COMPARATIVE SUSCEPTIBILITY OF FETAL AND MATERNAL GUINEA PIG EARS TO THE OTOTOXIC EFFECT OF KANAMYCIN

by

Rickie R. Davis, M.S.

A THESIS

Presented to the Department of Medical Psychology and
the Graduate Division of the Oregon Health Sciences University
in partial fulfillment of
the requirement for the degree of

Doctor of Philosophy
July, 1981

APPROVED: [redacted]

....

[redacted] in Charge of Thesis)

....

(Chairman, Graduate Council)

Acknowledgments

As anyone who has attempted a study of this size knows this was not a singular effort. I would like to take this opportunity to thank some of those people who contributed to this effort. With respect and gratitude I thank Robert Brummett and Kaye Fox. I also want to thank Rich Walloch, John Bissonette, Robert Fitzgerald and Jack Vernon for serving on my review committee. A special thanks to Chris Cunningham for his help with some of the statistics and alot of the spelling.

Invaluable technical help was supplied by Tom Bendrick and Nancy Schuff. Donna Himes dissected all of the ears examined and counted the hair cells in the last experiment--a gigantic task. She also drew the beautiful Figures 31 a & b and 32 a & b. Jim Fenwick examined early drafts of this thesis and offered often needed encouragement. Probably everyone at Kresge Lab has had some input into this work and I thank you.

My wife, Sharon, deserves alot of the credit for this thesis. She was there through the good and bad. Her help has included everything from handing me micropipets to drawing figures. I appreciate her loyalty and encouragement.

I thank the Deafness Research Foundation and Schering Corporation for their support. The Medical Research Foundation of Oregon finacially supported the guinea pig colony during this research project. I am indebited to Pope and Talbot, Inc for allowing me to use their word processor.

To the many friends I've made during my tenure in Portland I say thank you.

Table of Contents

Table of Contents (continued)

Table of Contents (continued)

List of Figures

List of Figures (continued)

List of Figures (continued)

List of Tables

Introduction

The problem of infant deafness is not restricted to the inability to hear sound. Dysfunction of this one sensory system modifies the emotional, intellectual and communicative skills of the infant. Altshuler (1974) compares the psychological development of a hearing child with that of a deaf child. A mother's words can comfort, reassure and scold even before the child understands what the words mean. The hearing child is able to learn the difference between words said in anger and concern. The hearing child learns the subtleties of sarcasm and sincerity. Many of these nuances of verbal communication are lost on the deaf child.

Altshuler also emphasizes the importance of language acquisition between the ages of 18 months and 4 years. During this time the child is primed to learn a language. If the child does not learn a language during this period it becomes very difficult later to teach him a language. The deaf child often does not acquire a language until well past this critical period. Acquisition of abstract concepts is often retarded in deaf children because language learning is postponed. In contrast, deaf children born to deaf parents do not display these language difficulties--they learn manual communication at the same time and in the same manner as a hearing child would learn to speak (Brill, 1974).

Another problem faced by deaf children is the reaction by their hearing parents. Often the parents feel guilt about the

child's deafness. Or they may feel anger towards their partner. The deaf child may sense these feelings but not understand them. Once they know their child is deaf, parents may treat their child differently. They may stop using hand gestures or, when speaking to the child, place their mouths close to the child's eyes to encourage lip reading. These behavioral changes confuse the child and may actually hinder parent-child communication (Altshuler, 1974).

The deaf child becomes the deaf adult. It is known that over a lifetime the deaf individual earns less money and gains less social status than the average hearing individual (Schien and Delk, 1974).

It would clearly be to the individual's and society's benefit to prevent deafness. In order to prevent deafness the causes must be discovered and eliminated.

Generally, three causes of congenital deafness can be identified: genetic causes, exposure to teratogenic agents, and exposure to agents producing functional deficits. These classifications are based on Wilson's 1973 classifications.

A genetic cause of deafness is based within the cell's genetic material--DNA. It may be due to an extra chromosome, a recessive gene pair or a dominant gene. Genetic causes of deafness are transmitted to future generations following the laws of Mendelian genetics.

A genetic cause of deafness may be an additional effect of gross neural maldevelopment or the deafness may be related to

degeneration of the auditory system. Many genetic defects which produce pathological development of the nervous system also modify development of the ear (referred to as an aplasia type defect, Smith, 1973). An example is Patau's syndrome. An extra thirteenth chromosome is associated with gross neural defects which result in mental retardation. Some children appear to be born deaf. Most of these children die before one year of age.

In contrast, retinitis pigmentosa is an autosomal recessive genetic defect which modifies melanin production in the eye and ear. In addition to a degenerative blindness due to this defect, progressive deafness has been described (Hallgren, 1959). Retinitis pigmentosa belongs to a class called degenerative type defects (Smith, 1973). In this case deafness does not necessarily accompany central nervous system malformation--the auditory system develops normally followed by degeneration of the organ of Corti.

Teratogenic causes of deafness are due to chemicals or infective processes acting upon the fetus during a limited time period of fetal development. This sharply defined period of susceptability is often referred to as a "critical period." The same agent acting earlier or later in development will not produce the teratogenic result. Wilson (1973) describes this as a disruption or destruction of fetal cells which leads to a permanent morphological modification of the tissue or organ. A teratogen which has caused congenital deafness in humans as well as gross congenital limb defects is the sedative thalidomide. When thalidomide was ingested by pregnant mothers 34 to 38 days after their last

menstruation their offspring were born deaf (Schardein, 1976).

Another example of a teratogen is German measles (rubella virus). Mothers who contracted rubella during the first four months of pregnancy bore children who were often blind or deaf (Bordley,Brookhouser,Hardy and Hardy, 1967; Gregg, 1941, 1944; Swan, 1944).

Generally, teratogens exhibit a "critical period" during in utero development as described above. Teratogens do not usually produce the same type of an effect in the individual after organogenesis. For example thalidomide is a safe and effective sedative for adults, and rubella infections are minor discomforts for children and adults. However, the teratogenic effects of both of these agents are devastating for the fetus if present during the critical period. This critical period usually occurs during differentiation of the effected organ.

A drug or infective process which produces a deficit but does not exhibit a critial period is said to produce a functional deficit (Wilson, 1973). Agents producing functional deficits usually operate upon differentiated tissue. For this reason a functional deficit may be exhibited by adults and children. However, the fetus may be more sensitive to the effects of the agent and exhibit a larger functional deficit.

One group of drugs which may produce functional deficits is the aminoglycoside antibiotics. In adults these drugs destroy the auditory system receptor organ--the cochlear hair cell.

Aminoglycoside antibiotics. The aminoglycoside antibiotics

Table 1. The Aminoglycoside Antibiotics

Amikacin

Dihydrostreptomycin

Gentamicin

Kanamycin

Neomycin

Netilmicin

Sisomicin

Streptomycin

Tobramycin

include those listed in Table 1. These antibiotics are very important in the treatment of Gram negative bacterial infections. Without treatment with these antibiotics many people would die from their infections.

The site of antibacterial action of these drugs appears to be the bacterial ribosome (Davies and Davis, 1968). The ribosome uses RNA to assemble the enzymes which power the cell. These antibiotics specifically attach to a portion of the bacterial ribosome and cause a misreading of the RNA. Without the enzymes to carry out the chemical reactions needed for functioning, the bacterium soon dies. Thus the aminoglycoside antibiotics kill bacteria.

Unfortunately, the aminoglycoside antibiotics are not without adverse effects. Their use is limited by their toxicity. This toxicity is of sufficient magnitude to usually limit the aminoglycosides to life threatening infections. The aminoglycosides have three major forms of toxicity: neuromuscular blockade, nephrotoxicity and ototoxicity.

Muscle fibers are the functional units of the heart and the respiratory diaphragm, along with other vital organs. Paralysis of the respiratory diaphragm or heart quickly leads to death. Calcium ions are necessary for the proper contraction of muscle fibers. In high doses the aminoglycoside antibiotics antagonize calcium ions in sufficient quantity to inhibit muscle contraction (Pittinger and Adamson, 1972). This effect was first observed when surgeons sprinkled neomycin powder into the abdominal cavi-

ties of patients undergoing gastrointestinal tract surgery (Pridgen, 1956). The patient often stopped breathing because of the effects of the aminoglycoside on the respiratory diaphragm. This paralysis can be reversed by infusion of a calcium ion containing solution (Weinstein, 1975). Calcium antagonism by the aminoglycoside antibiotics usually only becomes a problem in extremely high aminoglycoside doses or in patients having electrolyte imbalances.

A much more common side-effect of the aminoglycoside antibiotics and one having dire consequences is nephrotoxicity (Appel and Neu, 1977). The proximal tubule of the nephron in the kidney specifically binds the aminoglycoside antibiotics. After a long exposure to the aminoglycoside antibiotics the nephron will die. As more of the kidney function is compromised by nephron destruction the aminoglycoside antibiotic is not eliminated as well from the circulation and higher levels become present in the blood. This is because mammals do not modify the aminoglycoside antibiotics nor do they eliminate these antibiotics except through the kidneys by glomerular filtration. As nephrotoxicity advances the patient progresses into renal failure and only blood dialysis can save the patient's life. If nephrotoxicity can be detected early enough, administration of the aminoglycosides can be halted and nephrotoxicity can be reversed (Appel and Neu, 1977).

Reversal of toxic effects is generally not the case for the third form of toxicity--ototoxicity. Ototoxicity to the aminoglycosides is generally permanent. It involves the specific destruc-

tion of hair cell receptors. (A summary of the anatomy of the mammalian ear is presented in Appendix B). The inner ear can be divided into two parts--the organ of balance and the organ of hearing. Therefore, ototoxicity can be subdivided into vestibulotoxicity or cochleotoxicity. In the present study ototoxicity refers to cochleotoxicity.

Ototoxicity of the aminoglycoside antibiotics in neonates and adults involves destruction of the outer hair cells of the cochlea (Hawkins, 1976). Destruction begins in the hair cells in the base of the cochlea (Prossen and Stebbins, 1980). These hair cells transduce high frequency sounds. If aminoglycoside antibiotic treatment continues, hair cell destruction continues up the cochlea involving hair cells which transduce lower and lower frequencies. Eventually most of the outer hair cells of the entire cochlea are destroyed. This produces sensorineural hearing loss localized at the outer hair cells.

Little is known about aminoglycoside ototoxicity in the fetus. Until very recently animal experiments involving prenatal exposure to the aminoglycoside antibiotics were poorly controlled. Experimental fetal ototoxicity will be reviewed later.

Case reports of fetal ototoxicity due to the aminoglycoside antibiotics in humans are difficult to interpret. In treating disease the drug is given at different times during pregnancy for differing lengths of time. Thus the interval of exposure for the fetus is unique for each fetus. In addition the dose of aminoglycoside antibiotic used in treating disease is determined indepen-

dently for each mother. Therefore, the amount of drug to which each fetus is exposed is unique. The disease process itself can quantitatively modify how the body absorbs, distributes and excretes the aminoglycoside antibiotics. For example, the bedridden individual will have very different pharmacokinetics than the ambulatory individual. These variables are rarely included in case reports of fetal ototoxicity.

Another problem with case reports is the lack of an untreated control population. The control population allows the researcher to compare the incidence of sensorineural hearing loss in the drug treated group with the incidence of sensorineural hearing loss in a normal untreated group. Researchers who discover profound hearing loss in infants from mothers treated with aminoglycoside antibiotics during pregnancy often do not do thorough neurological examinations on these infants. Examinations must be extensive to rule out genetic disorders or teratogenic agents other than the aminoglycoside antibiotic as causation for the hearing loss. Rarely are these examinations reported in the fetal ototoxicity literature. With the above criticisms in mind a review of the literature of fetal ototoxicity follows.

