala media are separated by Reissner's membrane. The scala media and scala tympani are separated by

the various cell types of the basilar membrane and the organ of Corti. The organ of Corti (organ of hearing) includes one row of inner hair cells, three rows of outer hair cells, supporting cells, the tunnel of Corti between the inner and outer hair cells, efferent and afferent innervation, and the basilar and tectorial membranes. Endolymph, a fluid similar to intracellular fluid, is found in the scala media, and contains a high concentration of potassium and low concentration of sodium. Perilymph, a fluid found in the scala vestibuli and tympani, has a composition similar to extracellular fluid, including a high concentration of sodium and low concentration of potassium.

Avian Inner Ear. In contrast to the coiled mammalian cochlea, the avian cochlea is a relatively short, narrow, slightly curved structure (Hodges, 1974). When the avian cochlea is sectioned transversely (Figure 1) the basilar papilla (organ of Corti equivalent) is cresent shaped and composed of tall and short hair cells and supporting cells situated on the fibrous basilar membrane (Tanaka and Smith, 1978). Tall and short hair cells have many features which are similar to mammalian inner and outer hair cells, respectively (Whitehead, 1986). The basilar papilla stretches between the superior and inferior fibrocartilaginous plates, separating the scala media from the ventrally located scala tympani. The basilar papilla is covered by the wedge-shaped tectorial membrane. The tegmentum vasculosum forms the lateral wall and roof of the scala media. The tegmentum vasculosum is a

Figure 1. Transverse section of a 19-day chick embryo cochlea at 60% of the base to apex distance. This section contains the basilar papilla (BP), tall hair cells (T), short hair cells (S), tectorial membrane (TM), superior fibrocartilaginous plate (SFP), inferior fibrocartilaginous plates (IFP), scala media (SM), scala tympani (ST), tegmentum vasculosum (TV), and cochlear nerve ganglion (CG). Toluidine Blue. x 140.

heavily vascularized structure composed of two highly specialized cell types, light and dark, which are believed to be specialized for active secretion (light cells) and absorption (dark cells). This structure is equivalent to the stria vascularis of mammals (Meyer, 1986). Ganglion cells of the cochlear nerve are located below the superior fibrocartilaginous plate, with myelinated axons extending to the habenula perforata, then distributing as unmyelinated axons across the basilar papilla to hair cells.

At the distal (apical) end of the membranous cochlea of birds is another sensory organ, the lagena (Lewis et al., 1985). The lagena consists of a vertically positioned macula covered by a gelatinous otoconial mass. The function of the lagena is unknown, but may be associated with hearing and maintenance of balance (Boord, 1969). Nerve fibers extend from the lagena to the cochlear and vestibular nuclei.

As in mammals, three semicircular canals are present in birds with a similar function of detection of angular acceleration (Meyer, 1986). Both ends of each semicircular canal open into the utricle. Each semicircular canal has a dilation (ampulla). Considerable variation exists in the overall configuration and position of the canals in different birds (Meyer, 1986). In the ampulla, a crescent-shaped elevation is present. As in mammals, the area perpendicularto the long axis of the canals are covered by sensory epithelium (ampullary crests). Projections parallel to the long axis of the canals are covered by nonsensory, tall

columnar cells (plana semilunata). These projections are common in the anterior and posterior semicircular canals of birds. These are infrequent in mammals. The utricle and saccule have sensory epithelium restricted to the macula. Both structures have similar function to their mammalian counterparts. Type I and II hair cells, similar to those found in mammals, are found in the ampullary crests and utricular and saccular maculae of birds.

Embryology of the Otocyst and Cochleovestibular Ganglion. The otocyst is the embryologic structure that becomes histologically specialized as it differentiates into the cochlea and vestibular system. It begins as a thickened area of surface ectoderm (otic placode) on both sides of the rhombencephalon in early embryogenesis. For example, the otic placode can be identified in human embryos at approximately 22 days (O'Rahilly, 1963). Adjacent structures, including the rhombencephalon (Harrison, 1936; Jacobson, 1963; Model, 1981; Stone, 1931; Waddington, 1937; Yntema, 1937), nearby mesenchymal cells, and neural crest cells, appear to influence the formation of the placode. Neural crest cells migrate close to the placode as it begins to thicken.

The epithelium of the otic placode continues to thicken, then begins to invaginate to form the otic pit and eventually the otic cup. The rudimentary cochleovestibular ganglion separates from the epithelium as a small cluster of cells that collect at the ventromedial aspect of the otic cup. The period during which separation occurs is short. In the chick

embryo, this occurs sometime between the 45th and 71st hours of development (Knowlton, 1967; Meier, 1978), but not after 72 hours (Whitehead and Morest, 1985a). Although separated from the otic placode, cells of this embryonic ganglion are mitotically active and continue to divide to form neurons until embryonic day 6 in the chick embryo (D'Amico-Martel and Noden, 1983). All of the neurons of the cochlea, and the vast majority of the vestibular neurons are derived from otic placode (D'Amico-Martel and Noden, 1983; Yntema, 1937). Some neurons in the vestibular ganglion are derived from the neural crest. Schwann cells and satellite cells in the ganglion are entirely of neural crest origin.

As the rim of the otic cup fuses, the otocyst (otic vesicle) is formed (Whitehead, 1986). This structure appears as a hollow, almost spherical, ball that forms the cochlear and vestibular membranous labyrinths. The epithelium of the otocyst, through a complex progression of cytodifferentiation, forms the numerous cell types that make up the membranous labyrinth (Whitehead, 1986). Initially, the otocyst is without innervation, but synapses occur as nerve fibers grow from the ganglion cells and actively enter the epithelium.

Because of differential growth, the spherical otocyst begins to contract in some areas and distend in others. An early appearing constriction delineates the dorsal utricular bulge from the ventral saccular region. The cochlea develops from the saccular region as a ventral evagination of thick pseudostratified columnar epithelial cells. The utricular

bulge envaginates a small area of thin cuboidal epithelium that forms the endolymphatic duct. Next, the utricular epithelium is pulled into dorsally located folds which cavitate as the semicircular canals.

Cochlear Embryology. The cochlear evagination formed from the saccular region elongates ventrally and medially to form the lagena in lower vertebrates, or as a straight tube housing the basilar papilla in birds. Its distal end contains the macula lagena. In mammals, the ventrally extending cochlear evagination becomes relatively long and begins to form coils. The connection with the saccule continues as the narrow ductus reuniens.

There appears to be considerable overlap in the times of generation of different cell types within the cochlea. However, there is a spatial gradient of hair cell and supporting cell cytodifferentiation that occurs along the length of the cochlea (Ruben, 1967). Cells at the apex undergo terminal mitosis before more basilar regions. Once the membranous labyrinth attains all of its principal components, the surrounding mesenchyme condenses to form the bony labyrinth (Knowlton, 1967).

Mature hair cells are not attached to the basilar lamina like supporting cells. As hair cells differentiate, they lose their basal cytoplasmic attachments (Orr, 1975), as well as their attachments to adjacent cells (Ginzberg and Gilula, 1979). Primitive hair cells in the cochlear epithelium lose their basal attachments and extend toward the luminal surface

as they withdraw their basal extensions. During later stages, nerve fibers enter the epithelium and attach to the withdrawing basal processes of these cells as they migrate to the surface. It is at this time hair cells become apparent by electron microscopy (Whitehead and Morest, 1985a; Whitehead and Morest, 1985b). These neuroepithelial junctions have the morphology of immature synapses, and synaptic bodies appear for the first time in the newly differentiated hair cells (D'Amico-Martel and Noden, 1983). Synaptic bodies, also called synaptic bars, are presynaptic differentiations in the form of invaginations (Ades and Engstrom, 1974).

Hair cells do not seem to require innervation for their differentiation and survival (Ard et al., 1985; Fell, 1938; Van de Water, 1976). However, hair cells may provide some trophic influence upon vestibulocochlear ganglion cells because the survival of ganglionic neurons in culture is dependent upon the presence of peripheral target tissue (Ard et al., 1985). Normal differentiation of the vestibular and cochlear hair cells and cartilaginous otic capsule do depend upon reciprocal interactions between the growing otocyst and surrounding mesenchyme (Van De Water, 1983).

Hair cells are differentiated from undifferentiated supporting cells when they are confined to the luminal surface and form stereocilia and kinocilia from precursors of the microvilli. The ciliary bundle of hair cells in the mouse matures when the kinocilia are lost (Kikuchi and Hilding, 1965). The tunnel separating inner and outer hair cells in

mammals forms as pillar cells differentiate from the rather tall supporting cells located between the immature inner and outer hair cells (Kikuchi and Hilding, 1965; Larsell et al., 1944; Lenoir et al., 1980). The tunnel forms as a slit of extracellular space between the tall supporting cells and expands as a triangular shaped structure that widens to form the tunnel of Corti. As the tunnel forms, the expanding bases of the pillar cells cause the course of some afferent and efferent fibers leading to outer hair cells to be redirected as they cross the tunnel (Ginzberg and Morest, 1984). With continued maturation, the bases of the tall supporting cells bordering the tunnel widen and the free sides become convex. The cells now become recognizable as pillar cells. At the same time, other cytodifferential changes in the organ of Corti, including the differentiation of the phalangeal cells, the appearance of the space of Nuel, the opening of the internal spiral sulcus, a thinning of the basilar membrane (Anniko, 1980), and an increase in the thickness and density of the tectorial membrane (Lenoir et al., 1980; Pujol and Marty, 1970) are occurring.

The basilar papilla (Fermin and Cohen, 1984) and organ of Corti (Hilding, 1968; Larsell et al., 1944; Li and Ruben, 1979; Nakai and Hilding, 1968; Pujol and Marty, 1970; Sher, 1971) do not differentiate synchronously throughout their lengths. In general, hair cells and supporting structures differentiate earlier in basal regions and later in the apical region. An exception to this general statement is the

relatively late differentiation that occurs in the extreme basal area in some species (Larsell et al., 1944).

It is unclear if a similar pattern of differentiation occurs in regards to innervation. Intraepithelial cochlear nerve fibers have been clearly identified in basal regions containing differentiated hair cells, but not in apical regions with undifferentiated hair cells (Larsell et al., 1944; Sobkowicz et al., 1975). Nerve fiber growth into the sensory epithelium may occur first in mid-basal regions (Sher, 1971). An examination of other morphologic features may reveal a slightly different pattern of differentiation. Results of an examination of the generation times of ganglion cells and hair cells from different parts of the cochlea indicates that the developmental patterns of ganglion cells and hair cells are different rather than similar (Ruben, 1967). The first hair and supporting cells undergoing terminal mitosis occupy the apical region while the first ganglion cells formed are located in the basal region. Subsequently generated neurons are located in apical locations. The pattern of ganglion cell generation match the pattern of hair cell differentiation, but not the pattern of hair cell generation. Presumably, the time between terminal mitosis and cytodifferentiation is longer for apical hair cells than basal cells.

Vestibular System Embryology. The vestibular system originates within the otocyst (Anniko, 1983). The developmental sequence of the vestibular system in birds is

very similar to that observed in man (Anson, 1934; Anniko, 1983). Morphogenesis of the vestibular system occurs in the chick embryo during embryonic days (ED) 3.5-6.5 (Knowlton, 1967).

The endolymphatic appendage develops from an outgrowth of the dorsal aspect (future utricular portion) of the otocyst. This structure develops into the endolymphatic duct and sac. The endolymphatic appendage is delineated from the future vestibular tissue of the otocyst by a downward directed edge, the future position of the utriculoendolymphatic valve (utriculosaccular chamber). The endolymphatic appendage elongates with general expansion of the otocyst as a whole. The endolymphatic appendage divides into the proximally located ductus and distally located expansion of the saccus (Bast and Anson, 1945). The endolymphatic sac continues to extend dorsally into the differentiating meningeal tissue. With continued fetal development, the dura containing the endolymphatic sac migrates downward forcing the distal end of the thinned part (isthmus) of the endolymphatic duct and the proximal part of the sac to change from a straight to curved configuration. This leaves the apex of the endolymphatic sac, which was dorsal in the fetus, directed dorsocaudally in the adult.

The semicircular canals appear as flattened, concave outpocketings of the utricular part of the otic vesicle. The formation order for the semicircular canals is anterior, posterior, and lateral (Bast et al., 1947). The central

aspects of the walls of these outpocketings eventually become apposed to each other at the base. The apposed embryonic wall tissue is reabsorbed creating the semicircular canal. One end of each canal dilates to form the ampulla.

The first anlage of all the later sensory end organs is a thickening of the medial wall of the otocyst near the statoacoustic ganglion called the macula communis (Anniko, 1983). Differentiation of the neuroepithelium of cristae and maculae start at about the same time (Altmann, 1950). In the chick embryo, neural connections between the statoacoustic ganglion and future hair cells have been identified after 78 hours of incubation (Proctor and Lawrence, 1959). The anterior cristae and macula sacculi become separate units first (ED 5.5-6) followed by the posterior cristae and macula neglecta (ED 6-6.5) and, finally, the lateral cristae, macula utricle and lagena, and the papilla basilaris (ED 6.5-7). Hair cells and supporting cells are separated earlier in the posterior and anterior cristae (ED 5-5.5) than in the lateral cristae, macula utriculi, neglecta, and lagena. Histologic differentiation of hair cells is characterized by argentophilic transformation of cytoplasm (ED 5.5-8), rounding of the nuclei, and the cells become pear shaped. Stereocilia are identified by ED 8 and become longer through ED 10. The cristae and macula have a rather mature appearance grossly by ED 10.

Synaptogenesis. Synaptogenesis of the cochlea involves both afferent and efferent innervation. Synapses between hair cells and the peripheral processes of the cochlear ganglion are immature when first formed. Both the nerve fiber and the hair cell undergo a series of structural modifications with the end result being a synapse. During early synaptogenesis in cats and rats, afferent nerves posses many terminal branches in areas below the hair cells or beyond the base of the hair cell toward the cochlear duct (Perkins and Morest, 1975; Whitehead and Morest, 1985a). In chick embryos, most of these extraneous branches disappear as the remaining nerve fibers develop large, preterminal swellings below the hair cells. During this period, hair cells exhibit an increased number of synaptic bodies in embryonic chicks (Hirokawa, 1978) and mice (Sobkowicz et al., 1982). This results in a distribution of synaptic bodies to sites not adjacent to the nerve endings. During final afferent synaptogenesis, the heterotopic distribution of synaptic bodies is replaced by a pattern in which all synaptic bodies are situated presynaptically adjacent to a synapse (Whitehead and Morest, 1985b). This change in distribution of synaptic bodies in the final phase of synaptogenesis is apparently due to a decrease of synaptic bodies not adjacent to the synapse (Favre and Sans, 1979; Sobkowicz et al., 1982).

Nerve fibers in late synaptogenesis lose their presynaptic swellings in birds (Whitehead and Morest, 1981) and mammals (Ginzberg and Morest, 1983; Lorente, 1937; Perkins

and Morest, 1975) and appear as gnarled endfeet. Some afferent terminals committed to synapses may degenerate or decrease the area of their synaptic contact (Pujol et al., 1980; Whitehead and Morest, 1985b) with some endings even competing for postsynaptic sites (Favre and Sans, 1979).

In birds and mammals, the hair cells have efferent innervation from crossed and uncrossed olivocochlear nerve fibers (Ginzberg and Morest, 1983). The establishment of efferent innervation may be the final neuronal development as the cochlea nears final maturation and occurs for the most part, after afferent innervation is well underway. In fact, the formation of efferent synaptic endings coincides with the period when cochlear function begins in the chick embryo (Rebillard and Rubel, 1981; Whitehead and Morest, 1985b).

In chick embryos, the efferent fibers extend from the medulla into the developing cochlea 3 to 5 days after the afferent fibers (Cohen and Fermin, 1978; Whitehead and Morest, 1985b). These efferent projections apparently follow existing afferent fibers to the hair cell regions (Whitehead and Morest, 1985a; Whitehead and Morest, 1985b). In the basilar papilla of chick embryos, small efferent synapses are formed on tall hair cells and their subjacent afferent dendrites, while large efferent endings contact short hair cells. Efferent synaptic endings form in chick embryos during the intermediate stages of afferent synaptogenesis. During this period, afferent fibers are remodeled into endings with mature sizes and shapes.

Efferent synaptogenesis in mammals occurs predominately at a postnatal time, after the inner and outer hair cells have differentiated (Ginzberg and Morest, 1983; Ginzberg and Morest, 1984). During postnatal development, efferent synapses with inner hair cells decrease and form synapses only on afferent dendrites (Pujol et al., 1980). Synchronously with the formation of efferent synaptogenesis, outer hair cells lose all synaptic bodies formed during early afferent synaptogenesis and develop subsynaptic cysternae opposite the efferent nerve terminal (Pujol et al., 1979).

Peripheral Auditory Electrophysiology. The variety of electrophysiologic methods used to assess cochlear function used produce a diverse assortment of research results to understand. The resting endocochlear potential (EP) consists of two components: a positive portion thought to be produced by an electrogenic sodium-potassium pump, and a negative component produced by potassium diffusion or a leakage of current from cells of the organ of Corti (Moller, 1983). The cochlear microphonic (CM) most likely originates from the hair cells, and recordings from the round window reflect the activity of mainly the outer hair cells in the base of the cochlea (Moller, 1983).

The compound action potential (CAP) is a sound dependent potential reflecting excitation and synchronization of populations of auditory nerve fibers (Dallos, 1981). The response is most prominent following transient stimuli such as clicks and the potential is composed of two peaks, named N_1

and N_2. The magnitude of the N_1 response is a function of both the stimulus intensity and the number of fibers firing synchronously. The N_1 amplitude and latency are thought to reflect the integrity of the cochlea and eighth nerve, whereas the N_2 component may originate from more central structures.

The auditory evoked brain response (ABR) consists of a series of waves during the first 10 msec following a click or pure tone stimulus (Jewett, 1970). The ABR is extracted from the background electroencephalogram activity by computer and represents sequential activation of the auditory neuroaxis in the brain stem. The N_1 and N_2 components of the CAP are the first two waves of the ABR.

The tuning curve is a plot of neurons firing above threshold at various frequencies (Dallos, 1981; Moller, 1983). The neural response at various frequencies is not the same. A tuning curve consists of a low-frequency tail portion that indicates hearing response only to high-intensity sounds and an extremely sharp tuned "tip" region. The latter is characterized by an extremely sudden transition from the high-threshold tail region to a narrow region where the nerves or receptor cells increase their firing rate abruptly at tones of low intensity near the characteristic frequency. At frequencies above the characteristic frequency, there is again an abrupt and rapid rise in the threshold of the neuron.

Onset of Cochlear Function. It is likely that no single change is responsible for the onset of hearing in birds due to the multitude of structural, biochemical, and physiologic

changes occurring simultaneously (Rubel and Parks, 1988). These events are well synchronized despite their diversity with the product being a functional inner ear. The avian basilar papilla is responsive to sound and develop auditory capabilities during the last half of embryogenesis. The earliest electrical potentials recorded from the basilar papilla of the chick embryo in response to sound stimulation were first seen at ED 10, but could only be elicited by a very intense stimuli (Saunders et al., 1973). This developmental stage is 2 to 3 days after the first definitive afferent nerve-hair cell synapses are formed (Cohen and Fermin, 1978; Hirokawa, 1978; Whitehead and Morest, 1985b). By ED 11-12, a neural component appears (Saunders et al., 1973). Averaged evoked responses can be recorded from the brain stem. Intensities over 100 dB are still required, however, and only relatively low-frequency, long-duration tone bursts are effective. This event coincides with at least one measure of biochemical differentiation. Whitehead et al. (1982) described hair cells of the basilar papilla beginning to demonstrate neuron-specific enolase (NSE) on ED 10. In chick embryos, central responses evoked by tone bursts can be recorded at the level of the first central synapse, the nucleus magnocellularis, as early as ED 11 (Vanzulli and Garcia-Austt, 1963). The nucleus magnocellularis is considered homologous to the mammalian anterioventral cochlear nucleus (Boord, 1969). The response was poorly organized and slow until ED 15 when the central responses became more

synchronized with a shorter duration in conjunction with the onset of click-evoked activity (Saunders et al., 1973). Also during this period, behavioral responses to sound can first be elicited (Jackson and Rubel, 1978). The amount and distribution of NSE increases dramatically during this time frame (Whitehead et al., 1982). Prior to ED 16-17, the auditory system is mainly capable of responding to loud, low-frequency sounds. This is followed by a decrease in threshold intensity (increased sensitivity) across all frequency ranges, primarily due to middle ear clearing (Rubel and Parks, 1988).

Morphologically, onset of click-evoked activity and surge in NSE coincides with a transition from mid to late synaptogenesis. During this time, the basilar papilla exhibits considerable remodeling of the afferent synaptic endings and the beginning of efferent synaptogenesis (Whitehead and Morest, 1985b). Sharpening of the tuning curve observed during prenatal development of the chick embryo correlates with establishment of efferent innervation of the short hair cells and the final stages of maturation of afferent synapses (Rebillard and Rubel, 1981). As mentioned previously, the differentiation of hair cells and the development of at least some neural features in both birds and mammals generally proceeds from basal to apical regions (Fermin and Cohen, 1984; Li and Rubin, 1979; Sobkowicz et al., 1975). It has been well established that once mature, the basal cochlea detects high frequencies while the more apical regions detect lower frequencies. However, the earliest

frequency responses generated in both birds and mammals have been low-frequency stimuli while high-frequency responses mature relatively late (Brugge et al., 1978; Konishi, 1973; Moore and Irvine, 1979; Rebillard and Rubel, 1981; Saunders et al., 1973). This creates a paradox between the structural maturation sequence of the organ of Corti and basilar papilla and the development of frequency selectivity. The frequency limitation probably is not the result of immaturity of sound conduction through the immature outer and middle ear because it holds true even when auditory stimuli are applied after clearing of the external and middle ears (Rebillard and Rubel, 1981). Evidence suggests that the frequency organization of the cochlea shifts during development (Rubel and Ryals, 1983). Chick embryos exposed to high intensity pure tones that cause discrete foci of hair cell damage have regions of hair cell loss that differ along the basilar papilla from basal to apical regions for each of the tones applied. From this it appeared that basal regions mediate lower frequency hearing during earlier stages of hearing development than during later stages.