We should begin by determining if the aminoglycoside antibiotics cross the placenta and are available in the fetal circulation. If no drug crosses into the fetal circulation the fetus should exhibit no postnatal ototoxicity. The aminoglycosides are relatively large molecules (in excess of 400 MW), ionized at body pH's and only slightly lipid soluble (oil:water partition

coefficient equals approximately 0.06). These data would indicate that perhaps the aminoglycosides don't cross the placental membranes (Goldstein,et al,1968). However, it has been known for the last thirty-six years that the aminoglycosides do cross into the fetal circulation. In 1945 Woltz and Wiley injected streptomycin into pregnant women in labor and were able to measure streptomycin in the blood of the umbilical cord of the newborn infants. Bernard, et al (1977a, 1977b) have shown that when tobramycin and amikacin are administered to pregnant women these drugs can be detected in the fetus at the time of therapeutic abortion. Yoshioka, et al (1972) detected peak gentamicin levels in umbilical cord blood that were 34% of the maternal gentamicin concentrations for a single injection given prior to birth. Weinstein, et al (1976) injected gentamicin into pregnant women prior to caesarean delivery. They detected peak fetal concentrations of gentamicin that were 42% of maternal peak concentrations.

The conclusion must be that aminoglycoside antibiotics do cross into the fetal circulation. However, the peak concentration of aminoglycoside antibiotic in the fetus is generally less than that of the mother.

Case reports. The above data indicate that the aminoglycoside antibiotics can cross the placental membranes from the mother's circulation into the circulation of the fetus. Examination of human reports of fetal ototoxicity in the literature is thus warranted. Almost all reports concerning human fetal

ototoxicity were compiled from mothers who received streptomycin or dihydrostreptomycin for tuberculosis treatment during their pregnancy sometime within the period from 1945 to 1965. Streptomycin primarily produces vestibulotoxicity in adult mammals (Hawkins, 1976) and dihydrostreptomycin produces primarily cochleotoxicity in humans and certain primates (Hawkins, 1976). Many studies of fetal ototoxicity do not test for vestibulotoxicity in a child who was exposed to streptomycin prenatally.

The earliest report on fetal exposure to aminoglycoside antibiotics was that of Watson and Stow (1948). They reported no detectable ototoxicity in the children of two mothers who had received streptomycin for treatment of tuberculosis. These mothers each had received two grams of streptomycin per day for an undisclosed period of time. The techniques used for detecting ototoxicity were not described. It appears from the report that the children were still very young at the time of the report--indicating that anything but a gross test of hearing would be very difficult.

In 1950 Leroux described a deaf child whose mother had received streptomycin during the last month of pregnancy. This child was of special interest because her mother had received a rather conservative total dose of streptomycin--about one third of that believed to be the minimum amount capable of producing ototoxicity.

Bolleti and Croatto (1950) presented the case of a girl who was diagnosed as being deaf at age two when she failed to learn to

speak. In retrospect it appeared she may have been deaf from birth. She also did not display the charteristic oscillation of the eyes (nystagmus) to caloric stimultion of the vestibular system. Her mother had received an undisclosed amount of streptomycin while pregnant .

Perhaps unaware of these reports in the French and Italian literature, Sakula (1954) discussed the problem of prenatal ototoxicity. His conclusion was that there is no ototoxic danger to the fetus of streptomycin therapy to the mother during pregnancy. He gave an example of a mother who received an undisclosed amount of streptomycin between weeks 10 and 30 of pregnancy. No ototoxicity was detected in the child but Sakula (1964) did not describe how ototoxicity was assessed.

Robinson and Camron (1964) examined hospital records for mothers who had received streptomycin during pregnancy for treatment of tuberculosis. They were able to identify one hundred mothers where streptomycin administration could be documented. Of the offspring of this group of mothers, two children were determined to have severe sensorineural hearing losses by conventional audiometry. This is the type of hearing loss expected due to aminoglycoside ototoxicity. These two children also lacked nystagmatic eye movements to caloric stimulation of the vestibular system. Robinson and Cambron (1964) described these two children in detail. Both were severely handicapped. However, the authors failed to explain if genetic disorders or other causitive agents were considered before implicating streptomycin as the causative

factor. Also the amount and timing of the streptomycin doses were not given for these two youngsters.

Conway and Birt (1965) identified from hospital records eighteen women and their children who received streptomycin during pregnancy for the treatment of tuberculosis. Using conventional audiometry and caloric stimulation of the vestibular system the authors were able to identify measurable hearing losses in four children out of the eighteen total. Six children in the group had abnormal vestibular responses to caloric stimulation. No matched control group was included in this study.

Varpela, Hietalhti and Aro (1969) examined the offspring of fifty mothers who had received streptomycin during pregnancy for tuberculosis treatment. Using conventional audiometry they were able to identify one case of sensorineural hearing loss in the fifty children. Two cases of vestibular dysfunction were observed in the fifty children by use of caloric vestibular stimulation. No control group was included.

Rassmussen (1969) tested thirty-six children and their mothers for ototoxicity. The mothers had received streptomycin during pregnancy for various times and various doses because of tuberculosis. Rassmussen found that two of the children had measurable hearing losses using conventional audiometry. He could not demonstrate vestibular pathology in the children using caloric stimulation. Five mothers had measurable high frequency hearing losses (4000 and 8000 Hz). One mother had an abnormal vestibular response. Again no untreated control group was included.

Nelson and Forfar (1971) examined the histories of perscription drug administration for mothers delivering congenitally defective infants. Nelson and Forfar (1971) examined the infants within the first few days of birth. They reported no correlation between the administration of streptomycin and any birth defects. However, it is doubtful that hearing or vestibular dysfunction could be detected this early in the infant's life without special techniques.

Jones (1973) reported the case of a pregnant woman given kanamycin for a kidney infection. When the kidneys became dysfunctional kanamycin treatment was stopped and ethacrynic acid, a powerful diuretic, was given to restore kidney function. Both the mother and the resulting infant were deafened. In this case the ototoxicity was probably due to the interaction between the aminoglycoside antibiotic and ethacrynic acid. This interaction has been demonstrated in adult guinea pigs by West, Brummett and Himes (1973) and is described as an extensive, swift and permanent destruction of the hair cells of the cochlea to a single injection of both drugs. A single injection of either of these drugs alone has little or no effect on the hair cells of the cochlea (Hawkins, 1976). To date, no experimental study of the aminoglycoside-ethacrynic acid ototoxic interaction in pregnant experimental animals has been published.

Reviewing some of the published reports of fetal ototoxicity Scheinborn and Angelillo (1977) conclude that a significant risk to the fetus exsists with long term streptomycin therapy. They

especially emphasize that the use of streptomycin during embryological development of the ear is contraindicated in patients with renal failure or toxemia.

In order to determine if the aminoglycosides produce teratogenic or functional deficits of the ear it is important to know how the ear develops.

Fetal development of the ear. Development of the ear is morphologically the same for all terrestrial mammals. However, each species has its own timetable for ear development. Many animals do not have functional ears until days after birth (Pujol and Hilding, 1973). In contrast the human ear is complete at midterm. Excellent reviews of mammalian auditory system development exist (Anson and Donaldson, 1981; Chondynicki, 1968; Pearson, et al, 1967; Rubel,1978; Stephens, 1972). The following is derived from their work.

The ear begins development very early in the life of the embryo. The otic placode, a thickening of cells on either side of the head, is present in the third week of human gestation. Growth of the otic placode causes it to invaginate into the auditory pit and eventually the placode grows back on itself, forming a closed sphere. The sphere formed breaks off from the surface ectoderm and is called the otocyst. The otocyst continues to grow and eventually invaginations at the equator begin to divide the otocyst into two portions. The superior portion will become the utricle and semicircular canals. The inferior portion will become the saccule and the cochlea. The inferior portion elongates and

begins to spiral. The spiraling continues until 2½ turns are present in the human embryo or 4½ turns are present in the guinea pig embryo.

Meanwhile at the base of the cochlea the cartilage adjacent to the cochlear duct, as the otocyst is now called, begins to break down forming the perilymphatic spaces. The cochlear duct is changing its cross sectional shape from round to oval and finally to its charteristic triangular shape.

The organ of Corti develops from the epithelium of the cochlear duct. Initially the organ of Corti is pseudostratified epithelium. Outer hair cells of the organ of Corti can be identified by the basal position of their nuclei. A little later in development Hansen's and Deiter's cells become identifiable. The hair cells develop the characteristic apical hairs and the spaces between the hair cells open up--these are the spaces of Nuel and the tunnel of Corti. Cells anchoring the tectorial membrane degenerate allowing the tectorial membrane to float free except for the anchoring by the hairs of the outer hair cells. Table 2 lists the development of the guinea pig and human ear.

The above process proceeds from the base of the cochlea to the apex. There is some evidence that release of the tectorial membrane by supporting cells signals the onset of normal auditory functioning (Pujol and Hilding, 1973).

Maturity of the fetal ear can be determined in two ways. The first way is anatomically. The assumption is that when the structure of the ear of the fetus is the same as the structure of

Table 2

Development of Middle and Inner Ear in Guinea Pigs and Humans

Guinea Pig (days)		Human (weeks)
	Blastocyst attaches to uterine mucosa	1
	Otic placode, middle ear begins development	3
20	Otocyst, VIII nerve gangleon cells developing	4
	Stapedial artery, VIII nerve and corda tympani begin developing; VIII nerve fibers from otocyst; precartilidge mesenchyme forming otic capsule	5
22	3 semicircular ducts forming; ossicles pre-cartilage	6
26	Saccule, utricle, maculae, crus commune, ductus reuniens, scala tympani and cristae ampulares begin developing	7
	One turn of cochlea; precartilage otic capsule; organ of Corti begins developing; tympanic membrane, endolymphatic duct, stapedius muscle begin developing.	8
32	2½ turns of cochlea (human); ossicles cartilaginous; otic capsule cartilage; scala vestibuli, begining of perilymphatic system; tympanic annulus and tectorial membrane begin developing	9
	Otolithic membrane of maculae and cupulae of christae; tensor tympani muscle	11
	First ossification center of malleus; tympanic annulus membrane bone; stapedial artery disappears in human	15
34	Cochlear duct is 4 turns (guinea pig); cartilage capsule	
35	Cochlear duct is 4 turns (guinea pig); perilymphatic spaces can be seen around turns 1, 2 and part of 3 in guinea; Merkel's cartilage regressing	15

Table 2 (continued)

	First ossification center of cochlea and incus	15
36	Hairs can be seen on hair cells; large tectorial membrane attached to hair cells; small fluid spaces in Corti's tunnel; cochlear duct is triangular in turns 1 and 2, oval in 3 and 4; perilymphatic spaces in turns 1 and 2	
	Malleus and incus ossifying; tympanic membrane and tympanic annulus well formed	16
45	Tall cells of internal sulcus shortened to release tectorial membrane; fiber tracts can be seen in basilar membrane; can stimulate system with electricity but not sound	
47	Semicircular canals, stapes and facial canal begin ossifying	18
49	Ossification of entire capsule has taken place	
50	Onset of ac cochlear potential (guinea pig)	
51-53	Onset of N_1	
50	Release of tectorial membrane; Corti's tunnel is obvious; stria vascularis has epithelium and capillaries	
52	Cochlear duct is completely formed	20
53	Corti's tunnel increases and so do Neul's spaces; tall cells atrophy	
	Meatal plate hollowed out	21
	Malleus ossified; semicircular canals ossified	26
65 (birth)	Membranous and osseous labyrinth developed; middle ear developed; stapes ossified	28
	Stapes ossified	35
72 (7 days old)	Mature cochlea	
	Birth (human)	36

structure of the ear of the fetus is the same as the structure of the normal adult, normal functioning has begun. This is not a good assumption because the ear may be functional before structurally complete (Javel, 1980). In contrast, even after adult-like structure has been attained physiological processes may not have matured enough to allow functioning (Javel, 1980). A second way of determining the maturity in the auditory system is electrophysiologically.