OVERVIEW OF OTOTOXICOLOGY

Prosen and Stebbins (1980) defined ototoxins and vestibulotoxins as agents that affect hearing and balance, respectively. Many toxic compounds, however, affect both systems and may be referred to as ototoxicants. Ototoxicants are agents which, when administered, lead to hearing

impairment and corresponding structural damage to the peripheral auditory system or precipitate an impaired sense of balance and structural damage to the peripheral vestibular system.

Ototoxic effects on hearing can be classified as conductive or sensorineural hearing deficits. Conductive hearing deficits are the result of malformation of the outer and/or middle ear sound wave conductive pathways to the cochlea. Thalidomide, quinine, vitamin A, and retinoic acid are a few of the examples of this type of ototoxicant (Granstrom, 1990; Schuknecht, 1974). Sensorineural hearing deficits are the most common type of ototoxic effect and are due to a loss of the sensory epithelium (hair cells) or damage to central nervous system auditory components.

By the 1880s, it was known that quinine, salicylates, and oil of chenopodium produced both temporary and permanent hearing impairment and vestibular disturbances (Hawkins, 1976). With the reports of the auditory and vestibular effects of the first aminoglycoside, streptomycin, in 1945, ototoxicity began to receive significant attention (Hinshaw and Feldman, 1945). Reports of both transient and permanent hearing loss following the use of the loop diuretic, ethacrynic acid, began to appear in 1965, shortly after it was introduced into clinical practice (Bosher, 1980a). Since the loop diuretics have a unique ototoxic mechanism of action separate from the aminoglycosides, this reemphasized the susceptibility of the inner ear to ototoxicants.

Since 1988, the drugs most commonly associated with ototoxicity are the aminoglycoside antibiotics, nonsteroidal anti-inflammatory agents, loop diuretics, and antineoplastic agents (Norris, 1988). Their structures are quite diverse, as are their ototoxicologic signs, primary otic target tissue, and the type of change they induce (Schacht and Canlon, 1988). Nonsteroidal anti-inflammatory drugs and antineoplastic drugs will be briefly reviewed because of their current clinical relevance and classic association with ototoxicity.

Nonsteroidal Anti-Inflammatory Drugs. This group of drugs includes aspirin, sodium salicylate, and methyl salicylate, as well as newer agents such as ibuprofen and naproxen. The nonsteroidal anti-inflammatory drugs such as naproxen and ibuprofen have a lower incidence of ototoxicity, but when it occurs, it is frequently irreversible (Miller, 1985; Chapman, 1982). The most common ototoxic symptoms are reversible tinnitus (a high-pitched ringing in the ears) and hearing deficits (Douek, et al., 1883: Miller, 1985). Little progress has been made on the mechanism of action of salicylates. Early research revealed mitochondrial vacuolation in cells of the stria vascularis and outer hair cells (Covell, 1936). Falbe-Hansen (1941) proposed that salicylates increased perilymph production, inducing a cochlear hydrops. However, histologic and/or ultrastructural changes have been rarely reported (Bernstein and Weiss, 1967; Deer and Hunter-Duvar, 1982; DeMoura and Hayden, 1968; Falk, 1974; Morrison and Blakley, 1978). Flattening and destruction

of the epithelial elements of the organ of Corti in the guinea pig have been reported (Aly et al., 1975).

Several biochemical mechanisms of action have been proposed for this group of drugs. The anti-inflammatory properties of this group of drugs results from the inhibition of cyclooxygenase, an enzyme catalyzing the first step in the synthesis of prostaglandins from arachidonic acid. Aspirin lowers the rate of prostaglandin synthesis in the cochlea as indirectly indicated by the lower levels of prostaglandins in perilymph (Escoubet et al., 1985). A causal relationship between cyclooxygenase inhibition and ototoxicity has been proposed (Brown and Feldman, 1978; Rybak, 1986), but evidence for this is unsubstantiated.

Salicylates may alter membrane potentials and cellular energy production resulting in altered propagation of cochlear and auditory nerve action potentials. Salicylates inhibit cochlear transaminase and dehydrogenase systems (Silverstein et al., 1967) and acetylcholinesterase activity in efferent nerve endings (Ishii et al., 1967). While ATP and phosphocreatine content of the stria vascularis and cochlear nerve increase, reductions in ATP concentrations of Reissner's membrane are reported after salicylate treatment (Krzanowski and Matschinsky, 1971). In addition, vasoconstriction has been found experimentally in capillaries of the suprastrial spiral ligament, the stria vascularis, the tympanic lip and basilar membrane (Hawkins, 1973).

Antineoplastic Agents. Ototoxic antineoplastic agents include cisplatin (De Conti et al., 1973), 6-aminonicotinamide (Herter et al., 1961), nitrogen mustard (Cummings, 1968), vincristine (Mahajan et al., 1981), vinblastine (Serafy et al., 1982), and misonidazole (Abratt and Blackburn, 1980). Cisplatin induces a histologic change consisting of outer hair cell loss in the basal turn of the cochlea (Nakai et al., 1982, Komune et al., 1981; Konishi et al., 1983) that is strikingly similar to that seen with aminoglycoside antibiotics. Ultrastructural changes include supporting cell damage and irregular stereocilia of inner and outer hair cells (Estrem et al., 1981). In addition, vestibular toxicity (Kobayashi et al., 1987; Tange and Vuzevski, 1984) and degeneration of marginal, intermediate, and basal cells of the stria vascularis have also been reported (Kohn et al., 1988; Tange and Vizevski, 1984). Cochlear changes in human patients with cisplatin-induced hearing loss include large, fused stereocilia and damage to the cuticular plate of the basal outer hair cells, with degeneration of spiral ganglion cells and cochlear neurons (Strauss et al., 1983; Wright and Schaefer, 1982). A proposed mechanism of action involves the inhibition of adenylate cyclase (Kopelman et al., 1988).

Some ototoxic antineoplastic compounds are rarely used today. 6-Aminonicotinamide produces a number of toxic side effects such as irreversible vertigo, hearing deficits, and tinnitus (Herter et al., 1961). The histologic changes in human beings and cats induced by nitrogen mustard are

characterized by severe loss of hair cells in the basal and middle turns of the cochlea with the apex being spared (Schucknecht, 1964, Cummings, 1968).

Vincristine produces degenerative effects in the spiral ganglion and organ of Corti hair cells (Serafy and Hashash, 1981) while vinblastine produces degenerative changes only in hair cells (Serafy et al., 1982). Apparently replacing the formyl group on vincristine with the methyl group on vinblastine has a sparing effect on the spiral ganglion. Only audiologic changes have been reported with misonidazole (Abratt and Blackburn, 1980).

Loop Diuretics. The loop diuretics are a widely used and important component of the therapeutic approach to a variety of disease processes. Loop diuretics include furosemide, ethacrynic acid, piretanide, bumetanide, azosemide, ozolinone, and indacrinone (Jacobson and Kokko, 1976). Although chemically dissimilar, members of the group have a common mode of action in producing diuresis, but, the specific ototoxic mechanism of action of the loop diuretics is still unknown.

The development of acute, reversible deafness following the intravenous use of ethacrynic acid in man was first described by Maher and Schreiner (1965). Each of the active loop diuretics has been shown to have an adverse effect on cochlear function in experimental animals and most have been shown to be ototoxic in humans (Rybak, 1986). One unique feature of furosemide is the lack of an ototoxic effect on pigeons suggesting a possible fundamental difference in inner

ears between birds and mammals (Schermuly et al., 1983).

In human beings, the clinical effects of loop diuretic-induced ototoxicity are those of hearing loss and vestibular disturbances (Michaels, 1988). These effects are usually reversible, but can become permanent (Klinke et al., 1981). High doses or repetitive administration of ethacrynic acid have been shown to produce permanent hearing loss and damage to the hair cells in the organ of Corti (Mathog et al., 1970) which may result from a direct action on the hair cells or be secondary to prolonged changes in the stria vascularis. Furosemide-induced hearing loss occurs most frequently in patients with concomitant renal disease and is associated primarily with intravenous administration at rates exceeding 4 mg/min (Gallagher and Jones, 1979).

The hearing loss associated with furosemide can have a latency of onset up to 6 months with progressively higher thresholds of hearing (Quick and Hoppe, 1975). Ethacrynic acid ototoxicity has a definite latent period after administration (Brown, 1975; Brown, 1981; Brown and McElwee, 1972). A possible explanation for the delay is that metabolism to a more toxic intermediate is necessary.

Experimentally, ethacrynic acid and furosemide produce a dose-related reduction in endocochlear potential and/or alteration in endolymphatic ion concentration with the concomitant reduction in cochlear microphonic and subsequent N_1 depression of auditory-evoked responses (Brown, 1975; Brown, 1981; Brown and McElwee, 1972; Brusilow, 1976; Kusakari

et al., 1976; Rybak and Morizono, 1982; Silverstein and Yules, 1971; Spoendlin, 1988).

Loop diuretics appear to block the potassium chloride cotransport system that moves potassium and chloride out of the stria vascularis into the endolymph (Marcus et al., 1983; Santi and Lakhani, 1983). This may explain the endolymph electrolyte abnormalities such as reduced concentration of potassium (Bosher, 1979; Rybak and Morizono, 1982) and chloride (Brusilow, 1976) and an elevation in sodium concentration (Brusilow, 1976) induced by the loop diuretics.

Although the mechanism of action of loop diuretics in producing ototoxicity is unknown, the proposed molecular effects fall into the following three general categories (Bosher, 1980a,b):

(1) Energy utilization. The stria vascularis is responsible for the positive direct current (DC) endocochlear potential and maintenance of the endolymph electrolyte balance. The generation of the endocochlear potential has been shown to be dependent upon a sensitive Na^+/K^+ membrane ATPase pump which led to the suggestion that loop diuretics may produce their ototoxic effect by inhibition of strial Na^+/K^+ ATPase activity (Prazma et al., 1972; Thalmann et al., 1973). This hypothesis is consistent with the alterations in strial ATPase and phosphocreatine dynamics observed during ethacrynic acid ototoxicity. However, <u>in vivo</u> studies do not support this hypothesis (Kusakari et al., 1976, 1977). The hypothesis that loop diuretic induce ototoxicity via

inhibition of strial adenylate cyclase is not generally accepted (Kusakari et al., 1976; Marks and Schacht, 1982; Paloheimo and Thalmann, 1977).

(2) Decrease in membrane permeability. Changes noted in the stria vascularis during ethacrynic acid ototoxicity suggest alterations in the permeability of the endolymphatic membranes. Direct evidence of changes in membrane permeability are lacking (Bosher, 1980a,b). However, Bosher (1980b) and Forge (1981) reported disruption and alterations in the limiting membrane of the surface coat of marginal cells, possibly reflecting a functional change of the endolymphatic surface membrane of strial marginal cells.

(3) Depression of oxidative metabolism (energy generation). As previously mentioned, stria vascularis metabolism is believed responsible for the endocochlear potential whereas the metabolic activity of the organ of Corti is thought to represent the cochlear microphonic potential. Ethacrynic acid has been shown to reduce the cochlear microphonic potential in two phases of decline (Prazma et al., 1972). The two phases were attributed to an initial change in potassium ion concentration inside the cochlear duct that altered the cochlear microphonic potential followed by a decrease in glycolysis in hair cells.

Because of low glycogen storage in the hair cell, altered microphonic potentials might be expected to persist for some time, even after ion transport within the cochlear duct has been reestablished. The effect of ethacrynic acid on the

organ of Corti could thus be independent of any effect on the stria vascularis (Miller, 1985) and this is supported by Horn et al., (1978) who suggested that the acute depression of the cochlear microphonic potential was due to a decrease in energy production within the organ of Corti. A reversible impairment in the electron transfer system of the hair cell similar to that seen in renal mitochondria (Cunarro and Weiner, 1978) has been proposed as the mechanism of furosemide ototoxicity (Tamura, 1978).

Evidence exists that ethacrynic acid is ototoxic in the parent form (Fox and Brummett, 1974) and as the cysteine metabolite (Brown, 1975). Koechel (1981) demonstrated a direct correlation between the release of ethacrynic acid from the cysteine conjugate in vitro and the ototoxic potential in guinea pigs. Koechel (1981) also demonstrated a direct correlation between a series of ethacrynic acid thiol adducts and their ototoxic potential. In addition, the cysteine conjugate of ethacrynic acid is 3.1 to 3.3 times more potent at suppressing the N_1 potential than ethacrynic acid alone (Brown, 1975). This may be explained by ethacrynic acid having a greater protein binding capacity than the cysteine conjugate, allowing more of the cysteine conjugate to diffuse across the blood/cochlear barrier (Fox and Brummett, 1974).

Furosemide concentrations within the inner ear can be reduced and the ototoxicity ameliorated or prevented by administration of probenecid, an inhibitor of organic acid transport (Rybak et al., 1984). This suggests that the

diuretic uptake mechanism in the kidney, consisting of organic acid active transport from the blood to its site of action, may also be the same uptake mechanism in the cochlea.

Loop diuretic-induced morphologic changes are reported in both the cochlear and vestibular systems. Cochlear changes occur primarily in the stria vascularis of the basal cochlea (Santi and Lakhani, 1983; Arnold et al., 1981). This change is characterized by edema between and within cells of the stria vascularis. With excessive doses of loop diuretics, there is also damage to the outer hair cells of the basal cochlea (Matz, 1976). Vestibular changes are characterized by cyst formation in the sensory epithelium of the posterior semicircular canal and the saccular macula (Matz, 1976). The earliest vestibular changes are visible only by electron microscopy, with later damage visible by light microscopy (Forge, 1980). The damage to the sensory epithelium correlates well with alterations in vestibular electrophysiology (Brummett et al., 1977).

Although numerous methods have been utilized in the investigation of loop diuretic-induced ototoxicity, the exact mechanism is still unknown. Of the proposed biochemical effects, alterations in energy production by the hair cells and energy utilization in the stria vascularis may account for the separate effects on the vestibular and cochlear systems, respectively, following administration of ethacrynic acid while alterations in the electron transfer system within hair cells may be responsible for furosemide ototoxicity.

AMINOGLYCOSIDE ANTIBIOTICS

The aminoglycoside antibiotics began to appear in 1957 with the introduction of kanamycin (Pancoast, 1988). This group of antibiotics provided the beginning of an effective method for treating gram negative infections. With the advent of newer agents in this group, such as amikacin, with activity against Pseudomonas aeruginosa, the aminoglycosides became the standard for the treatment of gram negative infections (Kawaguchi, 1976).

Aminoglycoside antibiotics are a widely used group of antibiotics, with about three million doses being given per year in the United States (Pancoast, 1988). This usage has grown despite the availability of expanded spectrum beta-lactam antibiotics with equal activities (Davies, 1986). This wide use has also brought out the problems of toxicosis, super infections, and bacterial resistance.

Interest in the toxic side effects has generated interest in the development of other antibiotics with the same spectrum of activity, but with less associated toxicity. Many of these antibiotics are now available (Levine et al., 1985; Moellering, 1986; Neu, 1983; Neu, 1986; Nichols, 1986).

Chemistry. Streptomycin, the first aminoglycoside discovered, was isolated from the fungus Streptomyces grisens (Table 1). Other members of the aminoglycosides were also discovered by soil searching techniques. In 1963, the gentamicin complex (gentamicin C_1, C_{1a}, C_2) was isolated from Micromonospora purpura (Siegenthaler et al., 1986). The

Table 1. The Year and Fungal Species or Parent Antibiotic From Which Aminoglycoside Antibiotics Were Derived (Siegenthaler et al., 1986).

Year	Antibiotic	Fungal Species or Parent Antibiotic
1944	Streptomycin	Streptomyces grisens
1949	Neomycin	Streptomyces fradiae
1957	Kanamycin	Streptomyces kanamyceticus
1963	Gentamicin	Micromonospora purpurea
1967	Tobramycin	Streptomyces tenebrarius
1970	Sisomicin	Micromonospora inyoensis
1972	Amikacin	Kanamycin A
1975	Netilmicin	Sisomicin

suffix "mycin" designates an antibiotic isolated from Streptomyces sp. while "micin" indicates an isolate from a Micromonospora sp. Later aminoglycosides were chemically synthesized. These include amikacin (the first synthetic), paromomycin, tobramycin, sisomicin, and netilmicin (Kawaguchi, 1976; Miller et al., 1976).

The core aminoglycoside molecule consists of two or more cyclic amino sugars bound by a glycoside linkage to a central hexose. All aminoglycosides are aminocyclitols, but not all aminocyclitols are close enough analogs to the aminoglycosides to possess a similar antibacterial activity. An example of this is the antibiotic spectinomycin.

The aminoglycosides are highly water soluble polar cationic compounds with optimal activity at a pH of 6 to 8. Their antibacterial activity is strongly curtailed by acid pH and divalent cations. The agents do not perform well in the acid environment of bronchial secretions or abscesses, or in the presence of necrotic tissue and large quantities of organic material containing divalent cations (Bodem et al., 1983). The aminoglycosides are generally ineffective against anaerobic organisms because the drugs require O_2-dependent, active transport into the bacteria (Verklin and Mandell, 1977).

Pharmacokinetics. Aminoglycosides are poorly absorbed from the gastrointestinal tract, and because of their polarity, they poorly penetrate intracellular space and cerebrospinal fluid (Siegenthaler et al., 1986). They are

well tolerated following intramuscular or intravenous injection. Once absorbed, the aminoglycosides have a volume of distribution that is approximately that of the extracellular fluid compartment (Edwards et al., 1981; Hall et al., 1977; Leroy et al., 1978; Schwartz et al., 1978; Walker et al., 1979). They are metabolically stable with 95% being eliminated via the kidneys.

Antibacterial Activity. The antibacterial mechanism of action of the aminoglycosides is only partially understood. The aminoglycosides bind to the surface of the bacteria and are taken into the cell by an active transport mechanism (Bryan and Van den Elzen, 1977). Once inside the cell, they bind to the 30S ribosomal subunit, causing a misreading of messenger RNA during the translation process and producing "nonsense proteins" (Bryan, 1984; Moellering, 1983; Siegenthaler et al., 1986). Aminoglycosides do not dissociate once they bind to ribosomes. The irreversible binding results in a halt to cellular protein synthesis and an interference with cellular metabolism dependent on active protein synthesis such as membrane function. This causes potassium, sodium, amino acid, and other essential constituents to leak out, resulting in bacterial death.

Nephrotoxic effects. The signs associated with aminoglycoside-induced nephrotoxicity are generally a rise in blood urea nitrogen (BUN) and serum creatinine (Davey and Harpur, 1987), indicating a fall in glomerular filtration rate (GFR). This can proceed to non-oliguric renal failure. The

renal effects of aminoglycosides are generally reversible and seldom lead to a fatal outcome. Although the clinical changes associated with aminoglycoside-induced nephrotoxicity are those of decreased GFR, the renal toxicosis does not appear to involve the glomeruli. The primary area affected is the renal proximal (S1-S2 region) convoluted tubules (Houghton et al., 1976; Kosek et al., 1974).

Aminoglycosides are eliminated unchanged in the glomerular filtrate and accumulate in the renal cortex at concentrations several times higher than that of the serum (Fabre et al., 1976; Giuliano et al., 1984). Most AA accumulate in proximal tubular epithelial cells (Kuhar et al., 1979; Van de Walle et al., 1981), but small amounts are also reported to accumulate in patches in distal tubular epithelium (Bergeron et al., 1986; Silverblatt, 1982). The accumulation of the compounds is not by diffusion because they are too hydrophilic to freely cross cell membranes, but rather due to a process of absorptive endocytosis (Silverblatt and Kuehn, 1979). Once taken up, aminoglycosides are rapidly incorporated into lysosomes (Silverblatt and Kuehn, 1979). Further studies have shown that before overt tubular necrosis, the aminoglycosides are almost associated with lysosomes (Giurgea-Marion et al., 1986; Josepovitz et al., 1985).

As aminoglycosides accumulate in the tubular epithelial cells, typical ultrastructural changes occur and consist of a progressive swelling of lysosomes (Houghton et al., 1976; Kosek et al., 1974). The lysosomal contents become

increasingly osmiophilic and lamellar in appearance (myeloid bodies). Enlarged lysosomes, many with lamellar profiles and electron-translucent centers, have been reported in cultured human skin fibroblasts after the addition of gentamicin to the culture media (Oshima et al., 1986). In addition, a decrease in the rate of phospholipid turnover (phospholipidosis) in proximal tubular epithelium occurs (Ramsammy et al., 1989a) in association with a marked reduction in lysosomal acid sphingomyelinase and phospholipase A_1 activities (Feldman et al., 1982; Laurent et al., 1982; Tulkens, 1986; Tulkens and Van Hoof, 1980).