It has been known for the past fifty years that the ear will generate electrical activity in response to sound (Wever and Bray, 1930). The earliest report of fetal electrophysiological measures was that of Rawdon-Smith, Charmichael and Wellman (1938) who recorded cochlear potentials from fetal guinea pigs. Since that time someone has rediscovered that auditory potentials can be recorded from fetal animals about once per decade (e.g. Chondynicki, 1968; Pujol and Hilding, 1973; Schibetta and Rosen, 1969).

The types of electrical measures made from the cochlea and techniques used to make these measures are described in some detail in Appendix B. The amplitude of the ac cochlear potential is dependent upon the presence of the outer hair cells. Since the outer hair cells are damaged by the aminoglycoside antibiotics, the generation of the ac cochlear potential in response to sound is a sensitive indicator of cochlear function.

Electrophysiological and anatomical measures of fetal ototoxicity have been made in experimental animals. Experimental

measures of fetal ototoxicity can be better controlled than human case reports. First it is easy to include a control group. This allows for direct comparison of subjects in the drug treated group with subjects which have not been treated. Second, the amount of drug administered and the time of drug administration can be controlled relative to fetal development. Third, anatomical and electrophysiological measures can be made of each mother and offspring. These measures can detect minute lesions of the auditory system. Fourth, experimental animals known to be disease free and of a common background can be utilized. This eliminates disease processes and genetic variability common in the human reports. In the treatment of human disease often more than one drug is used. Combinations of drugs can interact to change the observed effects of both drugs. The use of many drugs may mask the responses of the drug of interest.

Riskaer, Christensen and Hertz (1952) used rotation as the stimulus to measure vestibular ototoxicity to streptomycin in guinea pigs. The eyes display an oscillation called nystagmus after stimulation of the intact vestibular system by rotation or changes in intraaural temperature. Placing water of lower or higher temperature than the body into the ear canal induces convection currents in the semicircular canal perilymph which stimulates the ampulla. They also used the reflexive twitching of the pinna to loud sounds (Preyer pinea reflex) as a measure of cochlear ototoxicity. Pregnant mothers were injected with various doses of streptomycin or dihydrostreptomycin daily (200 mg/kg, 100

mg/kg, 50 mg/kg, 25 mg/kg or 10 mg/kg). The drug injections were started early in gestation but were not timed relative to conception. The injections were given daily until delivery, abortion or complications occurred. Those mothers receiving 200 or 100 mg/kg per day either aborted dead fetuses or died themselves. In the lower dosage groups some abortions were noted as well as some stillbirths. Of the surviving offspring no abnormal vestibular responses or abnormal Preyer pinna reflex responses were observed. Neither of these tests are capable of detecting anything except extensive ototoxic damage.

Alajmo (1952) injected pregnant guinea pigs with various doses and schedules of streptomycin. He lost a large number of offspring to abortion and stillbirth. Of those who lived Alajmo made no effort to test their auditory systems. He did find a small number of offspring who 30 days after birth demonstrated vestibular dysfunction to rotation stimulus.

In 1963 Ericson-Strandnic and Gyllesten injected pregnant mice with an almost lethal dose of streptomycin. Using anatomical measures they were unable to observe any gross or light microscopic changes in the central nervous systems of the fetuses that could be attributed to streptomycin.

Also using anatomical measures Mesollelo (1963) injected pregnant guinea pigs daily with 200 mg/kg of kanamycin from conception to delivery (approximately 65 days). Using cellular measures he was able to demonstrate changes in Deiter's cells, Claudius' cells, inner hair cells and outer hair cells in those

offspring whose mothers received kanamycin throughout the entire pregnancy. A group of guinea pig offspring whose mothers received kanamycin during only part of their pregnancy showed the same types of changes but to a lesser extent. Mesollelo did not include a control group with which the kanamycin group could be compared. The anatomical changes noted are difficult to correlate with functioning of the cochlea.

Akiyoshi, et al (1977) injected five pregnant guinea pigs with amikacin (100 mg/kg and 200 mg/kg), kanamycin (100 mg/kg and 200 mg/kg) and saline. Injections were given daily beginning on day seven of pregnancy and continuing through day sixty three of pregnancy (56 days total). Their assessment of ototoxicity in newborn pups was to observe the Preyer pinna reflex stimulated by tone bursts whose frequencies were between 500 Hz and 20 kHz. They also examined each pup's cochlea and vestibular system microscopically. In pups from mothers who had received injections of 200 mg/kg per day of kanamycin, three out of ten pups showed a loss of Preyer pinna reflex between 15 kHz and 20 kHz. They were able to microscopically detect a unilateral incomplete hair cell loss in three out of thirteen pups from mothers who had received 100 mg/kg per day amikacin. All of the pups whose mothers had received 200 mg/kg per day amikacin showed vestibular damage. Vestibular damage measured histologically was even greater in the pups whose mothers had received 100 mg/kg or 200 mg/kg per day of kanamycin than those whose mothers had received 200 mg/kg per day of amikacin.

Uziel, Romand and Bagsion (1977) studied ototoxicity in offspring whose mothers had been injected for eight consecutive days with either 200 mg/kg per day or 400 mg/kg per day of kanamycin. The period of injection was randomly assigned and determined post hoc by counting back from the date of birth. They used filtered clicks to evoke the ac cochlear potential in the offspring after birth. They were able to measure a decline of ac cochlear potential in 56% in the offspring whose mothers had received 400 mg/kg per day. In the offspring of the mothers who had received 200 mg/kg per day 32% of the offspring had measurable declines of ac cochlear potential. Uziel, et al (1977) found that the closer to the date of birth the drug course was given the greater the diminution of the ac cochlear potential. No mention was made that the effects of the kanamycin injections may have on modifying the length of gestation. Counting backwards from birth could be misleading. They question why some littermates were affected by kanamycin treatment while others were not. They speculate that differences in susceptability to kanamycin may be the reason. Again an injected control group was not included.

Experiment 1: Electrophysiology of maternal and fetal ototoxicity.

The first experiment of this study used electrophysiological techniques to measure ototoxicity. Some differences exist between this experiment and previously cited experiments. First, this experiment compared kanamycin ototoxicity in the fetus with kanamycin ototoxicty in the mother. Thus, while this study is

still interested in effects on the fetus it is also interested in comparing the ototoxic effects of the mother and the fetus.

Second, all pregnancies were timed from conception rather than retrospectively from date of birth. The time chosen for fetal exposure to aminoglycoside antibiotic was from the time that the outer hair cells first become identifiable until the ac cochlear potential develops (Table 2)--days 37 to 50 in the guinea pig (Pujol and Hilding, 1973). This study was interested in finding out if these just-developed hair cells were susceptible to aminoglycoside destruction. If the aminoglycosides produce a functional deficit one would suspect little ototoxic effect until the hair cells are present. That is why this time frame was chosen. This experiment was not an attempt to identify a "critical period."

Third, the electrophysiological technique used to identify ototoxicity was identical to that used to identify ototoxicity in adults. This technique has been shown to be extremely sensitive to hair cell loss and to be highly repeatable (e.g. Brummett, Brown and Himes, 1979). In order to be tested in this system offspring were allowed to survive 30 days after birth. This provided a 45 day period of stabilization for ototoxicity in the offspring and the mother prior to electrophysiological evaluation.

A fourth difference was inclusion of injected control animals. Control animals allowed for a baseline against which the treated animals could be compared. Control animals were especially important because the treated mothers were mature adults. By including control animals of the same relative age changes in

hearing due to aging could be equated. These aging changes are normal and in humans are called presbycusis.

Method

Subjects. Seven pregnant guinea pigs of the Topeka strain were used. Their pregnancies were timed in the following manner. A group of females was continuously housed with an adult male guinea pig. They were allowed to become pregnant and give birth in the presence of the male. The birth of the litter served as a time marker for the second litter because female guinea pigs will become pregnant immediately after delivery of a litter if housed with a male. Five days after delivery the mother and her offspring were removed from the presence of the male. This prevented any nonpregnant female from conceiving at a later time. At most a five day error out of a 65 day pregnancy could exist. Kaufmann and Davidoff (1977) used this technique successfully to time guinea pig pregnancies for study of placental anatomy and physiology.

Mothers were tested for the presence of the Preyer pinna reflex before being entered into the study.

Procedure. At thirty-seven days gestation in the second pregnancy five of the pregnant guinea pigs began receiving daily subcutaneous injections of kanamycin sulfate (Kantrex, Bristol, 333 mg/ml) at 200 mg/kg. Also begining day 37 of gestation the two other pregnant guinea pigs began receiving a daily 0.6 ml/kg subcutaneous injection of 0.9% NaCl solution. This volume of saline is equivalent to the volume of kanamycin received by the other guinea pigs. Injections continued for fourteen days through

day 50 of pregnancy. The pregnant guinea pigs were weighed and their doses adjusted daily.

The mothers were allowed to deliver this second litter normally. A thirty day stabilization period was given after birth. This allowed for stabilization of cochlear pathology.

After the stabilization period had elapsed each pup and mother were evaluated for the ac cochlear potential. This procedure, described in detail in Appendix B, was the standard procedure for assessing the ac cochlear potential. The procedure included measuring the amount of sound necessary to produce one microvolt of ac cochlear potential at 18 frequencies and the maximum output of the ac cochlear potential at two frequencies for each ear. During surgery respiration and body temperature were maintained as described in Appendix B.

Surface preparations of the right ears of the treated mothers were examined under a phase contrast microscope (Appendix B). Outer hair cells were counted in 0.1 mm segments of the organ of Corti from apex to base. In addition the cochleae of any guinea pig pups demonstrating an abnormally low isopotential sensitivity were also dissected and examined microscopically. The electrophysiological techniques used are recognized as being very sensitive for detecting hair cell loss. Therefore, the cochleas of guinea pigs showing normal isopotential functions and maximum outputs were not dissected nor were their hair cells counted.

Data analysis. The one microvolt isopotential data were averaged and graphed at each frequency for the pups and the

mothers. An analysis of variance was computed for the mothers and for the offspring. The analysis of variance used unweighted means which allowed for unequal numbers of subjects within each group. Since the aminoglycoside antibiotics differentially affect the test frequencies, stimulus frequency was included in the analysis as a repeated measure. Unilateral changes in the sensitivity of the cochleae of a single animal are often noted in treated animals. For this reason each cochlea was treated as an independent measure. This is a common practice in ototoxicity research.

Maximum outputs for saline and kanamycin mothers and pups were averaged at 1 kHz and 10 kHz. Student's t-test for unrelated groups was calculated comparing the maximum outputs of pups from mothers treated with kanamycin to those of pups whose mothers had recieved saline injections. Student's t-test for unrelated groups was also calculated for the maximum outputs of the two groups of mothers. The counts of remaining outer hair cells for the subjects examined were graphed in 0.1 mm segments. Innermost, middle and outermost rows of outer hair cells were individually graphed from apex to base.

Results

In all two control mothers and their five offspring were utilized in this experiment along with five treated mothers and their sixteen offspring.

Figure 1 shows the average one microvolt isopotential curves recorded from mothers treated with 200 mg/kg per day kanamycin for two weeks and those mothers injected with saline. The triangles

represent five treated mothers (ten ears) and the circles represent two saline mothers (four ears). Note especially the difference in the high frequencies between the control and treated mothers in Figure 1. The difference observed is the classic aminoglycoside ototoxic effect on the ear--affecting the high frequencies first. The analysis of variance confirms this analysis (Table 3). A significant main effect is noted for the kanamycin treatment. The aminoglycoside effect is demonstrated by the statistically significant interaction effect of kanamycin treatment by stimulus frequency. A puzzling significant effect is a within subject main effect of frequency. Keep in mind, however, that this is a repeated measure for each ear. Since the normal isopotential function of the guinea pig is not flat across frequency the analysis of variance computation is detecting normal changes in the isopotential function with frequency. Perhaps it would be more surprizing if a significant frequency effect was not noted. It should be pointed out that because of the small sample size in these groups of mothers, caution should be exercized in interpreting these statistical tests.

The maximum output of ac cochlear potential differed for the mothers who received kanamycin and those who received saline (Figure 2). At 1 kHz mothers who received kanamycin were able to produce a maximum output of 253 microvolts of ac cochlear potential and those who received saline were able to produce 738 microvolts of ac cochlear potential. This difference was statistically

Figure 1. The intensity of sound required to produce one microvolt of ac cochlear potential at eighteen frequencies between 100 and 32,000 Hz. These data represent 5 pregnant females who received 200 mg/kg per day kanamycin injections for 14 days and 2 pregnant females who received isotonic saline injections for 14 days. Note that sound intensity increases in the upward direction.

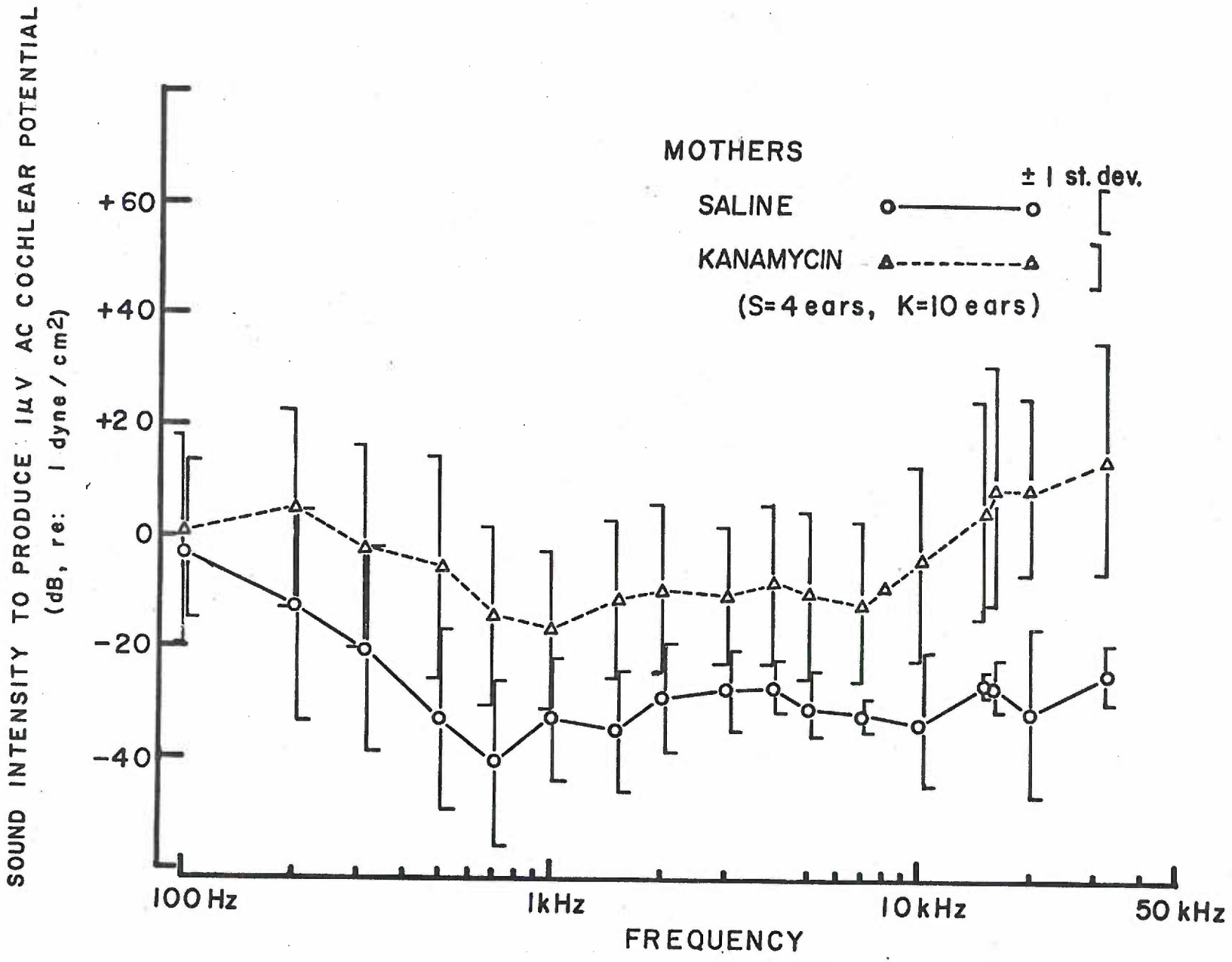

MOTHERS
± 1 st. dev.
SALINE
KANAMYCIN
(S=4 ears, K=10 ears)
SOUND INTENSITY TO PRODUCE 1μV AC COCHLEAR POTENTIAL
(dB, re: 1 dyne / cm2)
+60
+40
+20
0
-20
-40
100 Hz
1kHz
10kHz
50 kHz
FREQUENCY

figure 1

Table 3

Analysis of Variance for Isopotential Functions of Mothers in Experiment 1

Summary Table

(2-way, repeated measures on stimulus frequency, unequal N)

Source	Sums of Squares	df	Mean Squares	F	p
Between Subjects					
Kanamycin Treatment	27778.64	1	27778.64	10.78	p<.01
Error	30922.26	12	2576.85		
Within Subjects					
Stimulus Frequency	10877.74	16	679.86	5.96	p<.01
Treatment X Stim. Freq.	4023.44	16	251.46	2.20	p<.01
Error	21916.59	192	114.15		

Table 4

Maximum Outputs

A.C. Cochlear Potential

	Saline	Kanamycin		
Mothers				
1 kHz	738 (238)*	253 (312)	$p<.05$	($t=2.77$, $df=12$)
10 kHz	211 (90)	48 (66)	$p<.05$	($t=3.77$, $df=12$)
Offspring				
1 kHz	1306 (441)	1520 (463)	NS	($t=.96$, $df=40$)
10 kHz	325 (132)	415 (125)	NS	($t=1.96$, $df=40$)

All measurements are microvolts of ac cochlear potential.

*Figure in parentheses is standard deviation

NS=Not significant

Figure 2. Maximum outputs of the ac cochlear potential for mothers and their fetuses. These data were measured at 1 kHz and 10 kHz in mothers who had received kanamycin injections during pregnancy or saline injections during pregnancy. Their fetuses were tested in the same manner. The difference in output between the control mothers and control offspring is probably due to the effects of age on the mothers ears. The organ of Corti loses hair cells as a part of the aging process. This is called presbycusis in humans.

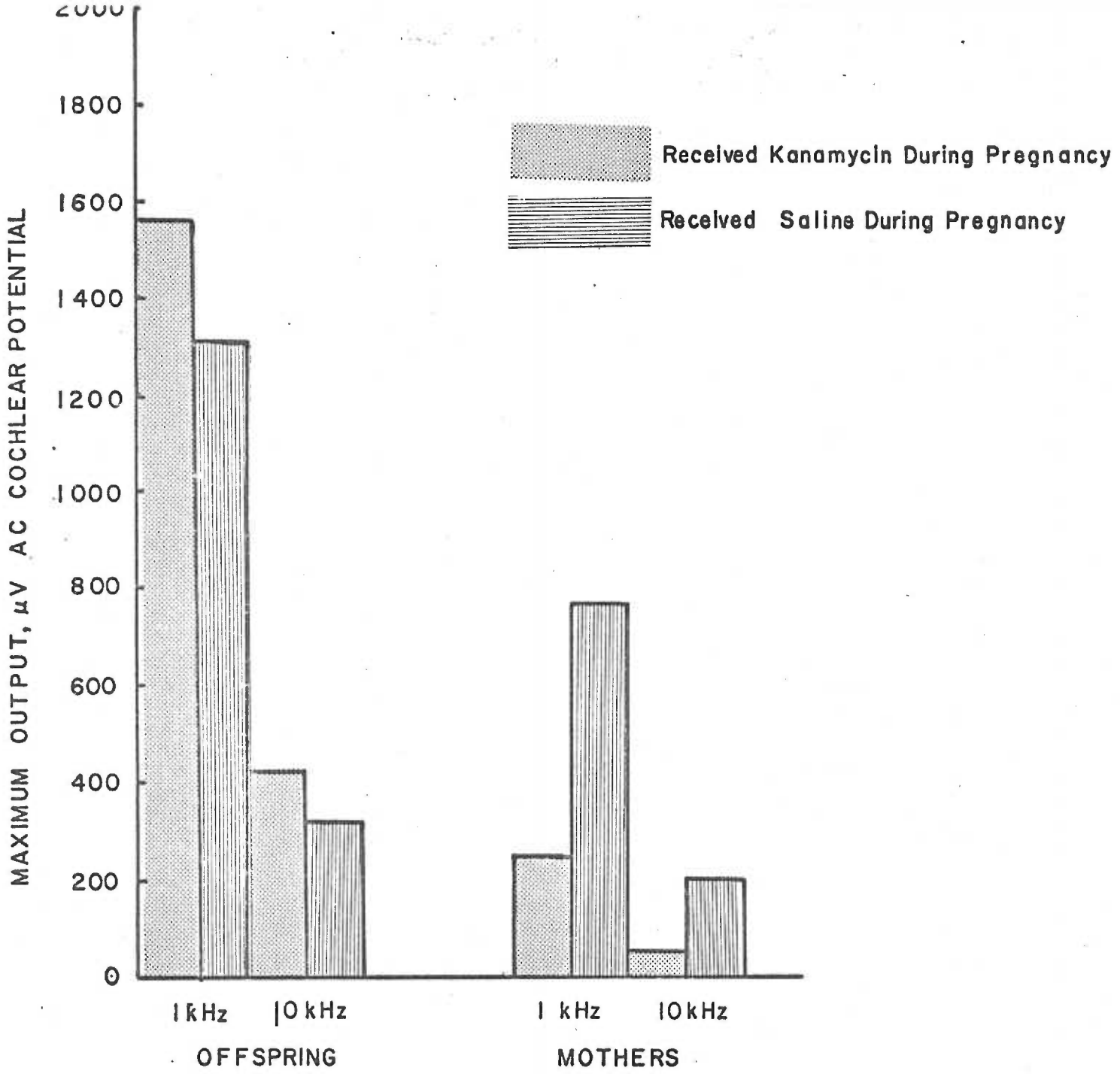

2000
1800
1600
1400
1200
1000
800
600
400
200
0
MAXIMUM OUTPUT, μV AC COCHLEAR POTENTIAL
Received Kanamycin During Pregnancy
Received Saline During Pregnancy
1kHz
10kHz
1 kHz
10kHz
OFFSPRING
MOTHERS

Figure 2

significant ($p<.05$, Table 4) The kanamycin treated mothers were only able to generate about one third of the maximum output of those mothers treated with saline. At 10 kHz the difference between the two groups of mothers is even greater. Mothers who received kanamycin were able to produce a maximum ac cochlear potential of 48 microvolts at 10 kHz while the saline group was able to produce 211 microvolts of ac cochlear potential. This difference was statistically significant ($p<.05$, Table 4). The mothers receiving kanamycin were only able to generate one fourth of the maximum output of ac cochlear potential of the saline injected mothers. This indicates the injections of kanamycin during pregnancy adversely affected the cochleae of the mothers. The maximum outputs of both groups of mothers were lower than those normally recorded in ototoxicity studies in this laboratory using young adult guinea pigs (see control animals in experiment 3). This diminished maximum output is probably due to the age of the mothers. Mammals lose cochlear hair cells throughout their life time and this age related loss is commonly known as presbycusis in human beings.