The cytotoxicity of aminoglycosides has been attributed to mitochondrial dysfunction (Simmons et al., 1980; Mela-Riker et al., 1986), perturbation of neoglucogenesis (Michalik and Bryla, 1987), impairment of protein synthesis (Bennett et al., 1988), and interactions with plasma membrane enzymes such as Na^+, K^+-ATPase (Williams et al., 1984) and phosphatidylinositol-specific phospholipase C (Schwertz et al., 1984). However, it is unclear whether these are a cause or a result of cellular dysfunction and/or necrosis. There exists good evidence of the relationship between the extent of tubular damage and the extent of inhibition of phospholipid catabolism (Toebeau et al., 1986). The close relationship between aminoglycoside-induced phospholipidosis and tubular necrosis is further demonstrated by the protective effects of anionic polypeptides against nephrotoxicity (Gilbert et al., 1989; Ramsammy et al., 1989b; Beauchamp et al., 1986)

The mechanism by which aminoglycosides cause inhibition of lysosomal phospholipase is not clear. Stearic hinderance resulting in a lesser accessibility to the substrate (i.e., phosphatidylcholine) has been proposed as one mechanism. This is supported by the association between aminoglycoside-induced inhibition of phospholipase activity toward phosphatidylcholine (Laurent et al., 1982; Brasseur et al., 1984) and drug binding to lipid bilayers (Laurent et al., 1982; Brasseur et al., 1984). As a second mechanism, aminoglycosides may decrease the availability of acid phospholipids that some phospholipases (Mingeot-Leclereq et al., 1988) and lipases (Kariya and Kaplan, 1973) required to function properly. This occurs by the antibiotics impairing lysosomal catabolism of phosphatidylcholine (possibly other zwitterionic phospholipids) by sequestering acid phospholipids and creating less favorable conditions for the hydrolysis of neutral phospholipids. A third mechanism is direct inhibition of lysosomal hydrolases by the drug (Feldman et al., 1982). Other amphophilic drugs, such as chloroquine that accumulate in hepatocytes and cause phospholipidosis, have been shown to directly inhibit lysosomal phospholipase A and C (Matsuzawa and Hostetler, 1980a,b).

The mechanism by which phospholipid accumulation in lysosomes causes cell death and tubular necrosis is not clear. Phospholipid overload in lysosomes could result in the rupture of the lysosomes and release within the cell of potentially harmful hydrolases and aminoglycosides themselves (Marin et

al., 1978). Necrosis of hepatic parenchyma has been attributed to the leakage of lysosomal enzymes into the cytosol (Wattiaux and Wattiaux-De Coninck, 1981). Prolonged exposure of cultured proximal tubule cells to gentamicin produces an increased fragility of the lysosomes (Regec et al., 1989; Viotte et al., 1983).

Ototoxic effects. Ototoxicity is one of the major side effects of aminoglycoside antibiotics (Govarts et al., 1990). The incidence of symptomatic ototoxicity in humans using aminoglycosides has been estimated at 2% with up to 10% having subclinical hearing deficits (Wang et al., 1984). The aminoglycosides vary widely in their ototoxic potential. Comparative animal studies (Aran et al., 1982; Arpini et al., 1979; Bamonte et al., 1986a,b; Igarshi et al., 1978; McCormick et al., 1985; Parravicini et al., 1982; Sato et al., 1983), mostly using guinea pigs, yield a cochleotoxic ranking for the aminoglycosides of: netilmicin < amikacin = dibekacin < gentamicin = tobramycin = kanamycin. From the same studies, a vestibulotoxic ranking of aminoglycosides is netilmicin < tobramycin = dibekacin < gentamicin < streptomycin.

The electrophysiologic effects of aminoglycosides include a shift in and attenuation of the input-output (sound intensity versus amplitude of response) curves for the auditory nerve compound action potential (CAP) and cochlear microphonic (CM) potential. These changes indicate that hair cell damage since the CM potential most likely originates from the hair cells. Shifts in CM and N_1 threshold and an

elevation of threshold of single auditory fibers in the high frequency range indicate the aminoglycosides decrease hearing sensitivity (Rybak, 1986). During aminoglycoside ototoxicity, tuning curve tips appear to be blunted with hypersensitivity of the tails (Kiang et al., 1976; Robertson and Johnstone, 1979) and a decreased rate of spontaneous activity is observed (Kiang et al., 1970). Auditory function and morphologic changes may be asymmetrical (Brummett, 1980; Johnsson et al., 1981).

Many studies have been performed on the histologic and ultrastructural changes associated with aminoglycoside-induced ototoxicity. Most changes are rather nonspecific degenerative changes. The earliest cochlear lesions produced by aminoglycoside antibiotics in experimental animals and humans appear in the inner row of outer hair cells of the basal turn (Rybak, 1986). With continued exposure, the cochlear changes extend apically, then to the inner hair cells (Barmonte et al., 1986b; Brown and Feldman, 1978; Gratacap et al., 1985; Hawkins, 1976). Federspil (1978) described the sequence of degeneration of cellular components of the organ of Corti as hair cells, Deiter's cells, pillar cells, Hensen's cells and Claudius' cells. Degeneration may occur in the stria vascularis simultaneous to the organ of Corti (Gratacap et al., 1985). Delayed degeneration of the afferent neurons can follow destruction of the inner hair cells (Kiang et al., 1976). The initial ultrastructural finding in hair cells is fusion of the stereocilia to form giant hairs (Theopold,

1978). Lysosomes increase in number and size, especially in the infracuticular region of the cell (Gratacap et al., 1985, de Groot et al., 1990; Lim, 1986).

Vacuolization of epithelial cells of Reissner's membrane at the endolymphatic surface has been described (Schuknecht et al., 1974). The appearance of melanin granules in Reissner's membrane of guinea pigs after kanamycin administration has also been reported (Gratacap et al., 1985). The significance of this change is unknown.

The stria vascularis regulates the resorption and secretion of the fluid and electrolytes that constitute endolymph (Schacht and Canlon, 1988). Several strial changes, especially of the marginal and intermediate cells, have been reported associated with aminoglycoside-induced ototoxicity. Extracellular edema (Gratacap et al., 1985) with reduced basal extensions and apical microvilli have been described (Hawkins, 1973). Marginal cell gap junctions show alterations suggesting a turnover of these structures with new intercellular junctions being established (Forge and Fradis, 1985). Ultrastructurally, increased numbers of liposomes have been described in marginal cells, suggesting disturbances in lipid metabolism (Forge and Fradis, 1985). Dumas et al. (1981) reported early strial changes of increased numbers of lysosomes in marginal and intermediate cells and myeloid bodies in vascular endothelial cells. These changes preceded severe outer hair cell lesions.

Johnsson (1974) reported a correlation in localization and severity of hair cell loss and cochlear neuron degeneration. However, neuronal degeneration is apparently more directly associated with degeneration of pillar and Dieter's cells (Johnsson, 1974). However, pillar cells provide only temporary protection, since in the long term, neuronal degeneration occurs even with intact pillar cells (Bichler et al., 1983; Leake and Hradek, 1988). Dendritic processes of first-order neurons degenerate much earlier than do axonal processes (Johnsson et al., 1981; Spoendlin, 1975). Retrograde degeneration of Type I fibers begins after degeneration of the end-organ is induced by neomycin (Spoendlin, 1975). Initially, Type II fibers remain intact, but eventually degenerate after an extended delay (Bichler et al., 1983; Leake and Hradek, 1988; Spoendlin, 1975). Ultrastructural changes within neurons begin with swelling and subsequent destruction of mitochondria, followed by damage to rough endoplasmic reticulum and ribosomes and an increase in the number of lysosomes and demyelinization (Koitchev et al., 1982; Spoendlin, 1975).

Vestibular changes associated with aminoglycosides have been reviewed by Hawkins and Preston (1975). Hair cells, especially Type I, are the principal target (Duvall and Wersäll, 1964; Kimura et al., 1988; Wersäll and Hawkins, 1962), although dark cells are also affected. Histologic and ultrastructural changes in the hair cells are similar to those reported in cochlear hair cells. Hair cells of the ampullar

crista and utricular and saccular macula differ in their susceptibility to aminoglycoside-induced injury (Lindeman, 1969). Dark cell areas are the major secretory tissues of the vestibular system and are similar to the cochlear stria vascularis (Iurato, 1967; Kimura, 1969). Vacuolation and shortening of the basal processes of these cells have been reported in the guinea pig (Hawkins and Preston, 1975), chick (Park and Cohen, 1982), and the cat (Pender, 1985).

Aminoglycosides have been shown to cross the placenta (Weinstein et al., 1976; Yoshioka et al., 1972) and previous reports do indicate that aminoglycoside antibiotics can produce in utero ototoxicity without simultaneously producing teratogenic effects. These reports include abnormalities of auditory and/or vestibular function in children whose mothers received streptomycin and dihydrostreptomycin for treatment of tuberculosis during pregnancy (Conway and Birt, 1965; Robinson and Cambon, 1964) or during chronic renal disease (Jones, 1973). In addition, experimental tobramycin-induced functional and histologic damage to the cochlea in newborn guinea pigs is also reported (Akiyoshi, 1978). Teratogenic-like effects have been produced in mouse cochlear explants exposed to aminoglycosides prior to the onset of morphogenesis (Anniko, 1981; Anniko and Nordemar, 1981).

Interactions between the aminoglycoside antibiotics and the loop diuretics in the production of ototoxicity has been reported clinically and reproduced experimentally. Permanent depression of cochlear function associated with hair cell

destruction occurs rapidly after the administration of a single dose of ethacrynic acid to an animal pretreated with a single dose of kanamycin (Brummett et al., 1975; Nakai, 1977; West et al., 1973). This rapid onset of ototoxicity is similar to that reported in human cases of ototoxicity (Russell et al., 1979a,b). The mechanism of this potentiation is unknown but is speculated to occur in the stria vascularis (Prazma et al., 1974) and/or hair cells (West et al., 1973). Although temporary alterations in vestibular function have occurred after the administration of ethacrynic acid and permanent effects on the vestibular system have been produced by the aminoglycoside antibiotics, there is no evidence that permanent vestibular changes occur as a result of an aminoglycoside-loop diuretic interaction (Mathog and Capps, 1977).

The interaction of loop diuretics and aminoglycosides in the production of ototoxicity appears somewhat specific to the loop diuretics, but not necessarily to the aminoglycoside antibiotics. Ototoxic interactions have been shown between the loop diuretics and nonaminoglycoside antibiotics viomycin, polymyxin B, capreomycin, and fortimicin, but do not occur with polymyxin E or vancomycin (Davis and Brummett, 1979).

Concurrent uremia has been implicated as increasing the sensitivity of patients to ototoxic effects of combined loop diuretic-aminoglycoside antibiotic therapy (Mathog and Klein, 1969; Meriwether et al., 1971). This may be the result of increased half-life of the cysteine conjugate of ethacrynic

acid (Schneider and Becker, 1966), a major and more toxic metabolite of the parent compound (Brown, 1975).

Early pharmacokinetic studies of aminoglycosides in animals suggested a concentrating effect of drug in the perilymph that resulted in higher levels than in plasma (Stupp, 1970; Voldrich, 1965; Vrabec et al., 1965). This accumulation was thought to have implications not only for the mechanism of ototoxicity but also the relative ototoxicity of different aminoglycosides. One of the hypotheses that led from these finding was the "toxic threshold" hypothesis (Stupp et al., 1973). With this theory, serum levels of antibiotic would have had to exceed a critical level before they could enter the perilymph. Ensuing studies, however, demonstrated a linear relationship between dose and perilymph level (Federspil et al., 1976; Tran Ba Huy et al., 1981,1983). A second hypothesis was that an accumulation of an aminoglycoside in perilymph might account for the nature of ototoxicity. This was suggested by the finding of perilymph concentrations of aminoglycosides being equal to serum concentrations after several hours, and exceeding them by several orders of magnitude after 20 hours (Brummett et al., 1978: Federspil et al., 1976; Federspil, 1978; Ohtani et al., 1982a). Also, perilymph half-life of the aminoglycosides was 10-12 times longer than that of serum and could be enhanced with repeated administration (Federspil et al., 1976; Toyoda and Tachibana, 1978). However, Tran Ba Huy et al. (1981), using extremely sensitive radioimmunoassay procedures,

described much lower peak perilymph concentrations of gentamicin than those previously reported and no evidence of accumulation. In addition, Ohtani et al. (1985) and Dulon et al. (1986) found no difference in perilymph kinetics after doses of netilmicin, amikacin and gentamicin previously shown to cause increased perilymph half-lives (Chung et al., 1982). A third hypothesis to attribute the mechanism of aminoglycoside ototoxicity to a unique pharmacokinetic characteristic was that perilymph drug concentrations followed a linear relationship with the administered dose (Dulon et al., 1986; Federspil et al., 1976; Ohtani et al., 1982a; Tran Ba Huy et al., 1983). This relationship appears to remain constant during the first few hours of drug administration, but losses correlation after long-term treatment (Ohtani et al., 1982a). Based on information regarding the pharmacokinetics of aminoglycosides in perilymph, the "accumulation theory" appears unable to explain why there is a vestibular or cochlear preference for toxicity of the aminoglycosides. This theory also fails to explain differences in toxicities of specific aminoglycosides, since there is no difference in pharmacokinetics between various aminoglycosides.

Data concerning endolymph pharmacokinetics are also controversial. Federspil et al. (1976) found no major difference between endolymph and perilymph concentrations of gentamicin, but, Tran Ba Huy et al. (1981, 1986) found much lower concentrations of gentamicin in endolymph compared to

perilymph. Also, gentamicin was shown to accumulate slowly in the endolymph and decay slowly after administration ceased (Tran Ba Huy et al., 1983). This implies that the endolymph may be considered a deep pool that drugs slowly accumulate in, but, also are only slowly eliminated from.

Notable results have been reported concerning the pharmacokinetics of aminoglycosides in regard to concentrations of drugs in tissues of the inner ear. Lengthening the drug administration from a single dose to 3 hours continuous infusion drastically decreases drug clearance from the tissue (Tran Ba Huy et al., 1986). This process might be interpreted as internalization and storage of the drug in the tissue of the inner ear. A positive correlation between the concentrations of different aminoglycosides in tissues of the inner ear and their ototoxic potential has been shown (Dulon et al., 1986). Biochemical assays on inner ear explant cultures (Schacht and Van De Water, 1986) and tissue homogenates (Moore et al., 1984) indicate the binding of aminoglycosides to inner ear tissue is a high-affinity, rapidly saturable phenomenon.

Numerous hypotheses relating to specific biochemical interactions of the aminoglycosides with target cells have been proposed as mechanisms of ototoxicity. Interpretation of these studies is frequently difficult since the changes observed must be differentiated between primary toxic effects and those changes that are secondary effects. One of the first proposals was the idea that the toxicity was caused by

inhibition of protein synthesis in the organ of Corti (Hawkins, 1976). Although aminoglycosides can inhibit protein synthesis in mammalian liver mitochondria (Buss et al., 1984), this same effect has not been demonstrated as a mechanism for specific destruction of outer hair cells (Ylikoski et al., 1974). It has also been proposed that aminoglycosides inhibit carbohydrate uptake and/or metabolism and energy utilization in outer hair cells. Kanamycin has been shown to inhibit respiratory enzyme activity in outer hair cells (Kaku et al., 1973) and selectively inhibit the Embden-Meyerhof pathway in the organ of Corti and kidney (Tachibana et al., 1976). This interference with carbohydrate metabolism may lead to inhibition of ATPase in outer hair cells. There are few glycogen granules reported in normal outer hair cells of the basal turn of the cochlea (Postma et al., 1976) which is the area most affected by aminoglycosides. Since severe depletion of glycogen granules from outer hair cells has been reported in animals treated with several aminoglycosides (Postma et al., 1976), it has been postulated that aminoglycosides may decrease glucose transport into the cochlea (Guarcia-Quiroga et al., 1978). This has been disproved in subsequent studies (Takada et al., 1983).

By using physicochemical models of drug-target interaction, associations between aminoglycosides and a variety of cellular components such as DNA, RNA, proteins, enzymes of energy metabolism and transport, gangliosides, mucopolysaccharides and various lipids have been identified as

inconsequential or secondary effects (Wang et al., 1984; Schacht and Weiner, 1986). These same models demonstrated that an interaction between aminoglycosides and membrane-bound polyphosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PIP_2) is critical in the mechanism of ototoxic action of the aminoglycoside antibiotics (Wang et al., 1984).

Work pursuing PIP_2 as a central factor in the toxic mechanism of action was summarized by Schacht and Weiner (1986) into the following five step hypothesized mechanism of action (Figure 2):

(1) The first step is an electrostatic interaction of the aminoglycoside with negatively charged components of the outer plasma membrane. The resultant displacement of calcium accounts for the acute effects of drug action and is reversible and antagonized by cations.

(2) The second step involves drug transport into the cell by an energy-dependent process and can be prevented by metabolic blockers.

(3) The third, and most crucial step according to Schacht and Weiner, is the binding of the drug to PIP_2. The formation of the drug-phospholipid complex has two effects: first, it prevents hydrolysis of PIP_2 which is the fundamental reaction in the phosphoinositide second messenger signal system; secondly, it disturbs membrane integrity and structure resulting in nonspecific permeability changes of the membrane to ions and possibly to drug itself, causing an enhanced entry of the drug into the cell.

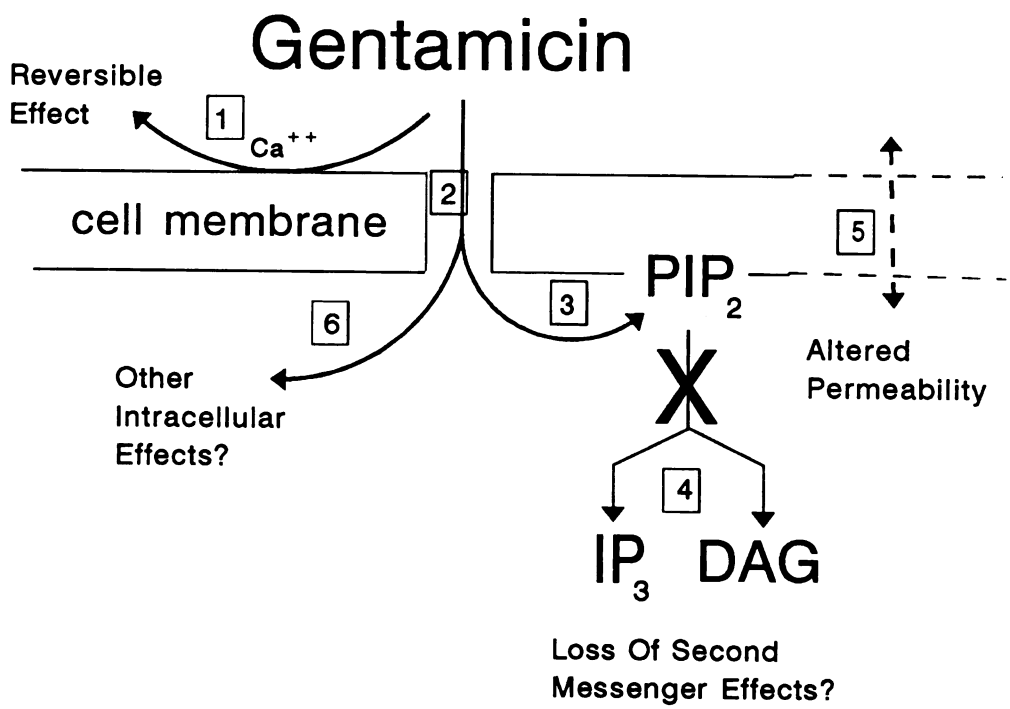

Figure 2. Diagram of proposed mechanism of aminoglycoside-induced ototoxicity. Steps include: (1) reversible binding of gentamicin with negatively charged outer plasma membrane, displacing divalent calcium (Ca^{++}); (2) transport of gentamicin into the cell and binding to phosphatidylinositol 4,5-bisphosphate (PIP_2); (3) lipid-drug complex formation prevents PIP_2 hydrolysis (**X**) to inositol triphosphate (IP_3) and diacylglycerol (DAG), a key reaction (4) in the phosphoinositide second messenger cascade; or (5) altered membrane integrity and permeability; (6) interference with other intracellular reactions (e.g. competition with divalent cations or polyamines). Modified from Schacht and Weiner (1986).

(4) Drug-phospholipid complex formation may also inhibit other reactions regulated by polyphosphoinositides such as the synthesis of prostaglandins.

(5) Once inside the cell, aminoglycosides may also interfere with further, unspecified intracellular reactions.

Polyphosphoinositides are acidic phospholipids of the plasma membrane (Berridge, 1984; Nizhizuka, 1984). A variety of neutral (e.g., lecithins) and acidic (e.g., phosphatidylserine) phospholipids provide the structural framework of cell membranes, regulate membrane function and mediate cell-to-cell and cell-to-external environment communications. The polyphosphoinositides, which are located on the cytoplasmic side of the plasma membrane, constitute a fundamental transmembrane signaling system for neuromodulators and hormones which use calcium as their ultimate messenger.

Investigation of the role of polyphosphoinositides in ototoxicity revealed all of the hair cell models studied (e.g., mammalian cochlea, otocyst in cell culture, and ear of the noctuid moth) contained polyphosphoinositides and that there was a high rate of turnover of these phospholipids (Anniko et al., 1981; Kaloyanides et al., 1980; Kilian and Schacht, 1980; Lodhi et al., 1979; Marche et al., 1983; Orsulakova et al., 1976; Schacht et al., 1977, Schacht, 1979; Stockhorst and Schacht, 1977; Tachibana et al., 1983). More importantly, these studies demonstrated, both *in vivo* and *in vitro*, that ototoxicity may be the result of phosphatidylinositol 4,5-bisphosphate (PIP_2) being a specific

binding site for aminoglycosides (Schacht et al., 1977; Schacht, 1979; Wang et al., 1984). The configuration of the negative charges of PIP_2 allows a specific three point attachment by the positively charged ototoxic aminoglycosides (Lodhi et al., 1979, Stockhorst and Schacht, 1977; Orsulakova et al., 1976). Hydrogen bonding further stabilizes the complex, and other cationic groups of the aminoglycoside may anchor the complex to secondary sites of the affected cell membrane. This binding inhibits metabolism of PIP_2 *in vivo* and *in vitro* (Orsulakova et al., 1976).

Electrophysiological and pharmacokinetic studies indicated aminoglycosides occupied at least two distinct cellular compartments during the sequence of ototoxicity (Takada and Schacht, 1982). The first site apparently corresponds to the acute and reversible (by calcium) phase of aminoglycoside ototoxicity and the second site is associated with the chronic and irreversible phase. These *in vivo* observations supported *in vitro* aminoglycoside-calcium antagonism studies using natural and artificial membranes (Lodhi et al., 1976). These results suggested the first compartment was the interaction of the aminoglycosides in a reversible manner with the outer plasma membrane. Since PIP_2 is found primarily on the cytoplasmic side of the plasma membrane, the water soluble, highly charged aminoglycoside molecule must be entering the cell as the second compartment. The uptake mechanism of hair cells is apparently an energy-dependent pathway (Takada et al., 1985). The nature of the

energy-dependent uptake system in the inner ear is unclear.

Immunocytochemical methods have been utilized in the localization of gentamicin and kanamycin in the cochlea at the light level (Tachibana et al., 1985; Veldman et al., 1987; Yaname et al., 1988). Initial studies by Tachibana et al. (1985) localized the ultrastructural "binding sites" of gentamicin to stereocilia, cuticular plates of hair cells, the head plates of Dieter's cells, cell filaments and the cones of pillar cells, tectorial membranes, basilar membranes, the matrix of the spiral limbus, plasma membranes, mitochondria, and the chromatin of various kinds of cells.

After intraperitoneal injection, gentamicin is detected by immunocytochemical staining within 1 to 2 days at the perilymphatic side of the basilar and Reissner's membrane (Veldman et al., 1987). From 3 days on, gentamicin was detected in the apical region of the outer hair cells, but only in the basal turn of the cochlea. At the same time, intracellular accumulations of gentamicin in the proximal tubular epithelial cells were also observed. de Groot et al. (1990) demonstrated gentamicin in the cochlea after 5-15 daily doses, with specific labeling restricted to the organ of Corti, in particular to the outer and inner hair cells, Deiter's cells, Hensen's cells and the tympanic layer cells of the basilar membrane. Specific labeling was not seen in other cochlear tissues such as the stria vascularis, Reissner's membrane, and the spiral ganglion.

Yanane et al. (1988) reported the detection of kanamycin in the organ of Corti 90 minutes after IV injection, using fluorescence detection. Specific staining was detected in the organ of Corti (Hensen's cells, inner hair cells slightly more than outer hair cells); membranous labyrinth facing the endolymph; pores (lacuna) of the osseous spiral lamina; Reissner's membrane; and tectorial membrane. Administration of furosemide after kanamycin did not alter the distribution of kanamycin from that when kanamycin was given alone. However, kanamycin was detected much quicker in cochlear tissues when combined with by furosemide.

Immunocytochemical methods have also been utilized in the localization of gentamicin and kanamycin in the cochlea at the ultrastructural levels (Tachibana et al., 1985; de Groot et al., 1990). Ultrastructurally, gentamicin was found in lysosomes and multivesicular bodies situated particularly in the infracuticular area and in small tubules and vesicles located in the infra- and supranuclear regions. No labeling was detected on the cuticular plate, stereocilia, mitochondria, nucleus, or golgi complex.

Using autoradiographic techniques, after a single administration, aminoglycosides were first detected in the stria vascularis and ligamentum spirale (Balogh et al., 1970). This was followed by appearance in the perilymph, Dieter's cells, and outer hair cells. After perilymphatic perfusion, maximum staining was seen over the inner and outer hair cells, basilar membrane, and nerve tissue of the spiral lamina

(Hawkins, 1973; Von Ilberg et al., 1971).

One problem encountered with studies attempting to localize aminoglycosides is the difficulty of retaining the drug in tissues during processing. Aminoglycosides are very hydrophilic molecules and easily displaced by processing in aqueous solutions when they are not firmly bound to structures within the specimen (Wedeen et al., 1983). Studies comparing freeze-drying techniques to aqueous fixation and embedding found marked loss of drug from the tissue (Von Ilberg et al., 1971).

Use Of Birds In Otologic Research

Over the past 20 years there has been extensive study of the avian auditory and vestibular systems resulting in a better understanding of avian otologic anatomy, pharmacology, physiology, and behavior. The avian ear is structurally less complex than the mammalian ear, yet there is close homology in function (Rubel and Parks, 1988). For these reasons, birds are utilized in a variety of studies of aminoglycoside-induced ototoxicity and sound-induced otic trauma.

Aminoglycoside-induced ototoxicity. Hatched chicks are sensitive to aminoglycoside-induced ototoxicity. Ototoxicity has been produced by administration of gentamicin (Cruz et al., 1987; Duckert and Rubel, 1990; Tucci and Rubel, 1990), kanamycin (Hashino and Sokabe, 1989; Hashino et al., 1991b), and streptomycin (Park and Cohen, 1982). The ototoxicity is characterized by hair cell degeneration which

characteristically begins in the basal part of the cochlea and proceeds toward the apex (Cruz et al., 1987; Duckert and Rubel, 1990). Significant hearing loss, predominantly in the high frequencies, has been produced in newly hatched chicks given gentamicin (Tucci and Rubel, 1990). Hearing loss, especially at low frequency ranges, continues to deteriorate up to five weeks following treatment. Severe hearing loss has also been demonstrated in parakeets treated with kanamycin (Hashino and Sokabe, 1989). Auditory neurons in the brain are affected secondary to aminoglycoside ototoxicity since gentamicin produces a reversible reduction of nerve cell body area in the nucleus magnocellularis (Lippe, 1991).

Fertile chicken eggs have been used to investigate ototoxicity in developing animals. Streptomycin injected into the center of eggs in a single dose of 166 mg per kg of egg produced a massive expansion of the apical surface of the hair cell (Pickles and Rouse, 1991). Stereocilia were reduced in numbers and often fused, and commonly, absorbed stereocilia were seen in isolated patches in the cuticular plate or around the margins of the cell. The development of some hair cells appeared to have arrested at approximately the time of injection.

Sound-induced otic trauma. The avian basilar papilla is affected by acoustic overstimulation in a manner very similar to the organ of Corti (Rubel and Ryals, 1982). Hair cell loss and reduced auditory capacity in neonatal chicks and parakeets after exposure to intense acoustic stimuli is reported

(Cotanche et al., 1987; Cotanche 1987a,b; Cousillas and Rebillard, 1985; Girod et al., 1989; Hashino et al., 1988; Rubel and Ryals, 1982; Ryals and Rubel, 1982; Saunders and Tilney, 1982). Noise-induced damage to the basilar papilla generally occurs in two regions. The primary region affected is a clearly defined "patch" occupying the abneural side of the papilla involving short hair cells (Henry et al., 1988; Marsh et al., 1988; McFadden and Saunders, 1989). The tectorial membrane is also greatly thinned or retracted over the patch immediately following exposure (Cotanche et al., 1987b). The second region affected is referred to as a "stripe" and is found in more apical (regions of higher frequency recognition) than the "patch". It may extend along the papilla for several hundred microns, but is only twenty to 40 microns wide. This "stripe" is located on the tall hair cell side of the junction between the mobile and immobile parts of the basilar papilla (Cotanche et al., 1987a). The damage observed in the "patch" is consistent with excessive mechanical motion in the 0.9 kHz region of the cochlea (Ryals and Rubel, 1982; Saunders and Tilney, 1982).

Hair cell regeneration. It has been generally accepted that there is no evidence of post-mitotic regeneration of auditory sensory cells in either birds or mammals (Ryals et al., 1988). However, both tall and short hair cells within the basilar papilla of chicks, parakeets, and quail have been shown to regenerate after aminoglycoside ototoxicity (Cruz et al., 1987; Duckert and Rubel, 1990; Hashino et al., 1991,

Lippe et al., 1991) and acoustic trauma (Corwin and Cotanche, 1988; Cotanche, 1987a, Cousillas and Rebillard, 1988; Henry et al., 1988; Marsh et al., 1990; Ryals and Rubel, 1988). Functional recovery of hair cells and regeneration of the tectorial membrane after aminoglycoside ototoxicity has also been shown in chicks (Cotanche, 1987b; Tucci and Rubel, 1990). Regeneration of hair cells occurs in adult quail following acoustic trauma (Ryals and Rubel, 1988), but little to no evidence of hair cell recovery after aminoglycoside ototoxicity is seen in adult chickens (Seidman et al., 1989). The hair cell precursor is still controversial but evidence suggests it is within the strata of supporting cells and undergoes a migratory process beginning at the basilar membrane and ending at the luminal surface (Duckert and Rubel, 1990; Girod et al., 1989).

CHAPTER 1

INSENSITIVITY OF THE CHICK EMBRYO TO THE OTOTOXIC EFFECTS OF AMINOGLYCOSIDE ANTIBIOTICS AND A LOOP DIURETIC

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ABSTRACT

The two-fold purpose of this research was to (1) determine if the cochlear hair cells of chick embryos were sensitive to the ototoxic effects of gentamicin, kanamycin, streptomycin, ethacrynic acid, and ethacrynic acid and gentamicin in combination and (2) compare the distribution of an ototoxic drug, gentamicin, in chick embryos to that of hatched chicks. Chick embryos were exposed to maximally tolerated doses of the drugs during days 10-17 of incubation, but no hair cell loss was detected in three locations along the basilar papilla. Therefore, the chick embryo appears to be resistant to drug-induced ototoxicity. The distribution of gentamicin, which was detected by immunocytochemical staining, in the basilar papilla of chick embryos which received 0.1 mg/egg on incubation days 10-18 was compared to that of hatched chicks injected at a dose of 5 or 100 mg/kg, subcutaneously on post-hatching days 1-9. Immunocytochemical staining for gentamicin was increased in the basilar papilla

of all treated chick embryos or chicks by 1 day after the first injection. The staining was significantly more intense ($p<0.05$) than staining of basilar papilla from controls after 3 days of treatment. Severe hair cell loss was detected in hatched chicks from the high dose group after 3 days of treatment. The apparent resistance of chick embryos to gentamicin-induced ototoxicity does not appear to be due to a lack of distribution of drug to the basilar papilla.

INTRODUCTION

Birds are used in otologic research because their inner ear is easily dissected and the development, morphology, and function of the inner ear is similar to that of mammals (Whitehead, 1986; Tanaka and Smith, 1978; Meyer, 1986;). The cochlea of birds is linear which makes it easier to preserve for morphologic and immunocytochemical analysis. Newly hatched chicks respond with hair cell loss, as mammals do, to several otologic insults, such as aminoglycoside antibiotics (Cruz et al., 1987; Hashino et al., 1991; Park and Cohen, 1982) and acoustic trauma (Rubel and Ryals, 1982). It is not known whether the aminoglycoside antibiotics can also produce hair cell loss in the chick embryo. The purpose of this investigation was to determine if chick embryos are sensitive to drug-induced ototoxicity, and to compare the distribution of an ototoxic drug (gentamicin) in chick embryos to that in hatched chicks.

MATERIALS AND METHODS

Fertile White Leghorn chicken eggs (Michigan State University, Poultry Research and Teaching Center, East Lansing, MI) were incubated (Petersime Incubator Model 5, Petersime Incubator Co., Gettysburg, OH) at 37°C and 50% humidity. Hatched chicks were housed in brooders (Petersime Brood-Unit, Petersime Incubator Co., Gettysburg, OH) at 30°C. Drugs were obtained as follows: gentamicin sulfate (G-3632, Sigma Chemical Co., St. Louis, MO); streptomycin sulfate (S-6501, Sigma Chemical Co., St. Louis, MO); kanamycin sulfate (Kantrim, 200 mg/ml, Fort Dodge Laboratories, Inc., Fort Dodge, IA); ethacrynate sodium (Sodium Edecrin, 50mg, Merck, Sharp, and Dohme, West Point, PA).

Three trials were performed in this study. Trials 1 and 2 were conducted to assess hair cell loss. In Trial 1, the effect of dose route on hair cell loss was examined and in Trial 2 the effect of various ototoxic drugs alone or in combination on hair cell loss was examined. Trial 3, the distribution of gentamicin to the basilar papilla was examined.

In Trials 1 and 2, three eggs were randomly assigned to each of the eleven groups (Table 2). In both trials, eggs were dosed on incubation days 10 through 17 with a maximally tolerated dose (see Appendix) of gentamicin, kanamycin, streptomycin, ethacrynic acid, or ethacrynic acid and gentamicin combined. Injection volumes were maintained between 0.1 to 0.2 ml for each treatment group. Both trials were terminated on day 18 of incubation.

Table 2. Routes of Injection and Dosages of Aminoglycoside Antibiotics and Ethacrynic Acid Used to Treat Chick Embryos.

Drug	Route	Dosage
Trial 1		
Gentamicin	Air Cell	0.01 mg/egg/day
Gentamicin	Allantoic Space	5.0 mg/egg/day
Gentamicin	Yolk Sac	1.0 mg/egg/day
Gentamicin	Submersion at 5°C	2.5 mg/ml for 10 minutes
Control (H_2O)	Air Cell	0.1 ml/egg/day
Trial 2		
Gentamicin	Air Cell	0.01 mg/egg/day
Kanamycin	Air Cell	0.1 mg/egg/day
Streptomycin	Air Cell	0.1 mg/egg/day
Ethacrynic Acid	Air Cell	1.0 mg/egg/day
Ethacrynic Acid (EA) and Gentamicin (G)	Air Cell	0.1 mg EA/egg/day + 0.01 mg G/egg/d
Control (H_2O)	Air Cell	0.1 ml/egg/day

Chick embryos were killed by decapitation. The calvarium was removed and the brain and lower jaw were removed. The skull was cut along the midline and the temporal bone was dissected away and placed in a small dish containing 2% glutaraldehyde in 0.1 M phosphate buffer. The connective tissue immediately posterior to the tympanic membrane was removed with the tympanic membrane and columella. The cartilage separating the round window and oval window was removed and the lateral cartilaginous wall of the cochlea was removed, exposing the membranous labyrinth of the cochlea. Additional cartilage was dissected away and the membranous labyrinth of the cochlea removed and placed in 2% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 4 hours. The tissue was then rinsed 3 times with 0.1 M phosphate buffer and stored in 0.1 M phosphate buffer at 4°C. Tissues were post-fixed with 1% (w/v) osmium tetroxide followed by uranyl acetate, dehydrated through graded ethanol, transferred to propylene oxide, and infiltrated with and embedded in epon/araldite resin. After curing for 60 hr at 60°C, thick sections (1 μm) were cut with a diamond knife on an LKB Ultrotome III (LKB-Produkter AB, Bromma, Sweden) and stained with toluidine blue stain. Transverse sections of the membranous labyrinth of the cochlea which were perpendicular to the longitudinal axis of the cochlea were made at three different levels of the basilar papilla. The levels were 20, 40, and 60% of the distance from the basal (or proximal) tip to the apex of the basilar papilla (Figure 3).

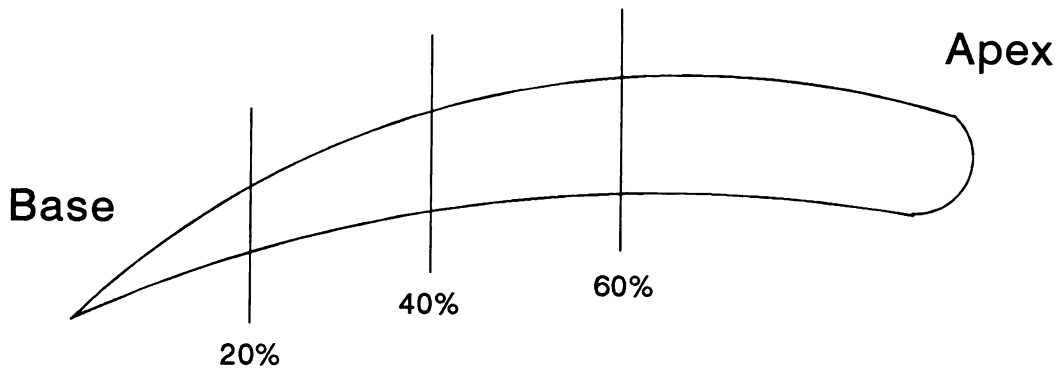


Figure 3. Diagram of the basilar papilla from a chick embryo or chick. Lines indicate level of transverse sections for hair cell counts as a percent from base to apex.

Samples of kidney tissue from each embryo were placed in 2% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 4 hours, then rinsed 3 times with 0.1 M phosphate buffer and stored in 0.1 M phosphate buffer at 4°C. Fixed renal tissue was then processed by standard procedures, mounted in paraffin and sections (7 μm) were stained with hematoxylin and eosin for routine evaluation.

Quantitative hair cell analysis at each of the three levels of the cochlea was completed by viewing each tissue section at a magnification of x1000. Tall and short hair cells were combined for total hair cell count. The following criteria were used to include hair cells in the count: presence of a well-formed cell body, extension of the cell to the cuticular plate, and identifiable stereocilia (Cruz et al., 1987). Three tissue sections/level were counted and the total number of hair cells per tissue section averaged for the mean hair cell count for each level.

Analysis of variance (completely random design) and least significant differences (posthoc) were used to evaluate for statistical differences ($p<0.05$) between the mean hair cell counts of the control and treatment groups.

In Trial 3, seventy-five fertile eggs were randomly divided into the following five groups: chick embryo,control; chick embryo, treatment; hatched chick, control; hatched chick, low-dose; and hatched chick, high-dose. Chick embryos received either deionized water (0.1 ml) or gentamicin (0.1 mg/egg) via an injection into the egg air cell on incubation

days 10 through 18. Hatched chicks were injected subcutaneously beginning one day after hatching for nine consecutive days with 0.1 ml of deionized water (control group), gentamicin at 5.0 mg/kg (low dose), or gentamicin at 100 mg/kg (high dose). Three randomly selected eggs or chicks were removed from each group one day after the 1st, 3rd, 5th, 7th, and 9th injections for the immunocytochemical evaluation of the basilar papilla. Trial 3 was terminated on day 19 of incubation for chick embryos and on posthatching day 10 for chicks.

Chick embryos were killed by decapitation and hatched chicks were anesthetized with CO_2 before decapitation. The basilar papilla was removed and processed as described above. Osmium tetroxide and uranyl acetate were omitted from the postfixation steps because of their potential adverse effects on cellular antigenic sites (Vardell and Polak, 1986). Immunocytochemical localization of gentamicin was determined using a commercial streptavidin-biotin system (Histostain-SP Kit, Zymed Laboratories Inc., South San Francisco, CA). Unstained sections of basilar papilla were etched for 3 minutes with sodium ethoxide (1:2 dilution) followed by three, 1 min, 100% ethanol rinses. Endogenous tissue peroxidase activity was then blocked with 3.0% H_2O_2 in water. Sections were rinsed with 0.05M Tris buffered saline (TBS), pH 7.2 at 24°C, and blocked with serum blocking solution for 10 min at room temperature (RT), and incubated with (1:300) goat whole antiserum to gentamicin (ICN Biomedical, Costa Mesa, CA)

diluted with 1% bovine serum albumin, 0.05M TBS, pH 7.2 at 4°C, for 14 h at 4°C in a humidified chamber. Sections were then washed twice for 7.5 min with TBS, and incubated for 10 min at RT with a biotinylated second antibody. Sections were then washed twice with TBS for 7.5 min and incubated for 5 min at RT with streptavidin-peroxidase conjugate. Sections were washed twice with TBS for 7.5 min and incubated for 5 min with the chromogen, 3,3 diaminobenzidine tetrahydrochloride (DAB) (Zymed Laboratories Inc., South San Francisco, CA). All sections were processed under identical conditions and treated with the same concentration of antibodies. Positive and negative control slides were included with each set of slides. Kidney from a hatched chick from the high-dose group was used as a positive control and kidney from a hatched chick from the control group was used as a negative control. The specificity of the anti-gentamicin antibody is shown in Figure 4. Absorption of anti-gentamicin antibody with an excess of gentamicin eliminated specific staining.

All sections of basilar papilla were viewed with an MTI Series 68 video camera mounted on a Nikon Microphot-FX microscope (Nikon Inc., Instrument Group, Garden City, NY) and the relative staining of DAB-stained sections of basilar papilla was analyzed with the aid of JAVA Image Analysis Software (Jandel Scientific, Corte Madera, CA). The entire basilar papilla bounded dorsally by the apical surfaces of hair cells and supporting cells, ventrally by the basilar membrane, and medially by the inner most tall hair cell border

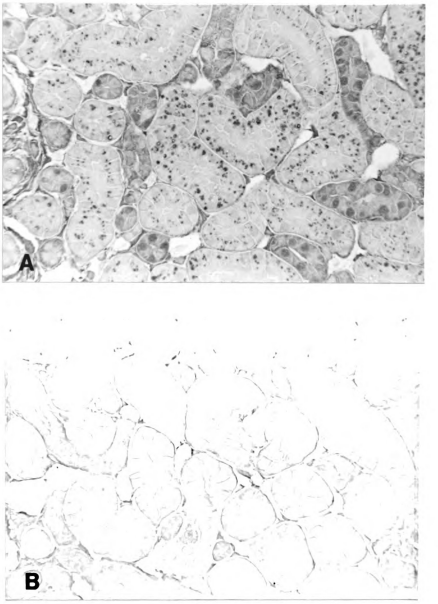


Figure 4. Photomicrograph of a section of kidney from a hatched chick treated for 9 days with gentamicin at 100 mg/kg with staining of gentamicin using the streptavidin-biotin method with DAB as the chromagen.