The presence of ototoxic damage is confirmed by Figure 3a-f. Figure 3a-f are cochleograms for the right ears of mothers who received kanamycin during their pregnancy. The left ear of mother 2213A was also counted because of extreme artifactual damage to the right ear due to dissection. Clearly hair cells are missing in the base region of the kanamycin treated mother's cochleae. Because they are missing from the base (the high frequency region)

this hair cell loss is probably due to kanamycin ototoxicity.

No ototoxic effect was noted for the pups born to those mothers receiving kanamycin. No treatment effects can be seen in either the one microvolt isopotential function (Figure 4) or main effects of analysis of variance (Table 5). There were no significant differences in the maximum output data (Table 4). A puzzling within groups interaction (Treatment X Stimulus Frequency) occurred. This interaction was probably due to the effects of a middle ear conductive disorder in three ears of the kanamycin group. It is general knowledge that a conductive hearing loss of the middle ear differentally reduces the sensitivity of the low frequencies the most in the early stages. Three pup ears from mothers who received kanamycin injections were identified which had changes interpreted as conductive hearing losses. Two of these guinea pig pups had mild low frequency losses with normal high frequency sensitivity. The surface preparations of these two ears were qualitatively reviewed and no abnormal hair cell loss was noted. The third pup had an extensive loss of sensitivity. This pup was from a mother had who received kanamycin injections during pregnancy and it required very high sound pressure levels to produce the one microvolt isopotential function. Figure 5a and b indicate that normal numbers of hair cells were present in this pup's ears. This would indicate that the unilateral loss of sensitivity was probably due to the inability of this guinea pig's middle ear to efficiently conduct sound to the inner ear. None of the changes described above was due to aminoglycoside administration.

Figure 3. Average number of outer hair cells present per 0.1 mm of organ of Corti for pregnant females treated with 200 mg/kg per day of kanamycin sulfate for 14 days.

Figure 3a. Cochleogram for right ear of 2213A. Although hair cell loss can be noted in outermost row of outer hair cells, extensive dissection artifact is present. Because of this damage the left ear of 2213A was also dissected and counted.

Figure 3b. Cochleogram for left ear of 2213A. Hair cell loss in base is clearly visable in innermost row of outer hair cells. The isopotential function for 2213A was not grossly abnormal.

Figure 3c. Cochleogram for right ear of 2197A. These data clearly indicate the complete loss of hair cells in the base due to kanamycin ototoxicity.

Figure 3d. Cochleogram of right ear for 2151A. Note greater loss of innermost row of outer hair cells over length of basilar membrane. Hair cell loss was greater in basal portion (high frequency region) of the cochlea.

Figure 3e. Cochleogram for right ear of 2083A. Again the innermost row of outer hair cells has been affected most by ototoxic drug exposure. Hair cell loss was greatest in the base region of the cochlea.

Figure 3f. Cochleogram for right ear of 2211A. Although dissection artifact is present the extensive loss of outer hair cells can be noted. Damage begins very high in this ear--about 4 or 5 mm from the apex.

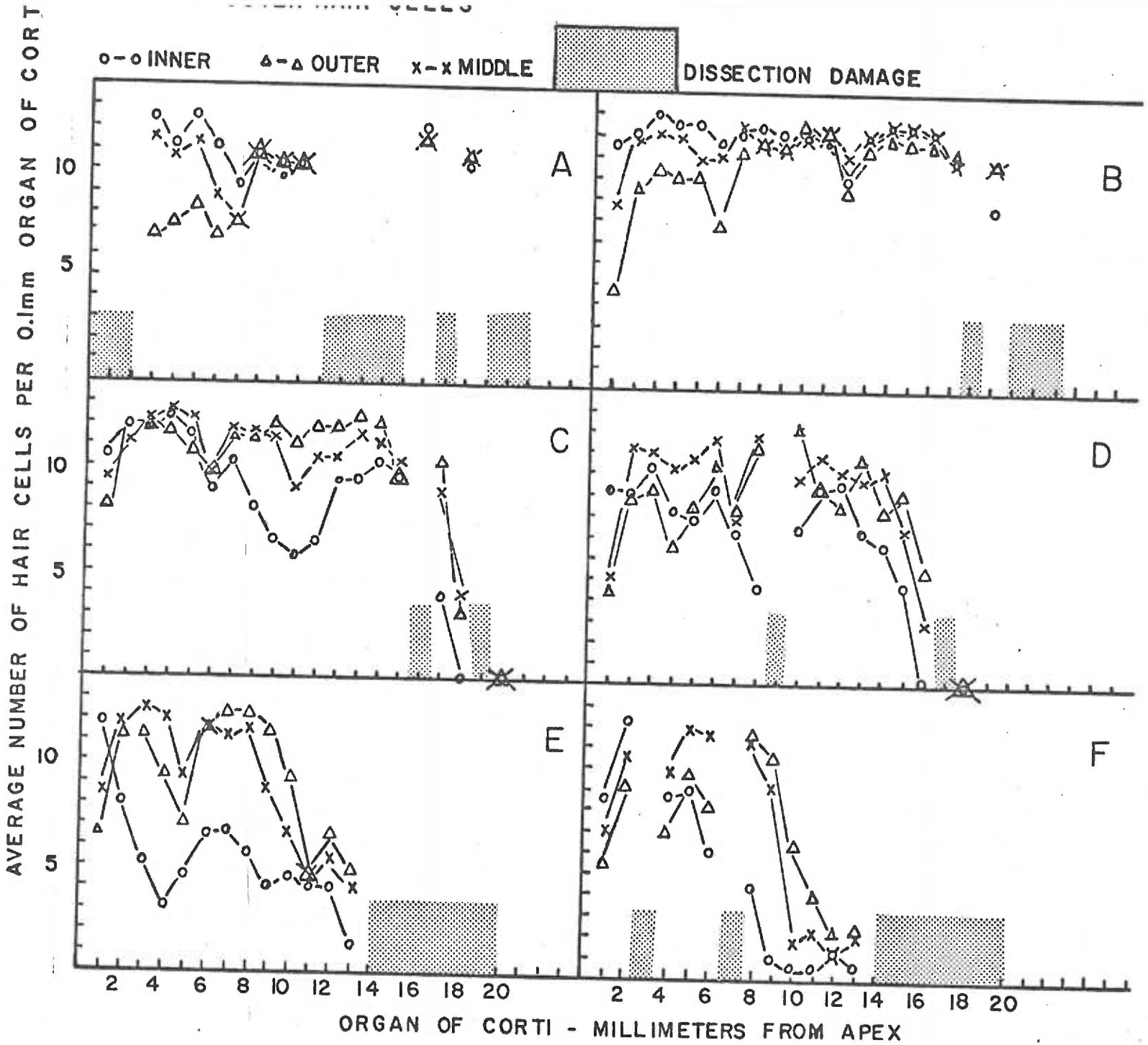

o–o INNER
Δ–Δ OUTER
x–x MIDDLE
DISSECTION DAMAGE
AVERAGE NUMBER OF HAIR CELLS PER 0.1mm ORGAN OF CORT
A
B
C
D
E
F
10
5
2 4 6 8 10 12 14 16 18 20
ORGAN OF CORTI - MILLIMETERS FROM APEX

Figure 3

Table 5

Analysis of Variance for Isopotential Functions of Offspring in Experiment 1

Summary Table

(2-way, repeated measures on stimulus frequency, unequal N)

Source	Sums of Squares	df	Mean Squares	F	p
Between Subjects					
Kanamycin Treatment	736.94	1	736.94	0.46	NS
Error	64396.21	40	1609.91		
Within Subjects					
Stimulus Frequency	13257.50	16	828.59	27.17	$p<.01$
Treatment X Stim. Freq.	922.67	16	57.67	1.89	$p<.05$
Error	19516.51	640	30.49		

NS=Not significant

Figure 4. The sound intensity necessary to produce one microvolt of ac cochlear potential in the offspring. One group was from mothers injected with 200 mg/kg kanamycin for two weeks during pregnancy and the other group was from mothers who had received saline injections for two weeks during pregnancy. Sound intensity increases in the vertical direction. No differences were detected between the two groups.

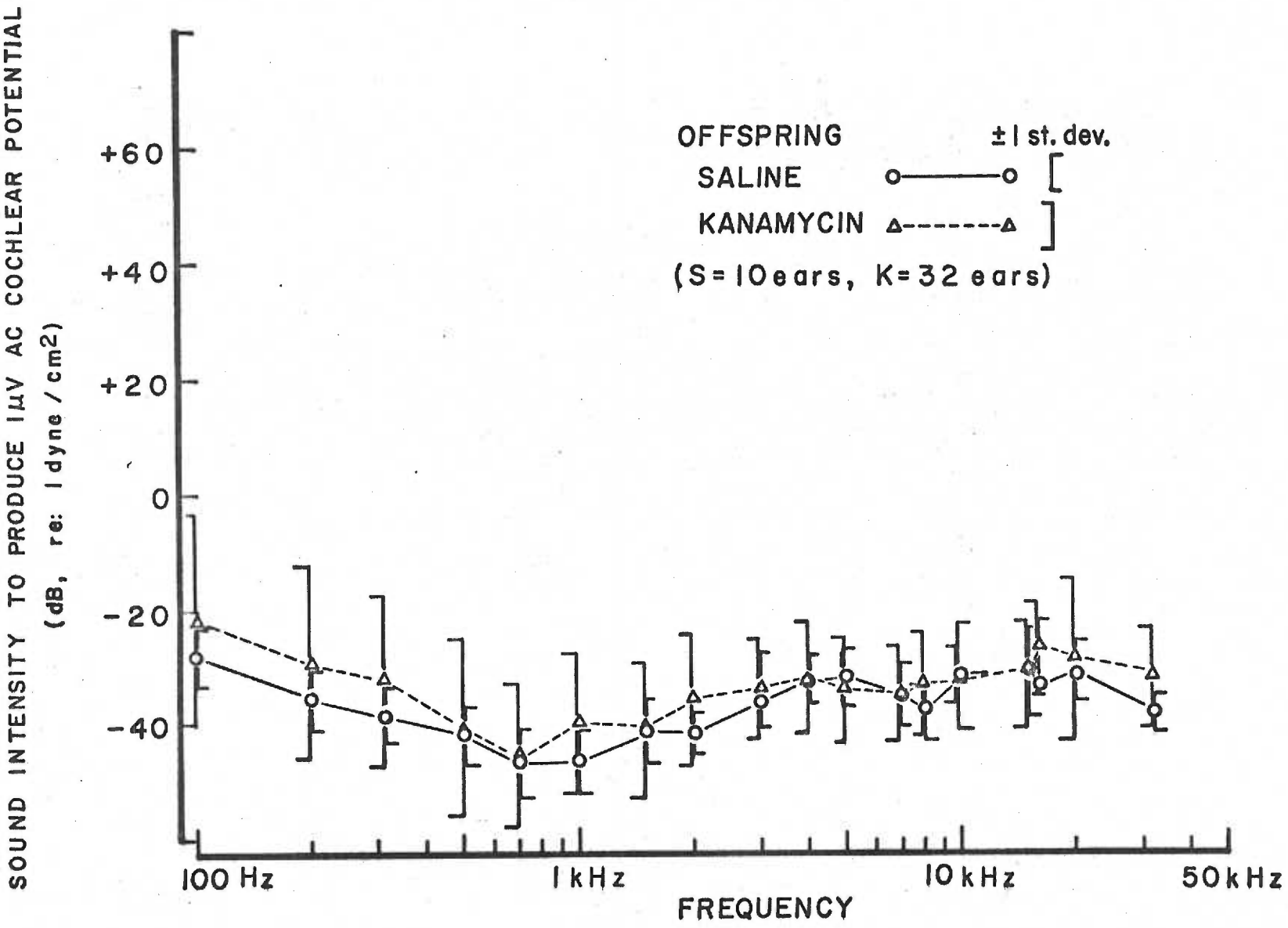

SOUND INTENSITY TO PRODUCE 1μV AC COCHLEAR POTENTIAL
(dB, re: 1 dyne / cm2)
+60
+40
+20
0
-20
-40
OFFSPRING
± 1 st. dev.
SALINE
KANAMYCIN
(S = 10 ears, K = 32 ears)
100 Hz
1 kHz
10 kHz
50 kHz
FREQUENCY

figure 4

Figure 5a. Cochleogram of the right ear of offspring 2124A. This ear had a normal isopotential function. This guinea pig's mother had received kanamycin for two weeks during pregnancy. This pup had demonstrated a unilateral loss of sensitivity in the left ear. A normal number of hair cells are present throughout the cochlea of this ear.

Figure 5b. Cochleogram of the left ear of offspring 2124A. This ear had a decreased sensitivity to sound as measured by the isopotential function. Normal numbers of hair cells in the base indicate that the loss of sensitivity was probabily due to mechanical losses in the middle ear rather than sensory cell losses in the inner ear due to ototoxicity.

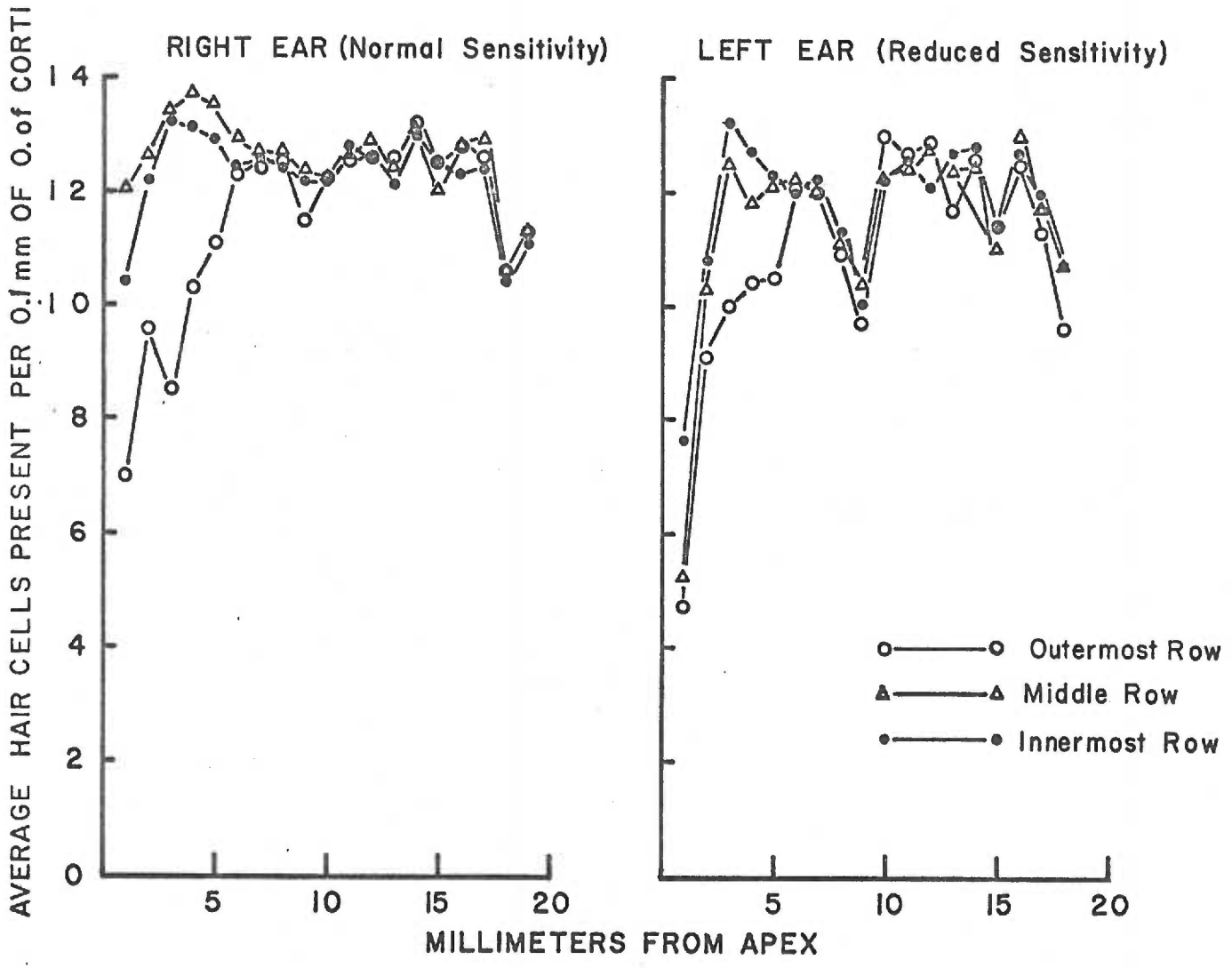


Figure 5a

Figure 5b

Discussion

One important finding of experiment 1 is the difference in ototoxicity shown between treated mothers and their offspring. Auditory systems of kanamycin treated mothers clearly differ from those of saline injected mothers. Yet offspring absorbing kanamycin through the placenta during pregnancy display no adverse effects when they are compared to control offspring.

This difference between fetal and maternal ototoxicity might be explained in two ways. First, the fetal ear may be immune to the effects of kanamycin. Perhaps tissue within the ear is not mature and does not bind the aminoglycoside antibiotics. This seems to be the theory Uziel, et al (1977) favor.

A second explanation is that the placenta blocks most of the aminoglycoside from entering the fetal circulation. The mother is operating on the middle, linear portion of the log dose-response curve for ototoxicity but the fetus receives only a small concentration of aminoglycoside and is operating on the subthreshold portion of the ototoxicity log dose-response curve. Based on human data reviewed earlier and guinea pig data (Andersen and Pedersen, 1971) this would be a reasonable hypothesis. It is known that the fetus has lower aminoglycoside antibiotic concentrations in blood than the mother (Akiyoshi, et al, 1974). One piece of data which has not been measured in fetuses is the level of kanamycin present in the perilymph of the fetal ear. Since perilymph bathes the hair cell bodies it is important to measure the concentration of kanamycin present.

In experiment 1 the pregnant mothers were chronically injected with kanamycin or saline. If more drug is administered before the body can completely eliminate or biotransform it the drug will accumulate. The elimination of kanamycin from adult guinea pig perilymph requires a very long time (Stupp, et al, 1967 reports detecting kanamycin in normal, adult guinea pig perilymph 24 hours after administration in some cases). The time needed to eliminate kanamycin from the perilymph of fetal guinea pigs is unknown.

Experiment 2: Levels of kanamycin in fetal and maternal body fluids.

Experiment 2 was designed to measure kanamycin concentrations in the blood and perilymph of the mother and blood, perilymph and amnionic fluid of the fetus after chronic and single injection schedules. This would allow a determination of accumulation in either the mother or the fetus or both. A preliminary experiment was conducted to determine the time for optimum sampling.

Method

Subjects. Eighteen pregnant guinea pigs of the Topeka strain had their pregnancies timed as described in experiment 1. All pregnancies were 50 days of gestation at the time of the experiment. Recall in experiment 1 that the pregnant guinea pigs were injected from day 37 to 50 of pregnancy. Therefore, gestation day 50 in this experiment represents the final day of injection in experiment 1.

Procedure. Pregnant guinea pigs were assigned to one of six

groups. Each group contained three mothers. A single subcutaneous injection of 200 mg/kg of kanamycin sulfate was given and the time recorded. The first fetal sample was taken at 1 hr, 2 hrs, 4 hrs, 6 hrs, 8 hrs or 24 hrs post-injection. These times were used to identify the individual groups of mothers and fetuses.

Thirty minutes before the first fetal sample was to be taken the pregnant guinea pig was anesthetized with Dial with urethane as described in Appendix B. Fur was clipped from around the pinna, the chest and the abdomen. Breathing and body temperature were supported as described in Appendix B.

A longitudinal 3 to 5 cm incision was made through the abdominal muscle layer. A cut was made through the lateral wall of one horn of the uterus exposing the amnionic sac with the fetus inside. The amnionic sac was opened. A double loop of surgical suture was tied around the umbilical cord and the cord was cut between the loops. The time of cord ligation was noted. A cut was made along the fetal sternum and a note was made indicating whether the heart was beating or not. A 3 ml syringe with a 21 gauge by 1 inch needle was inserted into the left side of the heart and approximately one milliliter of blood was withdrawn. The time of blood sampling was recorded. The time of removal of the blood sample in the first fetus corresponded to the group label. Thus, first fetal sample was taken at one of the six time intervals after the injection as listed above.

After blood sampling the perilymph of the fetus was sampled in the following manner. The left pinna was held by forceps and

removed with a scissor cut. A stereomicroscope was used to visualize the operation. The bone of the bulla was opened, the middle ear space cleared of fluid and a sample of fetal perilymph was taken. Perilymph was sampled in the following manner. A glass micropipet had previously been weighed to one ten-thousandth of a gram on a Metler analytical balance. The small tip of this micropipet was inserted through the round window membrane of the fetal cochlea. Gentle mouth suction was applied to the tubing attached to the pipet until a small sample (1 to 5 microliter) of perilymph was removed. Care was taken to avoid contaminating the outside of the pipet with blood, tissue, fur, skin oils, etc. The micropipet was then reweighed to determine sample volume. (In this study a simplifying assumption was used that the density of perilymph was the same as that of water:one milligram=one microliter. We know that this is not the case, however until perilymph density is known this error cannot be corrected.) The same procedure was repeated to remove a sample of perilymph from the right ear.

Each fetus was sampled in the same manner in turn. Finally, heart blood and perilymph samples were taken from the mother.