A. Note staining of tubular epithelial cells. x 544.

B. Same tissue section incubated with the gentamicin antibody preabsorbed with excess gentamicin. x 544.

was selected as the area of interest for image analysis. A mean integrated optical density (IOD) was determined for each section of basilar papilla by combining individual IODs from five randomly selected tissue sections on each slide. Each IOD was determined with reference to the average of the before and after measurements of the incident illumination. The integrated optical density is the sum of individual optical densities of each pixel in the area being measured (Joyce-Loebl, 1985).

The mean IOD from each basilar papilla from chick embryos or chicks in a treatment group (three total) were combined to determine the mean for each treatment or control group. Distribution of gentamicin to the basilar papilla of chick embryos and hatched chicks was examined by comparing the level of DAB staining versus time. The DAB staining level was quantified as the IOD. Student's T-test or analysis of variance (completely random design) and least significant differences (posthoc) were used to evaluate for statistical differences ($p<0.05$) between the mean hair cell counts of the control and treatment groups.

RESULTS

In Trial 1, no significant difference ($p<0.05$) was detected in hair cell counts of basilar papilla from chick embryos treated with gentamicin by different routes (Figure 5A). In Trial 2, there were no significant differences ($p<0.05$) in the hair cell counts of cochleas of chick embryos

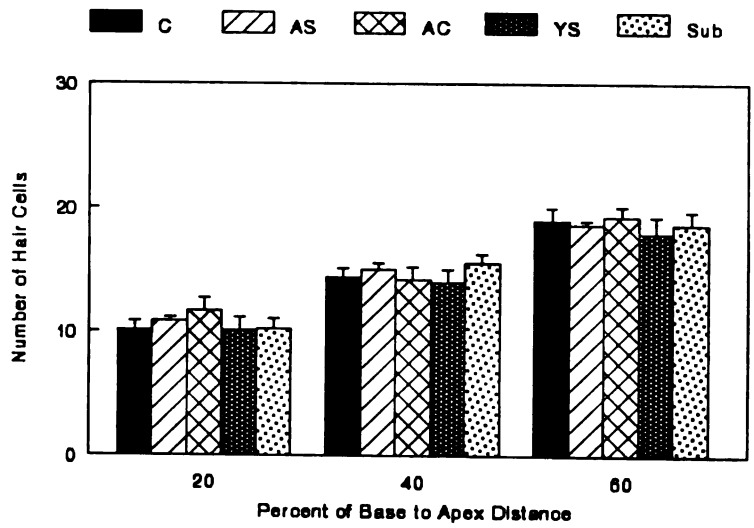
exposed to various ototoxic drugs (Figure 5B). Although no decrease in hair cell numbers was detected in chick embryos, mild to moderate renal tubular necrosis and mineralization were common findings in chick embryos receiving gentamicin, kanamycin, or streptomycin (Figure 6).

In Trial 3, after one injection, the basilar papilla had an increase in positive staining for gentamicin in all treatment groups (Figure 7A and B). However, this increased labeling did not become significant ($p < 0.05$) until after 3 successive doses. From the 3rd dose through the end of treatment (one day after the 9th dose), each treatment group had a significant ($p < 0.05$) accumulation of gentamicin in the basilar papilla (Figures 8 and 9). Accumulations of gentamicin within the basilar papilla continued to increase in all 3 treatment groups for the first 5 doses, then remained somewhat constant.

DISCUSSION

Marked hair cell loss can be produced by dosing newly hatched chicks with gentamicin (Cruz et al., 1987), kanamycin (Hashino et al., 1991), and streptomycin (Park and Cohen, 1982). Despite administration of ototoxic drugs by various routes, the chick embryo appears resistant to hair cell loss induced by aminoglycoside antibiotics and/or the loop diuretic ethacrynic acid. The insensitivity of the chick embryo to drug-induced ototoxic changes indicates that during the perinatal period, the chick cochlea undergoes a rapid

A.



B.

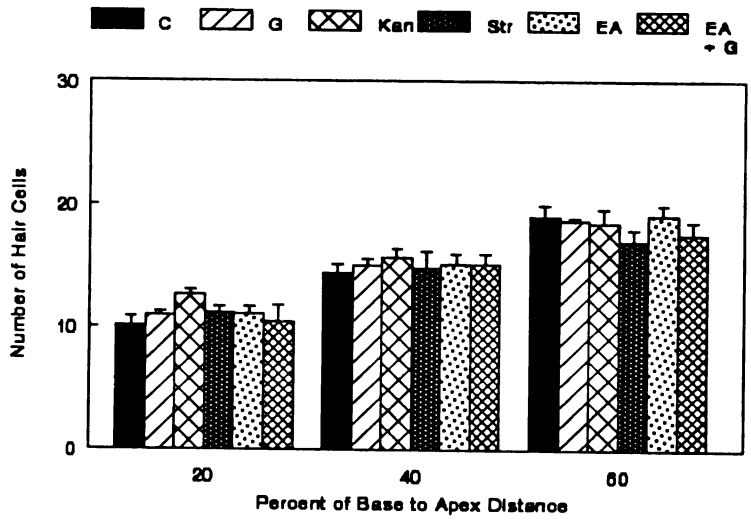


Figure 5. Effect of ototoxic drug treatment on mean (± standard deviation) hair cell numbers in 18-day old chick embryos.

A. Chick embryos exposed to gentamicin by injection into the air cell (AC), allantoic space (AS), yolk sac (YS), or by submersion (Sub). Control (C).

B. Chick embryos exposed by air cell injection to gentamicin (G), kanamycin (K), streptomycin (S), ethacrynic acid (EA), or ethacrynic acid and gentamicin combined (EA/G). Control (C).

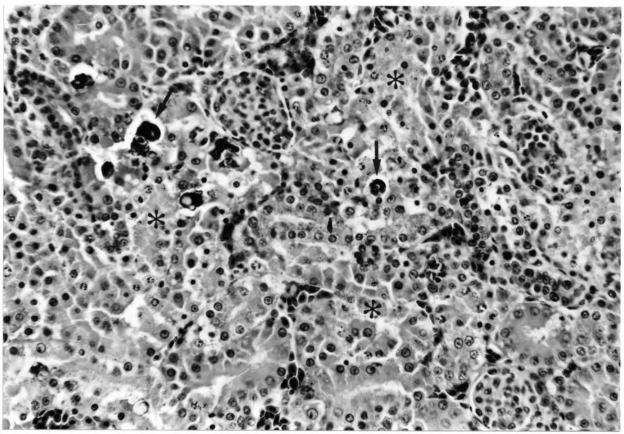
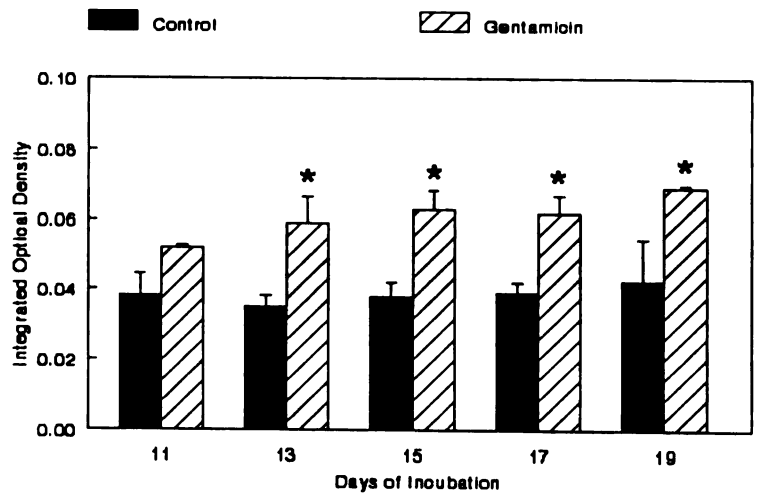


Figure 6. Photomicrograph of a section of kidney from a 19-day chick embryo treated with gentamicin by air space injection at 0.1 mg/egg/day on days 10 through 19 of incubation. Tubular epithelial necrosis (asterisks) and mineralization (arrows). Hematoxylin and Eosin, x 610.

A.



B.

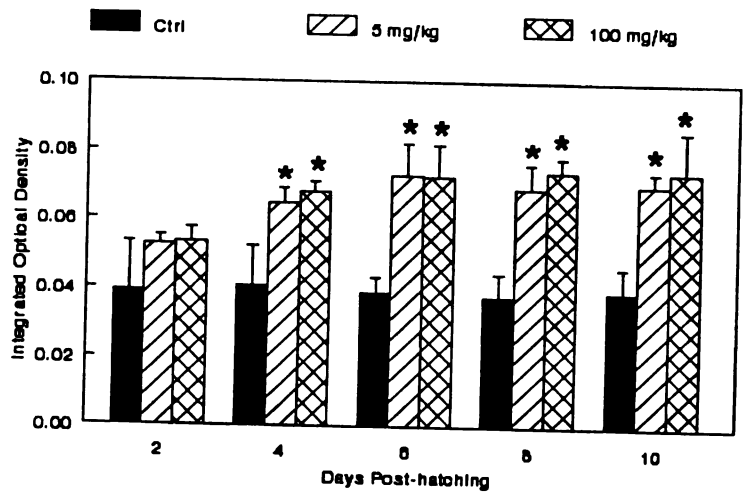


Figure 7. Relationship between integrated optical density (IOD) of basilar papilla sections stained for gentamicin and length of treatment. IOD level is directly related to staining intensity.

A. Chick embryos treated on days 10-18 of incubation with gentamicin or deionized water.

B. Hatched chicks treated on days 1-9 with gentamicin or deionized water.

* Signifies significant difference ($p<0.05$) from control.

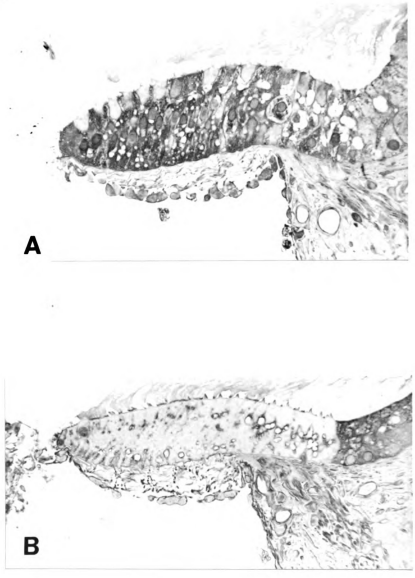


Figure 8. Photomicrograph of the basilar papilla of chick embryos treated with gentamicin for 5 consecutive days with staining of gentamicin using the streptavidin-biotin method with DAB as the chromogen.

A. Chick embryo, 0.1 mg/egg. Note diffuse staining of the basilar papilla. x 540.

B. Chick embryo control. Note absence of staining of the basilar papilla. x 540

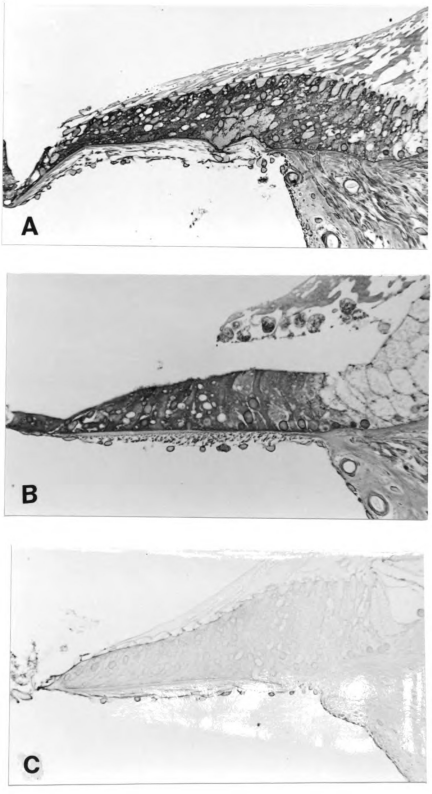
Figure 9. Photomicrographs of the basilar papilla of hatched chicks treated with gentamicin for 5 consecutive days with staining of gentamicin using the streptavidin-biotin method with DAB as the chromogen.

A. Hatched chick, 5 mg/kg. Notice diffuse staining of the basilar papilla. x 540.

B. Hatched chick, 100 mg/kg. Notice hair cell loss and diffuse staining of the basilar papilla and hair cell debris trapped in the tectorial membrane. x 540.

C. Hatched chick control. Notice absence of staining of the basilar papilla. x 540.

Figure 9



transition from a tolerant to a sensitive state.

A critical period of increased susceptibility to aminoglycoside ototoxicity during maturation of the cochlea has been shown to exist for guinea pigs (Uziel et al., 1979; Dumas and Charachon, 1982), rats (Osako et al., 1979; Carlier and Pujol, 1980; Lenoir et al, 1983; Marot et al., 1980), cats (Bernard, 1981), and mice (Henry et al., 1981). In mammals, this period and the onset and development of auditory function are closely related (Lenoir et al., 1983). The onset of cochlear function coincides with the last anatomical stage of maturation and involves mainly final maturation of outer hair cells, which are generally regarded as the most sensitive to aminoglycoside antibiotics (Pujol et al., 1978; Lenoir et al., 1980). The existence of an insensitive phase prior to onset of the critical period is controversial. Studies performed in vivo indicate that the rat cochlea is insensitive to kanamycin treatment between birth and postnatal day 10, followed by the critical period beginning on day 11 (Osaka et al., 1979).

The transition from an insensitive to sensitive period in postnatal mice is similar to that observed in this study as indicated by a rapid change from an insensitive chick embryo to a sensitive newly hatched chick. The chick critical period for the chick appears to be the first few days after hatching (Cruz et al., 1987; Hashino et al., 1991; Tucci and Rubel, 1990). Studies examining the onset and maturation of hearing in the chick embryo and hatched chick indicate that a progressive maturation of auditory response occurs between the

fifteenth day of incubation and the first few days post-hatching (Rebillard and Rubel, 1981; Saunders et al., 1973). As in mammals, drug-induced hair cell loss in the chick during the first several days after hatching does coincide with the period of final development and maturation of function of the cochlea.

The insensitivity of the chick embryo could be due to a lack of drug distribution to the basilar papilla. Indirect evidence of significant systemic distribution of gentamicin in the chick embryo after injection is denoted by renal tubular necrosis observed in chick embryos treated with gentamicin. This lesion was consistent with the nephrotoxic effects of aminoglycoside antibiotics (Laurent et al., 1990). In addition, immunocytochemical localization of gentamicin within the basilar papilla within 3 days indicates gentamicin is getting into the basilar papilla. It was not attempted in this study to relate the intensity of staining in the basilar papilla to an actual tissue concentration. If the basilar papilla of the chick embryo is accumulating gentamicin in concentrations comparable to those of the hatched chick from the high-dose group, then a lack of uptake into the hair cell or failure at a subsequent step in the mechanism of toxicity may be responsible for the insensitivity observed.

Immunocytochemistry has been utilized in the localization of gentamicin and kanamycin in the cochlea (Tachibana et al., 1984; Veldman et al., 1987; Yamane et al., 1988; de Groot et al., 1990). Gentamicin was detected in the guinea pig cochlea

within 3-5 days (Veldman et al., 1987; de Groot et al., 1990). This interval closely matches that in chick embryos and hatched chicks treated with gentamicin in this study. The trend of gentamicin starting to accumulate in the basilar papilla as soon as 24 hr after treatment in all 3 treatment groups is similar to the rapid distribution of kanamycin to the organ of Corti following intravenous administration (Yamane et al,. 1988). Kanamycin reaches the organ of Corti within 10 min via capillaries of the basilar membrane and spiral sulcus, and within 90 min by penetration through the perilymphatic space. The ventral surface of the egg air space is lined by the chorioallantoic membrane. This membrane is highly vascularized and drugs injected into the air space and subsequently absorbed, may quickly distribute systemically via the circulatory system of the embryo.

Specific labeling for gentamicin in the organ of Corti of guinea pigs included the outer and inner hair cells, as well as supporting cells such as Deiter's cells, Hensen's cells and the tympanic layer cells of the basilar membrane (de Groot et al., 1990. The pattern of gentamicin staining in this study certainly included supporting cells of the basilar papilla. Generally, hair cells did not appear to be labelled. To avoid interference with image analysis, tissue sections were not counterstained. This resulted in histologic detail not consistently allowing visualization of supporting cell borders. This precluded quantifying the lack of gentamicin staining in hair cells by image analysis.

The lack of morphologic alterations induced by aminoglycoside antibiotics in chick embryos of this study somewhat contrasts with the findings reported by Pickles et al. (1991). They reported a single dose of streptomycin injected in the center of an egg on days 7-11 of incubation resulted in 71% of embryos having hair cell morphologic changes. This compared to 18% affected on days 12-13 and none in those injected on days 14-15. Hair cells of chick embryos are clearly unambiguous by day 11 (Cohen and Fermin, 1978). Prior to this time, cytodifferentiation of the hair cell could be interfered with by a drug, resulting in a teratogenic (dysmorphogenic) effect rather than specific hair cell toxicity. Teratogenic effects have been produced in mouse cochlear explants exposed prior to the onset of morphogenesis on the 13th day of gestation (Anniko, 1981; Anniko and Nordemar, 1981). By 16 days of gestation, gross morphologic development has largely ended and exposure to drugs at this point resulted in selective hair cell toxicity.

In the hatched chick, high-dose group, severe hair cell loss began after only 3 injections. Embryos were treated with gentamicin for 6 additional days after significant amounts of gentamicin were detected in the basilar papilla. This suggests that if the chick embryos were within a period of sensitivity, adequate time was allowed for hair cell degeneration to be detected.

The chick embryo appears to be resistant to drug-induced ototoxicity and this resistance does not appear to be due to a lack of distribution of drug (gentamicin) to the cochlea. The following studies designed to examine the distribution of gentamicin within hair cells.

CHAPTER 2

ULTRASTRUCTURAL IMMUNOCYTOCHEMICAL LOCALIZATION OF GENTAMICIN AND PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE (PIP_2) IN THE COCHLEA OF THE CHICK EMBRYO AND THE NEWLY HATCHED CHICK

CHAPTER 2

ULTRASTRUCTURAL IMMUNOCYTOCHEMICAL LOCALIZATION OF GENTAMICIN AND PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE (PIP_2) IN THE COCHLEA OF THE CHICK EMBRYO AND THE NEWLY HATCHED CHICK

ABSTRACT

To study the insensitivity of chick embryos to aminoglycoside-induced ototoxicity, the uptake of gentamicin by cochlear hair cells in chick embryos and hatched chicks was examined. Because the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is critical in the mechanism of the ototoxicity, the intracellular distribution of gentamicin in relationship to PIP_2 in cochlear hair cells of chick embryos and hatched chicks was also examined. Chick embryos were dosed by air cell injection with gentamicin at 0.1 mg/egg/day during incubation days 10 - 18. Hatched chicks were dosed daily by subcutaneous injection with gentamicin at either 5.0 mg/kg (low-dose) or 100 mg/kg (high-dose) on post-hatching days 1 - 9. Ultrastructural immunocytochemical labeling for gentamicin and PIP_2 in hair cells was performed 1 day after the 1st, 3rd, 5th, 7th, and 9th doses of gentamicin. Mild, diffuse labeling for gentamicin was detected within endoplasmic reticulum of short and tall hair cells of chick

embryos after 7 days (incubation day 17). Moderate labeling of gentamicin was detected in hair cell lysosomes after 5 treatments in chicks receiving gentamicin at 5.0 mg/kg and after 1 treatment in hatched chicks receiving gentamicin at 100 mg/kg. These lysosomes were consistently located in the infracuticular region. There was an increase in the number of lysosomes with an occasional myeloid body coinciding with the accumulation of gentamicin within lysosomes in short and tall hair cells of chicks from both the low-dose and high-dose groups. Labeling for PIP_2 was detected in all chick embryos and hatched chicks in groups at each time point. There was consistent labeling of PIP_2 in the stereocilia, cuticular plate, chromatin, endoplasmic reticulum, cell walls, and tight junctions. Condensed PIP_2 labeling in lysosomes was infrequently observed and little variation in the amount was noted in tissue from treated chick embryos or chicks regardless of treatment group or treatment day. In hatched chicks from the high-dose group, short and tall hair cell loss was detected after 3 doses of gentamicin, and degeneration of dark cells in the tegmentum vasculosum was observed after 5 doses. No ultrastructural evidence of hair cell loss, hair cell degeneration, accumulation of gentamicin within lysosomes, or an increase in the numbers of lysosomes was detected in hair cells from chick embryos. Since PIP_2 and gentamicin were detected in the hair cells from chick embryos, but there was no evidence of drug-induced ototoxicity, the intracellular concentration of gentamicin was either

insufficient to induce toxicity or a step in the mechanism of toxicity is uncompleted.

INTRODUCTION

A period of insensitivity to aminoglycoside-induced ototoxicity has been reported in rats (Osako et al., 1979) and chick embryos (Fikes et al., 1992). This is followed by a sensitive period in both species (Osako et al., 1979; Carlier and Pujol, 1980; Cruz et al., 1987; Hashino et al., 1991; Park and Cohen, 1982) which coincides with the onset and maturation of hearing (Alford and Ruben, 1963; Bosher and Warren, 1971; Rebillard and Rubel, 1981; Saunders et al., 1973; Shnerson et al., 1982). In the mechanism of aminoglycoside-induced ototoxicity by proposed Schacht and Weiner (1986), aminoglycosides are taken into the hair cell by an active uptake process followed by the critical step of interacting with the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2). Gentamicin has been reported to be present in the basilar papilla of chick embryos by embryonic day (ED) 17 when treatment started on ED 10 (Fikes et al., 1992), but it is unknown whether gentamicin is actually taken into the hair cells. Aminoglycoside-induced ototoxicity may occur *in utero* in human beings (Scheinhorn and Angelillo, 1977) and animals (Akiyoshi, 1977), but if the mechanism of aminoglycoside tolerance observed in rat pups and chick embryos was understood, then *in utero* ototoxicity may be prevented. To determine the point at which the aminoglycoside

ototoxic mechanism of action may be uncompleted, the uptake of gentamicin into the hair cells of chick embryos and its intracellular distribution in relationship to PIP_2 was studied.