Perilymph samples were transferred to numbered test tubes containing premeasured volumes of phosphate buffer solution and frozen until assayed. The blood samples were centrifuged for ten minutes to remove blood clots and red blood cells. Serum was transferred to labelled polystyrene sample tubes.

Serum and perilymph were assayed for kanamycin using a stan-

dard radioenzymatic assay specific for aminoglycoside antibiotics (Appendix A). Any perilymph sample with a volume of less than 2 microliters or containing blood was eliminated from analysis.

Appendix A describes how the data were analyzed in order to convert data obtained from the assay into kanamycin concentrations.

Results

In all, 18 mothers and their 74 fetuses were utilized in this study.

Figure 6 is a graph of maternal and fetal blood concentrations measured throughout the 24 hour (1440 minute) sampling period. Maternal and fetal sampling times differ from each other slightly since each pregnant guinea pig contained various numbers of fetuses and each fetus required differing amounts of time to remove the samples. The 24 hour time period was divided into fifty minute periods and maternal and fetal data points were averaged within these fifty minute periods. The dashed and solid lines of Figure 6 represent average kanamycin concentrations in serum over 24 hours for the mothers and the fetuses respectively. The maximum concentration of kanamycin in serum occurred at about 120 minutes for the mother and about 200 minutes for the fetus. The peak concentration of kanamycin in serum was much less in the fetus than in the mother. The average maternal peak concentration was 235 micrograms of kanamycin per milliliter of serum with a standard deviation of 70 micrograms per milliliter. Average peak fetal concentration was 28 micrograms of kanamycin per milliliter of serum with a standard deviation of 18 micrograms

Figure 6. Serum concentration to a single injection of kanamycin in mothers and their fetuses measured over a twenty-four hour period. Data has been averaged by grouping data into 50 minute time periods.

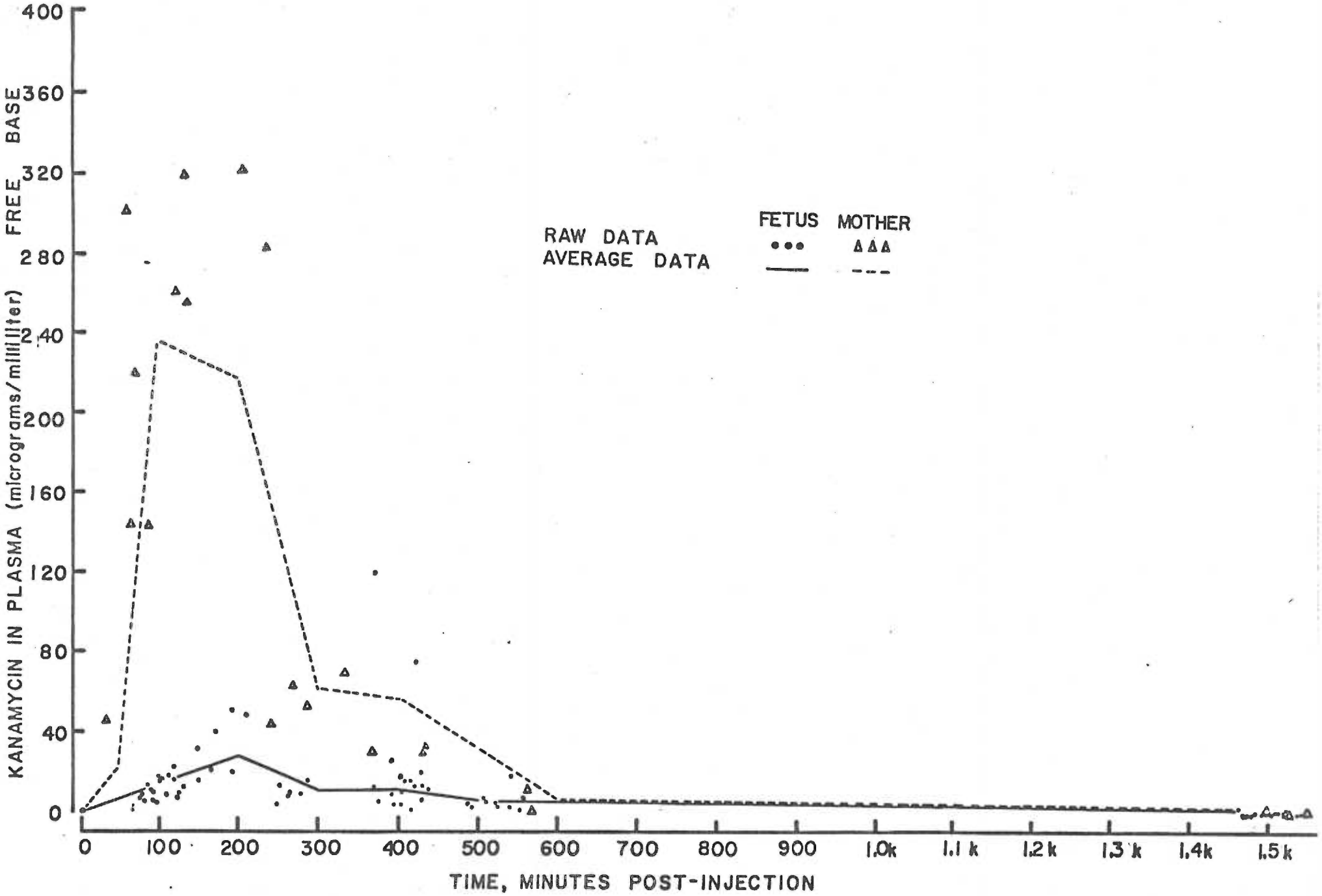

KANAMYCIN IN PLASMA (micrograms/milliliter) FREE BASE
400
360
320
280
240
200
160
120
80
40
0
RAW DATA
AVERAGE DATA
FETUS
MOTHER
0
100
200
300
400
500
600
700
800
900
1.0k
1.1 k
1.2 k
1.3 k
1.4k
1.5k
TIME, MINUTES POST-INJECTION

Figure 6

Figure 7. Perilymph kanamycin sulfate concentration to a single kanamycin injection, measured over time. Average values were derived by averaging 50 minute time periods. No twenty-four hour perilymph samples met the criterion of a volume greater than 2 microliters or being free of blood contamination.

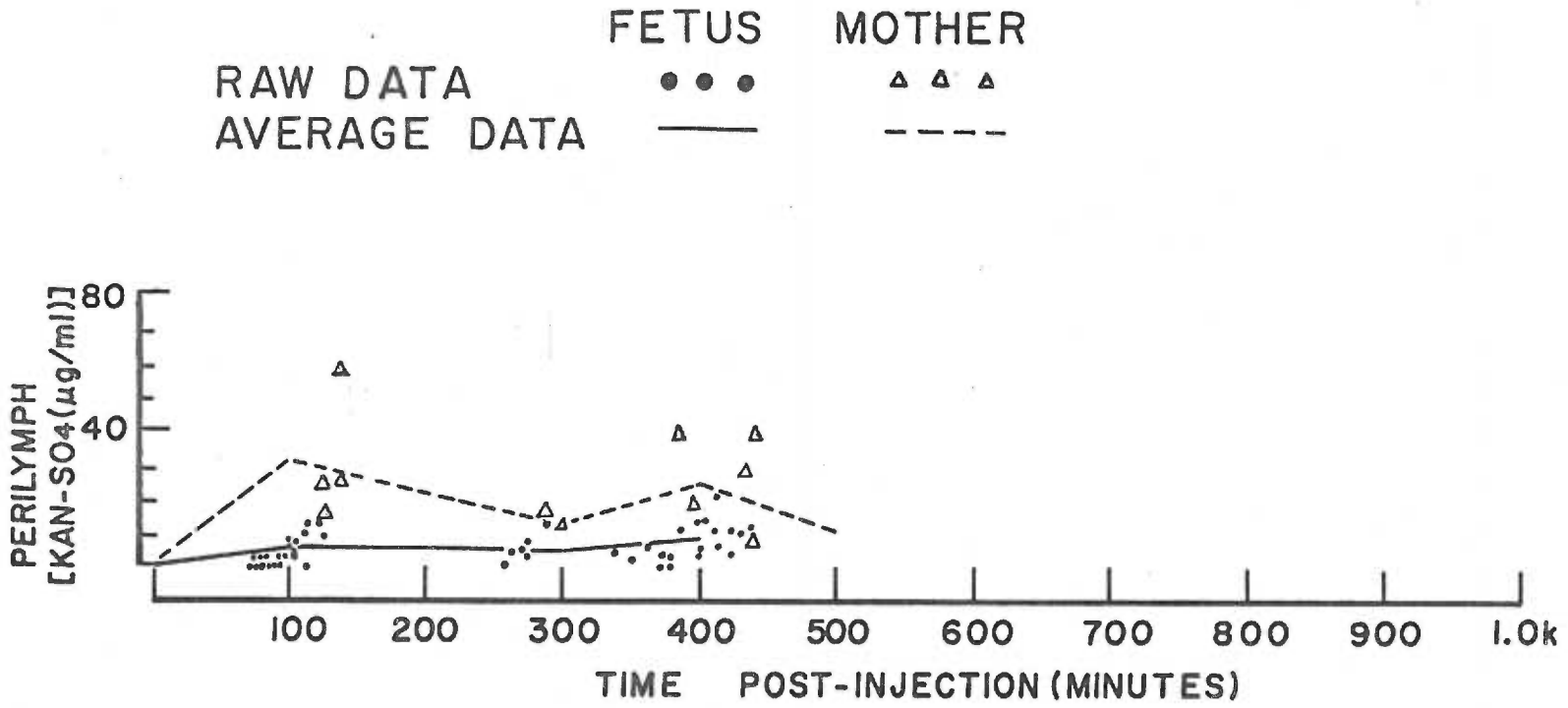

FETUS
MOTHER
RAW DATA
AVERAGE DATA
PERILYMPH [KAN-SO4(μg/ml)]
80
40
100
200
300
400
500
600
700
800
900
1.0k
TIME POST-INJECTION (MINUTES)

Figure 7

per milliliter.

Concentrations of kanamycin sulfate in perilymph are presented in Figure 7. Figure 7 was constructed in the same manner as Figure 6. Kanamycin sulfate concentrations in perilymph are extremely variable for both the fetus and mother. Because of the extreme variablility of the perilymph samples a single peak concentration could not be identified for either the mother or fetus. The range of maternal perilymph kanamycin sulfate concentarations was 7 to 58 micrograms per milliliter of perilymph. Fetal perilymph ranged from 0 to 42 micrograms kanamycin sulfate per milliliter of perilymph.

Discussion

These data provided a beginning for measuring levels of kanamycin in plasma and perilymph of fetuses and mothers. On the basis of these data the period chosen for sampling was two hours (120 minutes) post-injection.

As explained in the introduction to this section accumulation could occur in the fetus to aminoglycoside antibiotics if the fetus inadequately eliminated the antibiotic before a new injection of antibiotic was administered. The next experiment was designed to detect accumulation at one point in time.

Method

Subjects. Eleven pregnant guinea pigs of the Topeka strain were used. In the previous two experiments timing of one pregnancy could require 3 months or more. The pregnant guinea pigs used in this experiment were obtained from the Kresge Lab general

breeding colony and gestation stage was determined by abdominal palpation. This significantly decreased the time required to time a pregnancy. However, this procedure decreases the accuracy of predicting the date of gestation. As a check on this procedure, fetuses were weighed after the surgical procedure and found to be within the weight limits observed for fetuses of 50 days gestation in the previous two experiments.