MATERIALS AND METHODS

Fertile White Leghorn chicken eggs (Michigan State University, Poultry Research and Teaching Center, East Lansing, MI) were incubated (Petersime Incubator Model 5, Petersime Incubator Co., Gettysburg, OH) at 37°C at 50% humidity. After hatching, chicks were housed in brooders (Petersime Brood-Unit, Petersime Incubator Co., Gettysburg, OH) at 30°C. Chemicals were obtained as follows: gentamicin sulfate, G-3632, Sigma Chemical Co., St. Louis MO; 8% EM grade glutaraldehyde, Electron Microscopy Sciences, Fort Washington, PA; protein A-gold (10 and 20 nm label), E Y Laboratories, San Mateo, CA; goat whole antiserum to gentamicin, ICN Biochemicals, Costa Mesa, CA; and anti-PIP_2 monoclonal antibody (Professor Tohru Yoshioka, Waseda University, Tokorosawa, Japan).

Seventy-five fertile eggs were randomly divided into the following five groups: chick embryo, control group; chick embryo, treatment group; hatched chick, control group; hatched chick, low-dose group; and hatched chick, high-dose group. Chick embryos received either deionized water (0.1 ml) or gentamicin (0.1 mg/egg) via an injection into the egg air cell on incubation days 10 - 18. Hatched chicks were injected

subcutaneously beginning one day after hatching for eight consecutive days with deionized water (control group), gentamicin at 5.0 mg/kg (low-dose group), or gentamicin at 100 mg/kg (high-dose group). Three randomly selected eggs or chicks were removed from each group one day after the 1st, 3rd, 5th, 7th, and 9th injections.

Hatched chicks were anesthetized with CO_2 before decapitation. The cochlea and a section of kidney were removed and fixed as previously described (Fikes et al., 1992) except post-fixation with osmium tetroxide and uranyl acetate was omitted to avoid an adverse effect on antigenicity (Vardell and Polak, 1986). Tissues were dehydrated with ethanol, transferred to propylene oxide, and infiltrated with epon/araldite resin and embedded in the same resin. After curing for 60 hr at 60°C, a transverse thick section (1 μm), perpendicular to the longitudinal axis of the cochlea, was cut along a plane approximately 30% of the total length from the base to apex of the basilar papilla. This section was used to assess cochlear morphology. This area should be central to that reported as showing hair cell loss in chicks treated with aminoglycosides (Cruz et al., 1987; Hashino et al., 1991; Park and Cohen, 1982). Hair cell counts were completed by viewing each tissue section at a total magnification of x1000. Tall and short hair cells were combined for total hair cell count. The following criteria were used to include hair cells in the count: presence of a well-formed cell body, extension of the cell to the cuticular plate, and identifiable stereocilia

(Cruz et al., 1987). Three tissue sections/slide were counted and the total number of hair cells per tissue section averaged for the mean hair cell count for each slide. Three slides/group were then used to calculate the group mean and standard deviation.

For ultrastructural immunocytochemistry, thin sections (70-90 nm) were cut and collected on thin-bar nickel grids. All sections were cut with a diamond knife on an LKB Ultrotome III (LKB-Produkter AB, Bromma, Sweden). Post-embedding immunocytochemistry (Roth et al., 1978; Bendayan, 1982; Bendayan et al., 1987) was performed by floating grids on droplets of immunoreagents and buffers placed on strips of Parafilm (American Can Co., Greenwich, CT).

An indirect, four-step method was used to double label for PIP_2 and gentamicin simultaneously. In order to verify the somewhat arbitrary labeling of gentamicin in chick embryos with the double label technique, a single label method using a small (10 nm) label was used on the chick embryo tissue. In both techniques, 0.05M tris buffered saline (TBS) and 0.05% tween, 0.05M tris buffered saline (TTBS) were adjusted to pH 7.2 for use at the working temperature of either 4°C or 24°C (room temperature). For the indirect two-step method, grids were etched face down for 5 min with saturated sodium metaperiodate solution, rinsed with deionized water and pretreated for 30 min with 1% gelatin-TTBS. Each grid was transferred to a drop of gentamicin antiserum (1:450) in 1% BSA-TBS, for 16 hr at 4°C. After washing with TTBS, grids

were treated for 5 min with 1% gelatin-TTBS, followed by 30 min treatment with protein-A gold (10 nm) in TTBS. Each grid was rinsed with TTBS and deionized water. The sections were then stained with uranyl acetate and lead citrate and examined in a Philips 301 transmission electron microscope (Philips Electronic Instruments Co., Pittsburg, PA).

For double labeling, PIP_2 was labeled first by floating grids face up for 5 min with saturated sodium metaperiodate solution to etch, rinsed with deionized water and pretreated for 30 min with 1% gelatin-TBS. Grids were then floated face up on a drop of anti-PIP_2 monoclonal antibody (IgG_3, 0.05 ug/ml) in 1% BSA-TBS for 24 hr at 4°C. After washing with TBS, grids were treated for 5 min with 1% gelatin-TBS, and for 30 min with protein-A gold (10 nm) in TBS. Each grid was rinsed with TBS and deionized water. For labeling gentamicin, the grids already labeled on the face up side for PIP_2 were turned over and treated face down as described above for the indirect two-step method for gentamicin using a protein A-gold, 20 nm label, instead of 10 nm label. Following double labeling, sections were stained with uranyl acetate and lead citrate and examined.

Sections of kidney from chicks from the high-dose group which were given 9 doses were used as positive controls for gentamicin (Figure 10). The specificity of the anti-gentamicin antibody following absorption with gentamicin is shown in Figure 11. Nonspecific binding of the antigentamicin antibody was quite low (Figure 12).

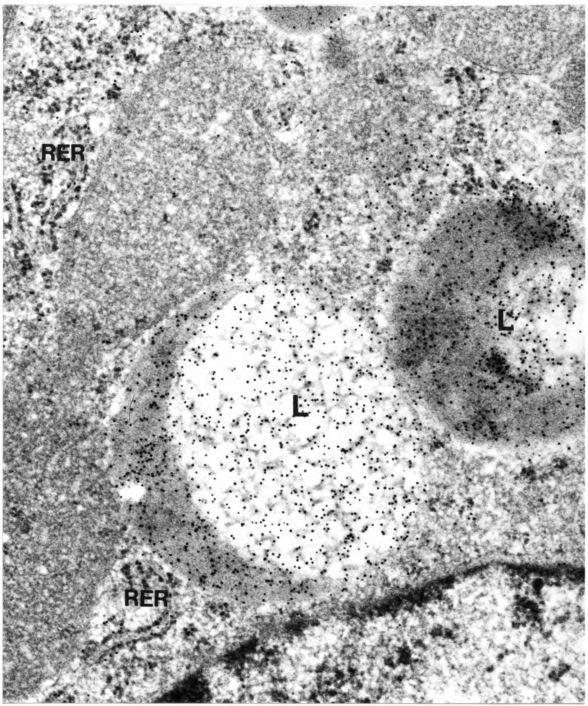


Figure 10. Transmission electron micrograph of a gentamicin immunolabeled section of kidney from a 10-day-old hatched chick after 9 days of treatment with gentamicin at a dose of 100 mg/kg. Labeling for gentamicin is restricted to lysosomes (L) and rough endoplasmic reticulum (RER). x 65,000.

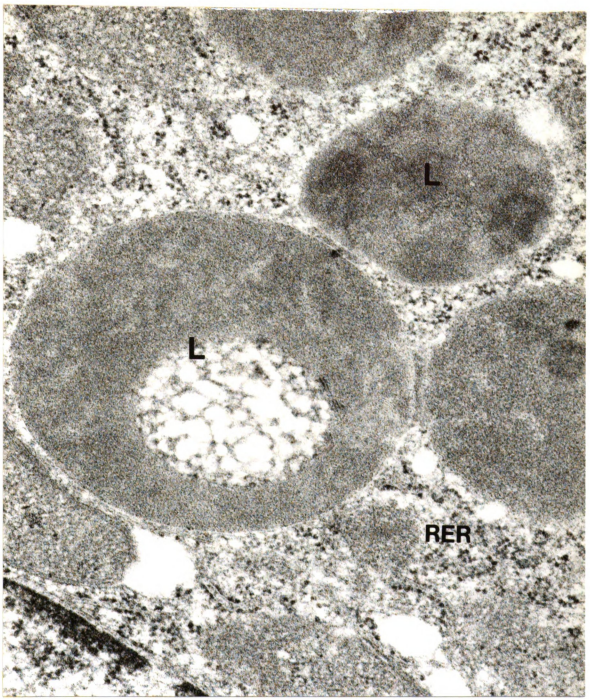


Figure 11. Transmission electron micrograph of a gentamicin immunolabeled section of kidney from a 10-day-old hatched chick after 9 days of treatment with gentamicin at a dose of 100 mg/kg following preincubation of anti-gentamicin serum with gentamicin. Same section as Figure 10. Note absence of staining of lysosomes (L), rough endoplasmic reticulum (RER). x 65,000.

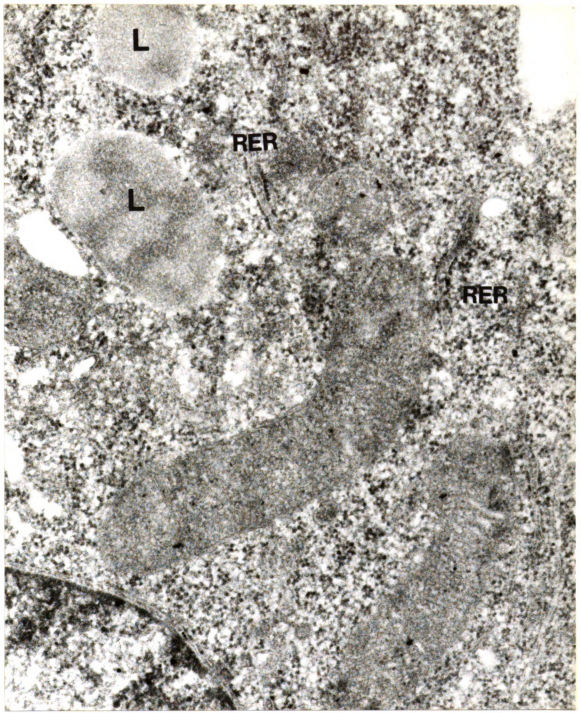


Figure 12. Transmission electron micrograph of a gentamicin immunolabeled section of kidney from a 10-day-old hatched chick, negative control, after 9 days of treatment with deionized water at 0.1 ml/day. No staining of lysosomes (L) and rough endoplasmic reticulum (RER). x 65,000.

The specificity of labeling with anti-PIP_2 antibody was demonstrated by an absorption test with inositol 1-monophosphate (IP_1), inositol 1,4-bisphosphate (IP_2), and inositol 1,4,5-triphosphate (IP_3). Since the epitopes of the anti-PIP_2 antibody are phosphate groups substituted at the 4- and 5-positions of the inositol head of PIP_2 (Miyazawa et al., 1988), IP_3 can act as an absorber for this antibody (Ito et al., 1991). Labeling of a negative control chick hair cell cuticular plate and stereocilia is demonstrated in Figure 13. When anti-PIP_2 antibody was absorbed with an excess of IP_3, immunolabeling was markedly abolished (Figures 14, 15). Preincubation of anti-PIP_2 antibody with IP_1 and IP_2 had no effect on labeling.

RESULTS

Labeling for gentamicin with the double labeling technique was negative through the first 5 days of treatment and equivocal after 7 and 9 days (Figure 16), but using the single labeling technique, gentamicin was detected in small amounts within short and tall hair cells of chick embryo cochlea after 7 injections (Figure 17). In hatched chicks from the low-dose group, there was an accumulation of gentamicin label within lysosomes which was detected after 5 injections (Figure 18). In the tissues from chicks in the high-dose gentamicin group, gentamicin was detected within lysosomes after one treatment (Figure 19). Gentamicin was rarely detected in mitochondria, golgi, or endoplasmic

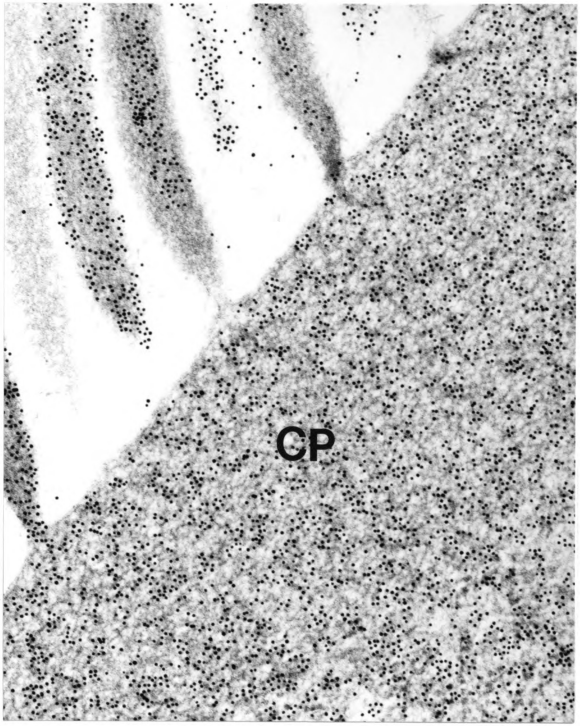


Figure 13. Transmission electron micrograph of a PIP_2 immunolabeled section of a tall hair cell from a 6-day-old hatched chick, negative control, cochlea after 5 days of treatment with 0.1 ml deionized water/day. Labeling for PIP_2 is restricted to stereocilia and cuticular plate (CP). x 88,400.

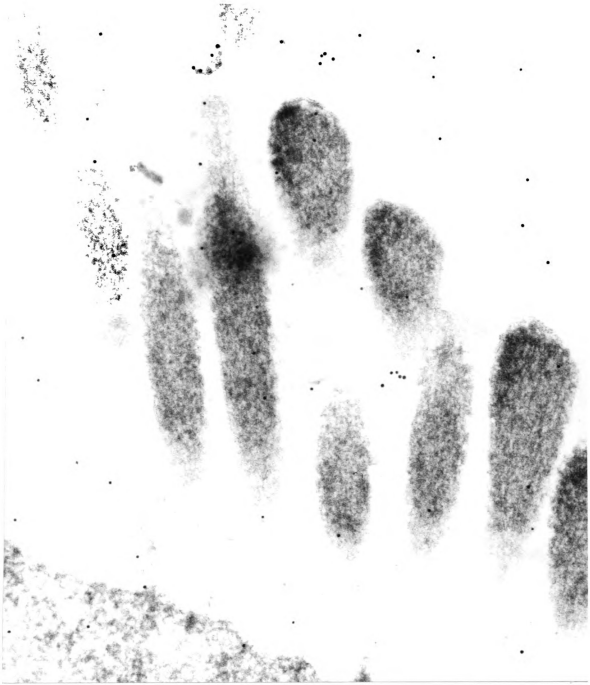


Figure 14. Transmission electron micrograph of a PIP_2 immunolabeled section of tall hair cell stereocilia from a 6-day-old hatched chick, negative control, cochlea after 5 days of treatment with 0.1 ml deionized water/day following preincubation of anti-PIP_2 antibody with an excess of IP_3. Same section as Figure 13. Note marked elimination of labeling of stereocilia. x 88,400.

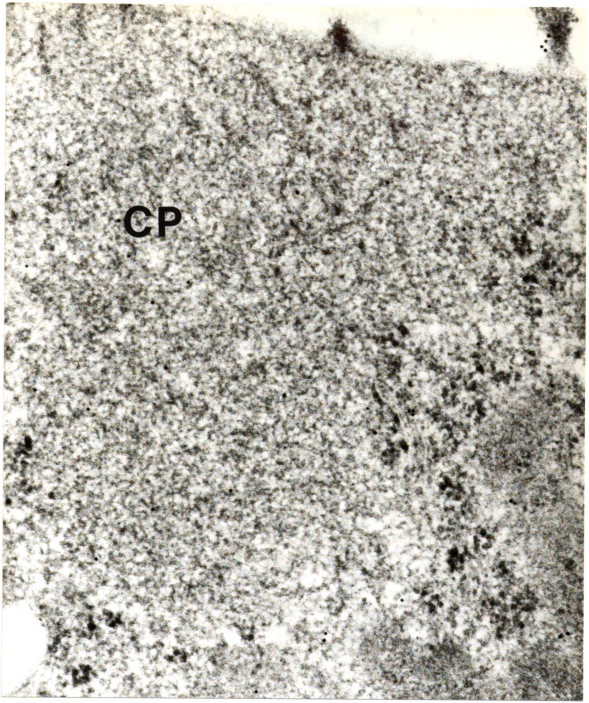


Figure 15. Transmission electron micrograph of a PIP_2 immunolabeled section of a tall hair cell cuticular plate (CP) from a 6-day-old hatched chick, negative control, cochlea after 5 days of treatment with 0.1 ml deionized water/day following preincubation of anti-PIP_2 antibody with an excess of IP_3. Same section as 13. Note marked elimination of labeling of cuticular plate. x 88,400.

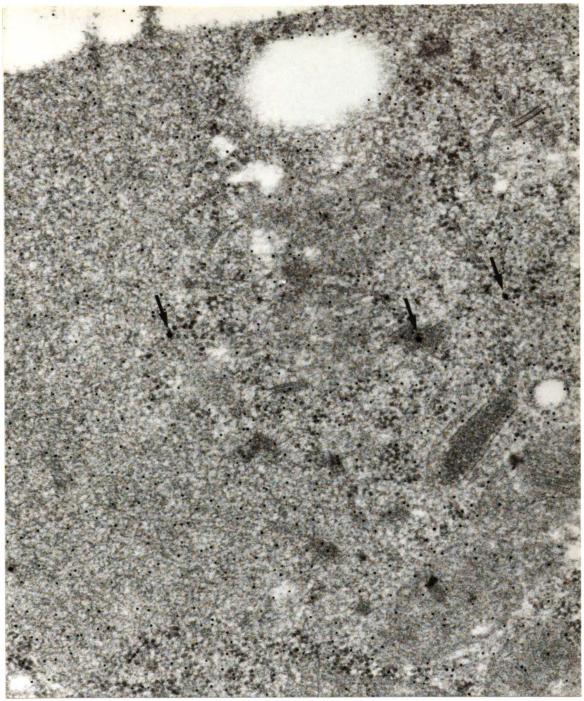


Figure 16. Transmission electron micrograph of a double immunolabeled section of a short hair cell from a chick embryo on incubation day 17 after 7 days of gentamicin treatment at a dose of 0.1 mg/egg. Notice diffuse labeling of PIP_2 with small label (10 nm), only equivocal labeling (arrows) of gentamicin with large label (20 nm). x 65,000.

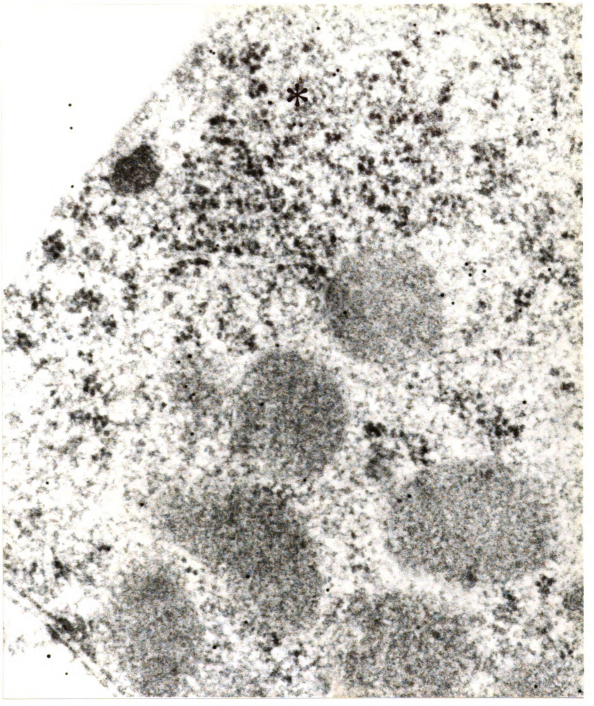


Figure 17. Transmission electron micrograph of a gentamicin immunolabeled section of a short hair cell from a chick embryo on day 17 of incubation after 7 days of gentamicin treatment at a dose of 0.1 mg/egg. Label (10 nm) for gentamicin in areas of rough endoplasmic reticulum (asterisk). x 88,400.

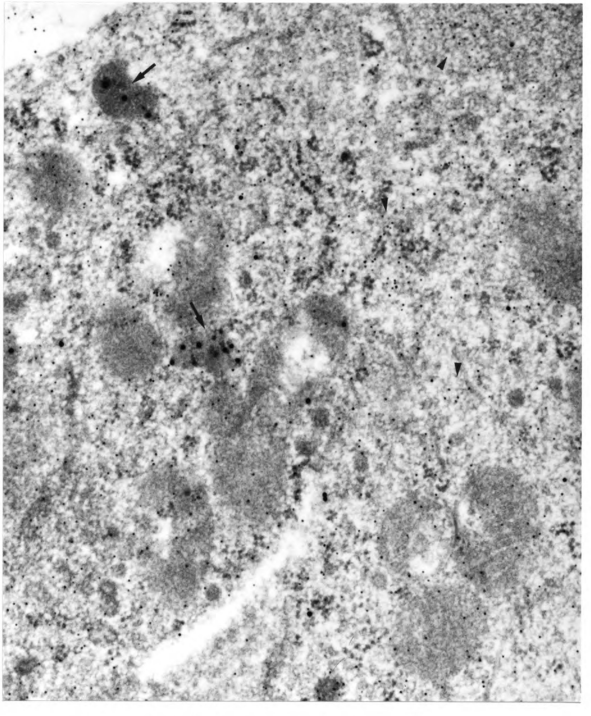


Figure 18. Transmission electron micrograph of a double immunolabeled section of a tall hair cell from a 6-day-old hatched chick after 5 days of gentamicin at 5.0 mg/kg. PIP_2 stained with small label (10 nm) and gentamicin stained with large label (20 nm). Note gentamicin staining within lysosomes (arrows). Diffuse PIP_2 staining (arrowheads) over cytoplasm. x 65,000.

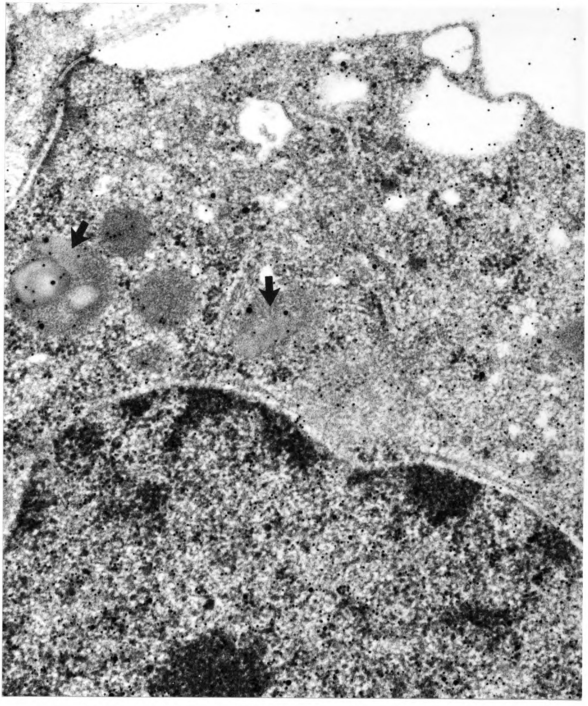


Figure 19. Transmission electron micrograph of a double immunolabeled section of a short hair cell from a 2-day-old hatched chick after 1 day of gentamicin treatment at 100 mg/kg. PIP_2 stained with small label (10 nm). Gentamicin stained with large label (20 nm). Note gentamicin accumulated within lysosomes (arrows). Some labeling of PIP_2 within lysosomes. x 65,000.

reticulum of the hair cells.

Label for anti-PIP_2 was detected in hair cells from chick embryos and from hatched chicks (Figures 17, 18, 19). In hair cells of chick embryos, PIP_2 was present in stereocilia, cuticular plate, nucleus, endoplasmic reticulum, cell wall and tight junctions. PIP_2 labeling within lysosomes was variable. Occasionally the PIP_2 labeling was concentrated (Figure 20). The distribution of PIP_2 within hair cells of chicks from the low- and high-dose gentamicin groups was essentially the same as described for the chick embryos.

Hair cell loss was detected in the hatched chick, high-dose group after three injections of gentamicin (Table 3). Once hair cell loss occurred, it did not appear to increase in severity over the remainder of the study. Both short and tall hair cells were lost, frequently leaving only an occasional hair cell (Figure 21). Extruded hair cells and cellular debris were located within and beneath the tectorial membrane (Figure 22). Intranuclear pseudoinclusions characterized by margination of chromatin and condensation of chromatin into focal aggregates were present in dark cells of the tegmentum vasculosum of chicks after 5 injections (Figure 23). This degenerative change was progressive and karyolysis was detected in chicks after 9 injections. No histologic evidence of hair cell degeneration or loss was detected in chick embryos from the control or treatment groups or in the hatched chicks from the control or low-dose groups.

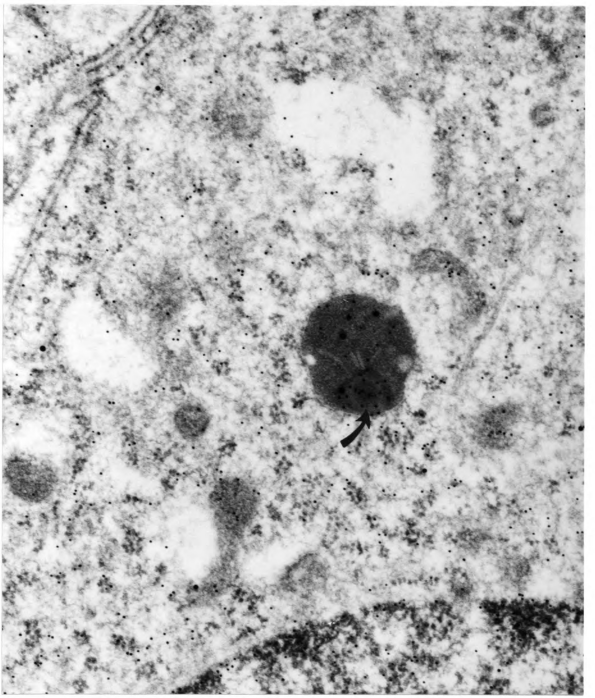


Figure 20. Transmission electron micrograph of a double immunolabeled section of a short hair cell from an 8-day-old hatched chick after 7 days of gentamicin treatment at a dose of 100 mg/kg. PIP_2 stained with small label (10 nm) and gentamicin stained with large label (20 nm). Labeling for gentamicin is present within lysosome. Focal area of labeling for PIP_2 within lysosome (arrow). x 65,000.

Table 3. Mean (± Standard Deviation) cochlear hair cell count at 30% of base to apex distance in [a]chick embryos and [a]hatched chicks treated with gentamicin.

Days of Gentamicin	Chick Embryo [b]Treatment	Chick Embryo [c]Control	Hatched Chick [d]Low-Dose	Hatched Chick [e]High-Dose	Hatched Chick [f]Control
1	13.00 ± 0.9	13.78 ± 0.8	12.67 ± 1.2	12.89 ± 1.0	13.24 ± 1.1
3	13.67 ± 0.6	13.24 ± 1.1	14.33 ± 0.9	2.67 ± 3.8	12.71 ± 1.4
5	13.55 ± 1.3	12.89 ± 1.3	13.00 ± 2.0	1.67 ± 1.2	13.67 ± 0.7
7	12.56 ± 1.1	13.56 ± 0.9	12.22 ± 1.4	3.17 ± 0.7	14.37 ± 1.6
9	13.00 ± 1.3	12.38 ± 1.7	12.56 ± 0.8	1.20 ± 2.3	14.04 ± 2.2

[a] Three/group
[b] 0.1 mg gentamicin/egg/day.
[c] 0.1 ml deionized water/egg/day.
[d] 5.0 mg gentamicin/kg/day.
[e] 100 mg gentamicin/kg/day.
[f] 0.1 ml deionized water/day.

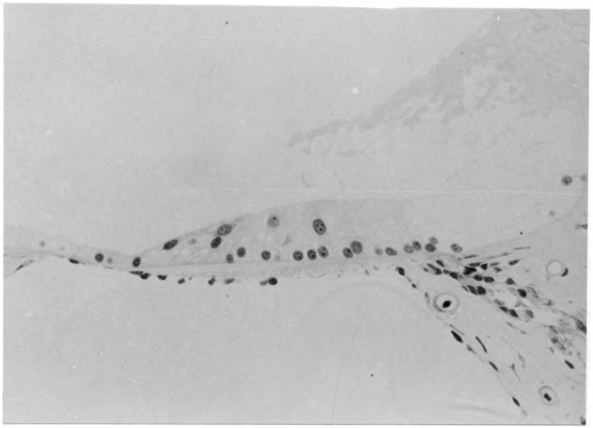


Figure 21. Photomicrograph of a section of basilar papilla from a 4-day-old hatched chick treated with gentamicin at 100 mg/kg for 3 days. Notice loss of short and tall hair cells (arrows). Toluidine blue. x 830.

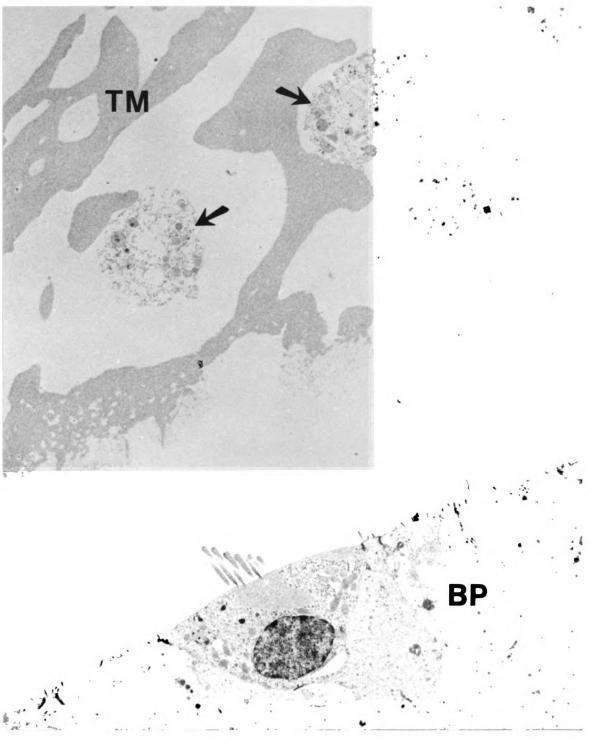


Figure 22. Transmission electron micrograph of a section of basilar papilla from a 4-day-old hatched chick treated with gentamicin at 100 mg/kg for 3 days . Basilar papilla (BP) and tectorial membrane (TM). Extruded, degenerating hair cells (arrows) trapped in the tectorial membrane. x 6,500.

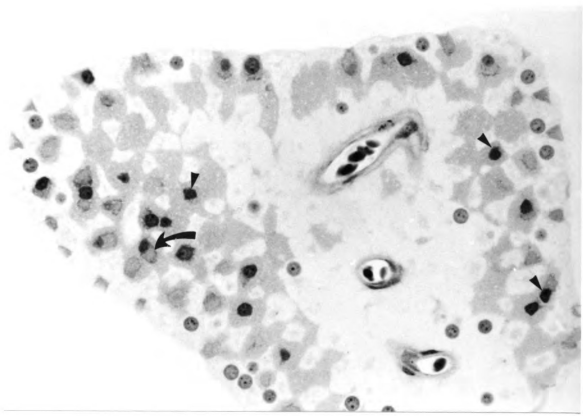


Figure 23. Photomicrograph of a section of tegmentum vasculosum from an 8-day-old hatched chick cochlea after gentamicin at 100 mg/kg for 7 days. Note pyknotic nuclei (arrowheads) and pseudoinclusion formation (arrow) in dark cells. Note adjacent normal appearing light cells. Toluidine blue. x 736.

DISCUSSION

The results of labeling gentamicin in the cochlea of chick embryos after 7 injections indicates some gentamicin is being taken into the cell. It was located in endoplasmic reticulum and other ribosomal dense areas which is consistent with the hypothesized intracellular movement of gentamicin before being concentrated into lysosomes (de Groot et al., 1990).

The accumulation of gentamicin within lysosomes of hair cells in this study is similar to the rapid incorporation into lysosomes seen in the kidney (Silverblatt and Kuehn, 1979). The accumulation of gentamicin within lysosomes occurred in conjunction with an increase in number of lysosomes and the formation of myeloid bodies. Not every lysosome labeled for gentamicin. This could be due to an increase in number of lysosomes as a cellular response to the toxic effects of gentamicin and not simply a mechanism to sequester the drug. The increase in the number of lysosomes is attributed to a drug-induced block in lysosomal phospholipid catabolism with accumulation of phospholipids (phospholipidosis) within lysosomes (Laurent et al., 1990). The increased number of lysosomes observed in hair cells were primarily restricted to the infracuticular region. Gentamicin-labeled lysosomes were always in this area, consistent with the findings of de Groot et al. (1990). In the hatched chick groups, labeling of gentamicin in lysosomes was generally equal between short and tall hair cells. Distribution of gentamicin to both inner and

outer hair cells of guinea pigs has also been reported (Hayashida, 1989). This suggests that a differential uptake between hair cell types does not account for previously observed increased sensitivity of outer hair cells.

Studies have shown that before overt renal tubular necrosis, the aminoglycosides are almost exclusively associated with lysosomes (Josepovitz et al., 1985; Giurgea-Marion et al., 1986), which act as the major intracellular storage site for gentamicin in spite of numerous changes in cell homeostasis (Giurgea-Marion et al, 1986). Although both hatched chick groups demonstrated ultrastructural evidence of gentamicin accumulation in lysosomes and phospholipidosis, only the high-dose group had hair cell loss. This may be explained by the premise that a critical threshold of phospholipid accumulation needs to be reached before it leads to cell death (Giuliano et al., 1984). This is supported in this study by the subjective observation of a larger number of lysosomes appearing over time in the hair cells of chicks from the high-dose group than those in the hair cells of chicks from the low-dose group. Although many phospholipids increase in conjunction with the phospholipidosis, apparently PIP_2 does not change during the early manifestations of nephrotoxicity (Feldman et al., 1982). Since focal increased labeling of lysosomes or the presence of myeloid bodies with anti-PIP_2 was rarely observed in this study, PIP_2 apparently does not change during the early manifestations of ototoxicity in hatched chicks.

Phosphatidylinositol 4,5-bisphosphate (PIP_2) was present in the youngest chick embryos of this study and was probably not a limiting factor in the production of ototoxic effects in the chick embryos. The similar labeling intensity of cochleas between chick embryos and hatched chicks is consistent with the finding that the turnover of PIP_2 within the cochlea is similar in fetal rats as adults (Anniko and Schacht, 1981).

Hair cell loss after 3 injections in the hatched chicks from the high-dose group was slightly earlier than the 8-10 days reported in several studies (Cruz et al., 1987); Lippe, 1991). This could be due to the higher doses of gentamicin (100 mg/kg) used in this study, in contrast to gentamicin at 50 mg/kg/day used by Cruz et al. (1987). This study also included an earlier time point than most studies.

The differences observed between the single and double labeling techniques for gentamicin in chick embryos of this study indicates the immunocytochemical techniques used may have limited sensitivity. The detection of gentamicin in the endoplasmic reticulum of hair cells by immunocytochemical methods was rare (de Groot et al., 1990) and was attributed to drug concentrations being below that which could be detected by the method. In the present study, the differences in sensitivity may be due to the different sizes of labels used to detect gentamicin in the two techniques. Smaller gold particles allow higher labeling densities and improved sensitivity (Horisberger, 1981; Lackie et al., 1985). The higher labeling density results from the greater accessibility

of the smaller particles to binding sites and lower steric interference as compared to larger gold particles. Larger particles give a lower staining density than smaller ones because of steric hinderance, lower mobility of large particles, and higher mutual electric repulsion of large particles (Doerr-Schott, 1989, Park and Park, 1989).

From the present study, it appears that gentamicin is getting into the hair cells of chick embryos near the end of the dosing schedule, but there was no evidence of ototoxicity, such as hair cell loss, increase in lysosome numbers, or accumulation of gentamicin in lysosomes. This raises several possibilities for explaining the insensitivity of the chick embryo to aminoglycoside-induced ototoxicity. Gentamicin uptake into hair cells had only begun 2 days prior to the end of this study indicating that the energy-dependent uptake mechanisms may not yet be fully functional. A second possibility is that once gentamicin is taken into the cell, bioactivation of gentamicin may not occur due to oxidative enzyme immaturity. It is generally regarded that the aminoglycosides are not metabolized (Laurent et al., 1990), and are therefore nephrotoxic and ototoxic as the parent compound. This tenet, however, may be incorrect in that recently, an oxidative metabolite was shown to be cytotoxic (Huang and Schacht, 1990). As a third possibility, gentamicin may be taken into the hair cell, but fail to complex with PIP_2.

In summary, this study demonstrated the presence of gentamicin within hair cells of chick embryos by day 17 of incubation, following 7 days of treatment. This was later than that of hatched chicks and no accumulation within lysosomes was detected. By day 11 of incubation, PIP_2 was present in the hair cells of chick embryos, suggesting it may not be the limiting factor in the chick embryo's insensitivity to aminoglycosides. However, the interaction of gentamicin with PIP_2 once it was taken into the cell still needs to be determined.

CONCLUSIONS AND FUTURE STUDIES

CONCLUSIONS AND FUTURE STUDIES

These studies were originally initiated in an attempt to use the chick embryo in screening tests for ototoxic compounds. Support for this hypothesis came from studies utilizing chick embryos in alternative "in vitro" ocular irritation tests instead of using rabbits in the traditional Draize test (Leighton et al., 1985) and studies demonstrating aminoglycoside antibiotics produce cochlear and vestibular hair cell loss in newly hatched chicks (Cruz et al,. 1987; Duckert and Rubel, 1990; Hashino and Sokabe, 1989; Hashino et al., 1991; Park and Cohen, 1982; Tucci and Rubel, 1990). During preliminary studies, it became apparent that chick embryos were tolerant to aminoglycoside-induced ototoxicity. With this background, the present research was directed toward verifying the insensitivity of the chick embryo to drug-induced ototoxicity and to begin a systematic characterization to understand this phenomenon.

The chick embryo is not a good animal to use in a screen for ototoxic compounds because of this insensitivity to the aminoglycosides and a loop diuretic (ethacrynic acid). This conclusion is based on the following three results described

in Chapters 1 and 2:

(1) Gentamicin is taken into the hair cell of chick embryos late in the treatment period (ED 17). The intensity of gentamicin labeling within short and tall hair cells was light and dispersed within areas of endoplasmic reticulum.

(2) No ultrastructural or histologic evidence of hair cell loss is detected in chick embryos treated with aminoglycoside antibiotics or aminoglycosides potentiated with a loop diuretic.

(3) No ultrastructural evidence of aminoglycoside-induced phospholipidosis was observed in chick embryo hair cells.

Additional noteworthy results described in Chapters 1 and 2 include:

(4) In hatched chicks, cochlear hair cell loss began after 3 treatments with gentamicin at 100 mg/kg.

(5) In hatched chicks treated with nontoxic and ototoxic doses of gentamicin, gentamicin labelling appeared as focal accumulations within lysosomes in the infracuticular region of short and tall hair cells, An increase in total number of lysosomes, both with and without gentamicin label, occurs in the infracuticular region.

(6) Gentamicin is distributed to the basilar papilla of the chick embryo after 3 days of treatment (ED 13). The rate of gentamicin appearance and its pattern of distribution in the basilar papilla of the chick embryo is similar to that of hatched chicks exposed to ototoxic doses of gentamicin.

This research has provided fundamental information regarding the basis of the insensitivity of chick embryos to ototoxicants, but three particular areas of research should be pursued.

First, quantitation over time of aminoglycoside drug (i.e. gentamicin) concentration in the basilar papilla in chick embryos compared to the quantitation over time of concentration in the basilar papilla in hatch chicks treated with ototoxic doses of drug would provide some direct information on the pharmacokinetics of drug movement in the chick embryo cochlea. Although gentamicin is distributed to the basilar papilla of chick embryos early in the dosing period (ED 13), the amount relative to that delivered to the basilar papilla of chicks receiving ototoxic doses is unknown. In the present studies, the IODs of the basilar papilla were similar between chick embryos treated with gentamicin at 0.1 mg/egg and hatched chicks treated with gentamicin at 5 and 100 mg/kg. A direct correlation between staining intensity and drug concentration would indicate that the concentration of gentamicin is similar in the basilar papilla of all three of these treatment groups. This also suggests that delivery of an aminoglycoside to the basilar papilla has little correlation with subsequent ototoxicity. Determination of this would provide some direct information on the pharmacokinetics of drug movement in the chick embryo cochlea. Although the basilar papilla of the chick embryo stained for gentamicin, the actual concentration in the structure may not

be high enough to serve as a pool for delivery to the hair cells.

A second area of research is to more clearly define the "width" of the window of transformation from an ototoxicant-tolerant state to a sensitive state. This could be investigated by sequentially moving the dosing period forward from one day after hatching into the embryonic period. The present work identified a tolerant state, but did not establish an incremental conversion to the sensitive state. If an incremental process is identified, later studies could be concentrated on exactly what unique events are occurring within that specific time frame.

A third area of future research is the examination of concentrations of gentamicin within isolated hair cells from in vivo treated chick embryos and hatched chicks. The present study demonstrated that hair cells from chick embryos began taking up gentamicin late in the treatment period (ED 17). However, the relevance of the amount identified is unknown. Comparison over time of drug concentration in the hair cells of chick embryos to that in hatched chicks given an ototoxic dose would provide some indication of the pharmacodynamics at the cellular level. Aminoglycosides may enter into the hair cell apical surface from the endolymph or the path of aminoglycosides into the hair cell may involve supporting cells, which may be a limiting factor. Regardless of the route of drug passage to the hair cell, the entire insensitive state in the chick embryo could be due to insufficient drug

uptake by a transiently immature transport mechanism, preventing intracellular cytotoxic accumulations.

Also, more general questions to consider in planning future studies include: are aminoglycoside insensitive hair cells also insensitive to noise-induced trauma and can hair cells sensitive to aminoglycosides be made insensitive? Before pursuing these questions, a better understanding of the mechanism of insensitivity would be helpful. However, concurrent investigation of the insensitivity to aminoglycoside-induced ototoxicity and these general issues may simultaneously provide information on the mechanism of the insensitivity and application of the knowledge in a more general situation.

Understanding the insensitivity of the chick embryo to aminoglycoside-induced ototoxicity is important from the aspect of what it offers in decreasing the incidence of this therapy limiting side effect. Also, since ototoxicity can be the limiting factor in the use of aminoglycosides, a means to avoid this may allow enhanced use of aminoglycosides in critical infectious disease cases.

Although the chick embryo is not a good model for screening for ototoxicity, the newly hatched chick does make a suitable model. At one to two weeks of age, the chick inner ear is still easily dissected. Literature currently exists on the sensitivity of chicks to aminoglycoside-induced ototoxicity and noise-induced trauma which supports the concept of using chicks as a screening test for ototoxicants.

This system would require further validation with other ototoxicants such as loop diuretics (ethacrynic acid), antineoplastic agents (cisplatin), nonsteroidal antiinflammatory drugs (aspirin), and antimalarials (quinine). The effects of these drugs in human beings is well characterized and similar results would be desired in the chick if it were to be used in an ototoxicity screen. Use of the adult chicken in otologic research is rarely reported in the literature. Although adult chickens may have similar sensitivities as young chicks to ototoxicants, the increased logistics of maintaining adult chickens and increased difficulty in dissecting and processing the inner ear tissue make it unlikely that adult chickens would be advantageous over chicks as an ototoxicity screen.

APPENDIX

APPENDIX

INTRODUCTION

Information regarding the toxicity of aminoglycoside antibiotics and loop diuretics is generally unavailable. It was therefore necessary to perform a series of studies to determine the lethal and maximum tolerated doses of the drugs using chick embryos for the studies described in Chapters 1 and 2.

MATERIALS AND METHODS

Fertile White Leghorn chicken eggs (Michigan State University, Poultry Research and Teaching Center, East Lansing, MI) were incubated (Petersime Incubator Model 5, Petersime Incubator Co., Gettysburg, OH) at 37°C with 90% humidity. Drugs were obtained as follows: gentamicin sulfate (G-3632, Sigma Chemical Co., St. Louis, MO); streptomycin sulfate (S-6501, Sigma Chemical Co., St. Louis, MO); kanamycin sulfate (Kantrim, 200 mg/ml, Fort Dodge Laboratories, Inc., Fort Dodge, IA); ethacrynate sodium (Sodium Edecrin, 50mg, Merck, Sharp, and Dohme, West Point, PA).

Fertile eggs were exposed to the aminoglycoside antibiotics gentamicin, kanamycin, and streptomycin and/or the loop diuretic, ethacrynic acid. Drugs were administered by the routes, time periods, and dose ranges listed in Table 4. For each trial, eggs were candled one to two times per day to assess embryo viability.

Table 4. Routes of administration, dose periods, and dosages of drugs used in dose response studies in chick embryos.

Drug	Route	Dose Period	Dosage
Gentamicin	Air Cell	ED 10 - 17	0.01 - 40 mg/egg/day
	Air Cell	ED 10 - 14	0.01 - 0.5 mg/egg/day
	Air Cell	ED 15 - 19	0.05 - 1.0 mg/egg/day
	Allantoic Space	ED 10 - 17	1.0 - 5.0 mg/egg/day
	Yolk Sac	ED 10 - 17	1.0 - 5.0 mg/egg/day
	Submersion at 5°C for 10 minutes	ED 10 - 15	2.5 - 25.0 mg/ml
Kanamycin	Air Cell	ED 10 - 17	0.01 - 100.0 mg/egg/day
Streptomycin	Air Cell	ED 10 - 17	0.001 - 5.0 mg/egg/day
Ethacrynic Acid	Air Cell	ED 10 - 17	0.001 - 1.0 mg/egg/day
Ethacrynic Acid (EA)	Air Cell	ED 10 - 17	0.001 mg EA/egg/day + 0.001 - 0.1 mg G/egg/day 0.01 mg EA/egg/day + 0.001 - 0.1 mg G/egg/day 0.1 mg EA/egg/day + 0.001 - 0.01 mg G/egg/day

Air cell injection was performed by candling the egg in a perpendicular plane and marking the limits of the air cell with a pencil. A mark was made at a point approximately equal distance from the limits of the air space. The area around the mark was disinfected with 70% ethanol and allowed to dry. A small hole was drilled through the shell at the pencil mark, but not through the shell membrane. The area around the hole was disinfected with 70% ethanol and allowed to dry. Eggs were injected by holding them in a perpendicular fashion and inserting a 25 gauge, 5/8th inch needle, attached to a 1 ml tuberculin syringe, approximately 1/4th of an inch into the hole. Following injection, the hole was sealed with melted paraffin. On subsequent injections, the egg was held in a perpendicular plane and the paraffin cap and surrounding area disinfected with 70% ethanol. The paraffin cap was scraped free and the ethanol allowed to dry. The egg was then injected and resealed as previously described.

For allantoic cavity injections, eggs were candled in a perpendicular plane and the limits of the air cell was outlined in pencil. A mark was made approximately 1/8 inch above the line over the air cell on the side opposite the developing embryo. The area around the mark was disinfected with 70% ethanol and allowed to dry. A small hole was drilled through the shell at the pencil mark, but not through the shell membrane. The area around the hole was disinfected with 70% ethanol and allowed to dry. Eggs were injected by holding them in a perpendicular plane and, using a 1 ml tuberculin

syringe fitted with a 25 gauge 5/8 inch needle, inserting the needle perpendicularly through the hole to the hub. Following injection, the hole was sealed with melted paraffin. On subsequent injections, the egg was held in a perpendicular plane and the paraffin cap and surrounding area disinfected with 70% ethanol. The paraffin cap was scraped free and the ethanol allowed to dry. The egg was then injected and resealed as previously described.

For yolk sac injections, eggs were candled with the long axis in the horizontal plane to locate the yolk sac. A pencil mark was made on the shell over the yolk sac about half-way from the small end of the egg to the apex of the curvature of the shell. The area was disinfected by applying 70% ethanol and allowed to dry. A small hole was drilled through the shell but not through the shell membrane. The area around the hole was disinfected with 70% ethanol and allowed to dry. Injection was performed by holding the long axis of the egg in the horizontal plane and using a 1 ml tuberculin syringe fitted with a 25 gauge 5/8 inch needle, the needle was passed perpendicularly through the hole to the hub

RESULTS

Gentamicin Studies

Air cell injection of gentamicin at a dose between 5 to 40 mg/egg/day produced death of chick embryos after 2 treatments of the highest dose group after 2 treatments and after 6 treatments of the lowest dose (Table 5). No chick

embryo lived longer than ED 18. Air cell injection of gentamicin at a dose between 0.01 mg to 1.0 mg/egg/day caused a dose related mortality. Administration of gentamicin at a dose of 1.0 mg/egg/day resulted in chick embryo death beginning on ED 14 (Table 6). Gentamicin at a dose of 0.5 mg/egg/day produced chick embryo death beginning on ED 17 and at a dose of 0.1 mg/egg/day caused chick embryo death beginning on ED 19. Death of chicks receiving gentamicin at a dose of 0.01 or 0.05 mg/egg/day was infrequent.

Gentamicin air cell injection during ED 10-14 or ED 15-19 at a dose between 0.01-1.0 mg/kg/egg was performed to compare the effect of injection early in incubation (ED 10-14) and late in incubation (ED 15-19) on mortality. Although chick embryo death was noted during both time periods, the time of injection had little effect on mortality (Table 7).

Allantoic space injection of gentamicin also produced chick embryo death. Chick embryos given gentamicin at a dose of 5.0 mg/egg/day in allantoic space starting on ED 10 began dying by ED 18. Doses of gentamicin between 1.0 to 4.0 mg/egg/day caused death between ED 18-20 (Table 8).

Yolk sac injection of gentamicin at a dose of 5.0 mg/egg/day caused chick embryo death beginning on ED 16 with no chick embryos surviving past ED 17 (Table 9). Administration of gentamicin at a dose of 3.0 mg/egg/day was lethal beginning on ED 17 with no chick embryos surviving past ED 19. A single chick embryo receiving gentamicin at a dose of 1.0 mg/egg/day died on ED 19.

Table 5. Mortality in embryonated chicken eggs injected into the air cell with gentamicin at 5.0 to 40 mg/egg/day on incubation days 10 through 17.

Group	Incubation Day								
	10	11	12	13	14	15	16	17	18
A	[1]2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
B	3/3	3/3	3/3	3/3	3/3	3/3	2/3	2/3	0/3
C	3/3	3/3	2/3	2/3	1/3	0/3			
D	3/3	3/3	3/3	2/3	2/3	1/3	0/3		
E	3/3	2/3	2/3	2/3	0/3				
F	3/3	2/3	1/3	0/3					
G	3/3	3/3	2/3	0/3					

[1]Live chick embryos remaining/chick embryos at start.
Group A: 0.1 ml deionized water/egg.
Group B: 5 mg/egg.
Group C: 10 mg/egg.
Group D: 15 mg/egg.
Group E: 20 mg/egg.
Group F: 30 mg/egg.
Group G: 40 mg/egg.

Table 6. Mortality in embryonated chicken eggs injected in the air space with gentamicin at 0.01 to 1.0 mg/egg/day on incubation days 10 through 17.

Group	Incubation Day									
	10	11	12	13	14	15	16	17	18	19
A	[1]7/7	6/7	6/7	5/7	5/7	5/7	5/7	5/7	5/7	5/7
B	7/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7	6/7
C	7/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7	6/7
D	7/7	7/7	7/7	7/7	7/7	6/7	6/7	6/7	6/7	3/7
E	6/6	6/6	6/6	6/6	6/6	6/6	5/6	2/6	1/6	1/6
F	6/6	6/6	6/6	6/6	5/6	2/6	1/6	0/6		

[1]Live chick embryos remaining/chick embryos at start.
Group A: 0.1 ml deionized water/egg.
Group B: 0.01 mg/egg.
Group C: 0.05 mg/egg.
Group D: 0.10 mg/egg.
Group E: 0.50 mg/egg.
Group F: 1.00 mg/egg.

Table 7. Mortality in embryonated chicken eggs injected in the air space with gentamicin on incubation days 10 through 14 or days 15 through 19.

Group	Incubation Day										
	10	11	12	13	14	15	16	17	18	19	20
Dosed during embryonic days 10-14											
A	[1]5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5
B	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	5/6	5/6	5/6
C	6/6	6/6	6/6	6/6	6/6	6/6	5/6	5/6	5/6	5/6	2/6
D	4/4	4/4	4/4	4/4	4/4	3/4	2/4	2/4	2/4	1/4	0/4
E	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Dosed during embryonic days 15-19											
F	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4
G	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5	4/5	1/5
H	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	2/5	0/5	
I	4/4	4/4	4/4	4/4	4/4	4/4	4/4	2/4	0/4		

[1]Live chick embryos remaining/chick embryos at start.
Group A: 0.01 mg gentamicin/egg.
Group B: 0.05 mg gentamicin/egg.
Group C: 0.1 mg gentamicin/egg.
Group D: 0.5 mg gentamicin/egg.
Group E: 0.01 mg gentamicin/egg.
Group F: 0.05 mg gentamicin/egg.
Group G: 0.1 mg gentamicin/egg.
Group H: 0.5 mg gentamicin/egg.
Group I: 1.0 mg gentamicin/egg.

Table 8. Mortality in embryonated chicken eggs injected with gentamicin into the allantoic space on incubation days 10 through 17.

Group	Incubation Day											
	10	11	12	13	14	15	16	17	18	19	20	21
A	[1]4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	1/4	1/4
B	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	3/4	1/4	0/4
C	4/4	4/4	4/4	4/4	3/4	3/4	3/4	3/4	1/4	0/4		
D	3/3	3/3	3/3	3/3	3/3	3/3	2/3	2/3	2/3	1/3	0/3	
E	4/4	3/4	3/4	3/4	3/4	3/4	3/4	3/4	0/4			

[1]Live chick embryos remaining/chick embryos at start.
Group A: 1 mg gentamicin/egg.
Group B: 2 mg gentamicin/egg.
Group C: 3 mg gentamicin/egg.
Group D. 4 mg gentamicin/egg.
Group E: 5 mg gentamicin/egg.

Table 9. Mortality in embryonated chicken eggs injected with gentamicin into the yolk sac on incubation days 10 through 17.

Group	Incubation Day												
	10	11	12	13	14	15	16	17	18	19	20	21	H+1
A	[1]3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	2/3	2/3
B	4/4	3/4	3/4	3/4	3/4	3/4	3/4	2/4	2/4	2/4	0/4		
C	4/4	4/4	4/4	4/4	4/4	4/4	4/4	2/4	0/4				

[1]Live chick embryos remaining/chick embryos at start.
Group A: 1 mg gentamicin/egg.
Group B: 3 mg gentamicin/egg.
Group C: 5 mg gentamicin/egg.

Chick embryos were also exposed by submersion of the egg in solutions of gentamicin. Because of the temperature differential between the warm egg and the cold water, solutions are readily taken into the egg. In order to determine what temperature of solution would result in an adequate amount of solution uptake by the egg, a trial using 1.0% gentamicin solutions maintained at 4.5-6.0°C, 11-16°C, or 20-21°C was completed. Three eggs/group were weighed every two minutes during the submersion and the cumulative weight gain recorded (Table 10). Submersion in the gentamicin solution at 4.5-6.0°C for 10 minutes resulted in a cumulative gain of 0.405 ± 0.034 grams while submersion for 10 minutes at 11-16°C or 20-21°C resulted in a cumulative weight gain of 0.101 ± 0.021 grams and 0.191 ± .001 grams, respectively. In a second submersion trial, eggs were submerged for 10 minutes in 5-7°C solutions of deionized water, or gentamicin at 2.5, 12.5 , or 25 mg/ml. Mortality occurred after 1 day of treatment in all groups including the deionized water group, with no chick embryo living past ED 16 (Table 11).

Kanamycin Study

Air cell injection of kanamycin beginning on ED 10 was lethal in all chick embryos receiving 100 mg/egg/day after one treatment and in chick embryos receiving 10 mg/egg/day after two treatments (Table 12). Deaths began to occur on ED 17 in chick embryos receiving kanamycin at a dose of 1.0 mg/egg/day while no deaths occurred in chick embryos receiving doses between 0.01 or 0.1 mg of kanamycin/egg/day.

Table 10. Mean cumulative weight gain (grams) of embryonated chicken [1]eggs submerged in a 1.0% gentamicin solution maintained at different temperatures.

Group	Cumulative Minutes of Submersion				
	2	4	6	8	10
A	0.119 ± .190	0.223 ± .051	0.307 ± .051	0.360 ± .037	0.405 ± .034
B	0.050 ± .053	0.079 ± .029	0.091 ± .028	0.097 ± .034	0.101 ± .021
C	0.097 ± .045	0.124 ± .013	0.148 ± .003	0.174 ± .005	0.191 ± .001

[1] Three eggs per group.
Group A: Solution temperature maintained at 4.5-6.0°C.
Group B: Solution temperature maintained at 11-16°C.
Group C: Solution temperature maintained at 20-21°C.

Table 11. Mortality in embryonated chicken eggs dipped in gentamicin solutions on incubation days 10 through 17.

Group	Incubation Day										
	10	11	12	13	14	15	16	17	18	19	20
A	[1]4/4	2/4	1/4	1/4	1/4	1/4	0/4				
B	4/4	3/4	2/4	1/4	0/4						
C	4/4	2/4	0/4								
D	4/5	2/5	1/5	0/5							

[1]Live chick embryos remaining/chick embryos at start.
Group A: Deionized water.
Group B: 2.5 mg gentamicin/ml.
Group C: 12.5 mg gentamicin/ml.
Group D: 25 mg gentamicin/ml.

Table 12. Mortality in embryonated chicken eggs injected in the air space with kanamycin on incubation days 10 through 17.

Group	Incubation Day									
	10	11	12	13	14	15	16	17	18	19
A	[1]5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
B	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
C	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	
D	4/4	4/4	4/4	4/4	4/4	4/4	4/4	2/4	1/4	
E	4/4	4/4	0/4							
F	4/4	0/4								

[1]Live chick embryos remaining/chick embryos at start.
Group A: 0.1 ml deionized water/egg.
Group B: 0.01 mg kanamycin/egg.
Group C: 0.1 mg kanamycin/egg.
Group D: 1.0 mg kanamycin/egg.
Group E: 10.0 mg kanamycin/egg.
Group F: 100.0 mg kanamycin/egg.

Streptomycin Study

Injection of streptomycin beginning on ED 10 produced death of chick embryos receiving 10.0 mg/egg/day on day 11 with no chick embryos surviving past ED 12 (Table 13). Deaths began to occur in chicks receiving streptomycin at a dose of 5.0 mg/egg/day on ED 12, with no chick embryos surviving past ED 14. Deaths began to occur on ED 17 in chick embryos receiving streptomycin at a dose of 1.0 mg/egg/day while little effect was noted in chick embryos receiving a dose between 0.001 to 0.1 mg of streptomycin/egg/day.

Ethacrynic Acid Studies

Injection of ethacrynic acid (EA) beginning on ED 10 was lethal to all chick embryos receiving a dose of 1.0 mg/egg/day after 1 treatment (Table 14). Two chick embryos receiving EA at a dose of 0.1 mg/egg/day died on ED 14. No chick embryos receiving EA at a dose of 0.001 or 0.01 mg/egg/day died.

A second trial was designed to determine a maximal tolerable dose of gentamicin and EA together. Combinations of EA at a dose of 0.001 mg/egg/day with gentamicin at a dose between 0.001 to 0.1 mg/egg/day caused death beginning on ED 17 in chick embryos receiving gentamicin at a dose of 0.1 mg/egg/day (Table 15). A few chick embryos receiving combinations of EA at a dose of 0.01 mg/egg/day with gentamicin at doses between 0.001 to 0.1 mg/egg/day died early in the treatment period with no relationship to the dose. Combinations of EA at 0.1 mg/egg/day with gentamicin at 0.001 and 0.01 mg/egg/day resulted in chick embryo mortality during

Table 13. Mortality in embryonated chicken eggs injected in the air space with streptomycin on incubation days 10 through 17.

Group	Incubation Day								
	10	11	12	13	14	15	16	17	18
A	[1]7/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7
B	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
C	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
D	5/5	5/5	5/5	4/5	4/5	4/5	4/5	4/5	4/5
E	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	2/5
F	4/4	4/4	4/4	3/4	2/4	0/4			
G	4/4	4/4	2/4	0/4					

[1]Live chick embryos remaining/chick embryos at start.
Group A: 0.1 ml deionized water/egg.
Group B: 0.001 mg streptomycin/egg.
Group C: 0.01 mg streptomycin/egg.
Group D: 0.1 mg streptomycin/egg.
Group E: 1.0 mg streptomycin/egg.
Group F: 5.0 mg streptomycin/egg.
Group G: 10.0 mg streptomycin/egg.

Table 14. Mortality in embryonated chicken eggs injected in the air space with ethacrynic acid (EA) on incubation days 10 through 17.

Group	Incubation Day								
	10	11	12	13	14	15	16	17	18
A	[1]8/8	8/8	8/8	7/8	7/8	7/8	7/8	7/8	7/8
B	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
C	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
D	6/6	6/6	6/6	6/6	4/6	4/6	4/6	4/6	4/6
E	6/6	0/6							

[1]Live chick embryos remaining/chick embryos at start.
Group A: 0.1 ml deionized water/egg.
Group B: 0.001 mg ethacrynic acid/egg.
Group C: 0.01 mg ethacrynic acid/egg.
Group D: 0.1 mg ethacrynic acid/egg.
Group E: 1.0 mg ethacrynic acid/egg.

Table 15. Mortality in embryonated chicken eggs injected in the air space with ethacrynic acid and gentamicin on incubation days 10 through 17.

Group	Incubation Day								
	10	11	12	13	14	15	16	17	18
A	[1]8/8	8/8	8/8	7/8	7/8	7/8	7/8	7/8	7/8
B	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
C	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
D	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	2/4
E	5/5	5/5	5/5	4/5	4/5	4/5	4/5	4/5	4/5
F	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
G	4/4	4/4	4/4	3/4	3/4	3/4	3/4	3/4	2/4
H	4/4	4/4	3/4	2/4	2/4	2/4	2/4	2/4	1/4
I	4/4	4/4	4/4	3/4	2/4	2/4	2/4	2/4	2/4

[1]Live chick embryos remaining/chick embryos at start.
Group A: 0.1 ml deionized water/egg.
Group B: 0.001 mg ethacrynic acid, 0.001 mg gentamicin/egg.
Group C: 0.001 mg ethacrynic acid, 0.01 mg gentamicin/egg.
Group D: 0.001 mg ethacrynic acid, 0.1 mg gentamicin/egg.
Group E: 0.01 mg ethacrynic acid, 0.001 mg gentamicin/egg.
Group F: 0.01 mg ethacrynic acid, 0.01 mg gentamicin/egg.
Group G: 0.01 mg ethacrynic acid, 0.1 mg gentamicin/egg.
Group H: 0.1 mg ethacrynic acid, 0.001 mg gentamicin/egg.
Group I: 0.1 mg ethacrynic acid, 0.01 mg gentamicin/egg.

ED 12 to 14 with remaining chick embryos surviving until ED 18 (end of the observation period).

DISCUSSION

Each of the drugs used in these dose response studies caused death of chick embryos. This allowed easy determination of a maximally tolerated dose to be used for other toxicity studies using the chick embryo. There was no difference between the results of injection of gentamicin into the air space early in incubation to late in incubation. Thus, when doses do not cause death within one or two days, lethality may be dependent upon a specific stage of development. This could be explained by the onset of enzyme function to bioactivate a protoxicant or it may reflect increasing dependence on an organ system such as the kidneys which may be damaged by the drugs. Also, submersion did not appear to be a method of exposure that provided a consistently predictable dose. Results, even those from the negative control group indicated this method had considerable draw backs.

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