All mothers were required to produce the Preyer pinna reflex response to a vocalization in order to be included in this experiment.

Procedure. Pregnant guinea pigs were assigned to one of two groups. One group, containing seven females, received a daily subcutaneous injection of 200 mg/kg kanamycin sulfate for 14 days from gestation day 37 to 50. They were weighed and their doses calculated and adjusted daily. This group was equivalent to the mothers treated and evaluated electrophysiologically in experiment 1.

The second group of females received a single subcutaneous injection of 200 mg/kg kanamycin sulfate on day 50 of pregnancy. This group was equivalent to the single injection mothers of the previous experiment.

All guinea pigs were anesthetized with Dial with urethane one and one half hours after their last (or only) injection of kanamycin. Anesthesia, surgical preparation, respiration and temperature support were the same as the preliminary experiment in this series.

Surgery was the same as the last experiment. Appendix A describes how blood and perilymph samples were assayed and how data derived from the assay was mathmatically manipulated to determine the concentrations of kanamycin.

Data analysis. A Student's t-test for unrelated groups was calculated to determine if kanamycin concentration levels measured in serum and perilymph of the mothers and fetuses differed significantly between pregnant guinea pigs injected for two weeks and pregnant guinea pigs injected only once.

Results

Three of the seven multiple injected pregnant guinea pigs contained a large number of dead fetuses. Their data were discarded. Only living fetuses and mothers containing living fetuses were used for this experiment. This left four mothers in each of the two groups.

Table 6 lists kanamycin concentrations measured in maternal serum and perilymph. One grouping is the guinea pig mothers who received multiple injections of kanamycin, the other is the guinea pig mothers who received a single injection of kanamycin. Raw data are presented to indicate the variability observed. Although mothers in the single injection group had almost twice the serum concentration of kanamycin of the multiple injection group (716 micrograms per milliliter versus 441 micrograms per milliliter) this difference was not significantly different (Table 8) because of the variability in both groups. The kanamycin concentrations in maternal perilymph also exhibit a large variability but the

means of the two groups were very close. An attempt was made to determine if the groups could have extreme body weight differences. This could be a source of variability since kanamycin was given on a per weight basis. The multiple injection mothers averaged 1020 grams and the single injected mothers averaged 1329 grams. The difference between weights in the two groups is statistically significant ($t=3.32$, $df=6$). The lower average weight for the multiple injected mothers could be due to toxic effects of the kanamycin injection. An attempt was made to explain variability on the basis of the number of pups in each mother. However, the average number of pups did not differ greatly--about 3 in each group.

Average maternal perilymph kanamycin concentrations were 25.8 micrograms per milliliter for those mothers receiving two weeks of multiple injections and 28.3 micrograms per milliliter for those mothers injected only once. Figure 8 shows that maternal serum concentrations and perilymph concentrations of kanamycin relate to each other. A correlation coefficient of +.63 was obtained for the data in Figure 8.

Table 7 is a listing of kanamycin concentrations for fetal serum, perilymph and amnionic fluid. Again the two groupings represent measurements made in fetuses from mothers who received multiple injections and fetuses from mothers injected only once. Fetuses from mothers who received multiple injections have an average of 22.4 micrograms of kanamycin per milliliter of serum while the fetuses from the mothers injected only once have 20.8

Table 6

Maternal plasma and perilymph levels of kanamycin

Multiple injection

ID No	Serum Levels	Perilymph Levels	Number of Pups	Maternal Wt in grams
011681	327.1*	11.3**	5	1133
010781-8	607.6	61.1	4	905
010781-5	583.6	24.2	3	1220
121181	245.3	6.5	1	823
Average	440.9	25.8		1020.3
St.Dev	(181.9)	(24.7)		(186.9)
		Single injection		
022481	947.8*	35.7**	5	1328
021881	539.6	13.2	3	1521
041681	761.8	38.5	3	1079
041481	616.7	25.8	3	1389
Average	716.5	28.3		1329.3
St.Dev.	(179.6)	(11.4)		(185.3)

* micrograms of kanamycin base per ml of serum

** micrograms of kanamycin sulfate per ml of perilymph

Fetal plasma, perilymph and amnionic fluid levels of kanamycin

Multiple injection

Maternal ID No.	Serum Levels	Perilymph Levels	Amnionic Fluid Levels
	23.4*	7.4**	-----
N=5	21.5	8.4	23.7*
	18.8	6.2	18.6
	21.0	16.6	33.2
	21.5	6.5	29.0
010781-8	16.3	2.9	34.8
N=4	28.1	22.7	35.4
	32.1	21.4	19.9
	36.6	---	37.1
010781-5	11.9	9.6	29.2
N=3	13.0	13.0	22.5
	16.5	13.8	33.5
121180	30.8	3.0	25.6
N=1			
Average (S_D)	22.4 (7.6)	10.9 (6.6)	28.5 (6.4)
N=	13	12	12

Single injection

022481	17.5	2.3	0.2
N=5	17.1	3.0	0.7
	20.5	7.4	0.2
	22.8	10.2	11.1
	20.6	---	17.6
021881	21.5	1.8	24.1
N=3	12.2	7.3	0.0
	23.1	---	0.0
041681	18.4	7.5	1.8
N=3	19.7	12.0	2.1
	19.3	5.3	1.2
041481	27.6	---	1.4
N=3	20.7	1.1	0.7
	29.6	11.5	1.2
Average (S_D)	20.8 (4.3)	6.3 (3.9)	4.5 (7.6)
N=	14	11	14

* micrograms of kanamycin base/ml fluid
** micrograms of kanamycin sulfate/ml perilymph

Table 8

Statistical Tests

Serum	df	Calculated t	
Multiple injected mothers vs Single injected mothers	6	1.94	NS
Fetuses from multiple injected mothers vs Fetuses from single injected mothers	25	0.33	NS
Mothers vs Fetuses	33	13.46	$p<.05$
Perilymph			
Multiple injected mothers vs Single injected mothers	6	0.14	NS
Fetuses from multiple injected mothers vs Fetuses from single injected mothers	21	2.00	NS
Mothers vs Fetuses	29	4.39	$p<.05$
Amnionic Fluid			
Fetuses from multiple injected mothers vs Fetuses from single injected mothers	24	8.63	$p<.05$

micrograms of kanamycin per milliliter of serum. This difference is not statistically significant (Table 8). Measurements of perilymphatic kanamycin concentrations are variable in the fetuses. Fetuses from mothers injected daily for two weeks had an average of 10.9 micrograms of kanamycin sulfate per milliliter of perilymph while fetuses from mothers injected only once had an average of 6.3 micrograms of kanamycin sulfate per milliliter of perilymph. This difference was not statistically significant (Table 8).

The data presented in Figure 9 compare fetal serum concentrations and fetal perilymph concentrations for kanamycin. A correlation coefficient of only +.33 was calculated for the data of Figure 9. This indicates only a slight relationship between serum concentrations and perilymph concentrations.

The one difference which was significant when comparing the fetuses from the mothers who received multiple injections with the fetuses from the mothers injected only once was the kanamycin concentration in amnionic fluid (Table 7). The average amnionic fluid concentrations of kanamycin in fetuses from mothers receiving multiple injections was 28.5 micrograms per milliliter compared to an average of 4.5 micrograms per milliliter of amnionic fluid in the fetuses from mothers injected but once. In the fetuses from mothers injected daily for two weeks the level of kanamycin in the amnionic fluid was almost as high as the level of kanamycin in the fetal blood.

Figure 8. Maternal serum concentrations of kanamycin versus maternal perilymph concentrations of kanamycin sulfate. A correlation coefficient of +.63 indicates a relationship between serum levels of kanamycin and perilymph levels of kanamycin in the mother.

Figure 8

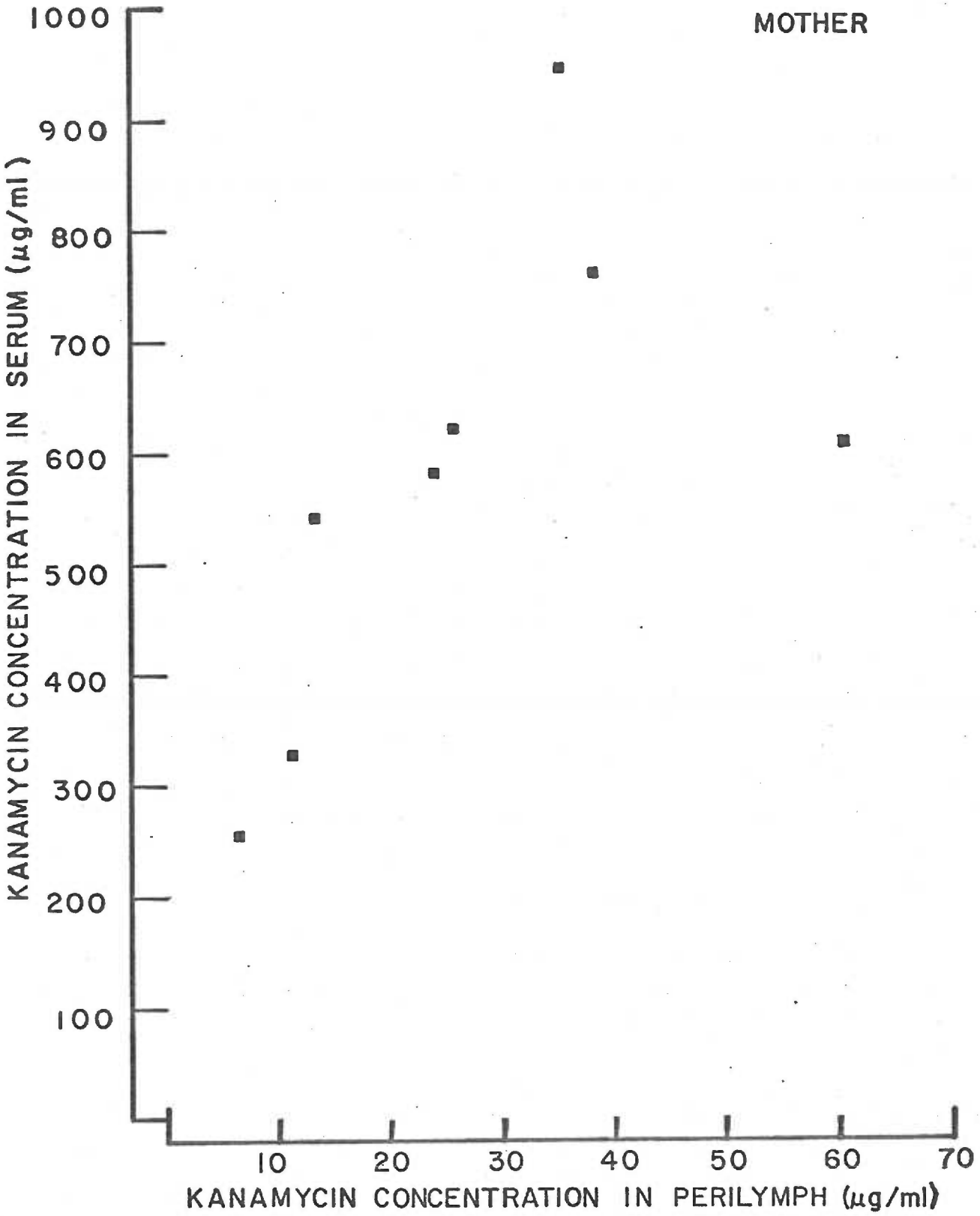
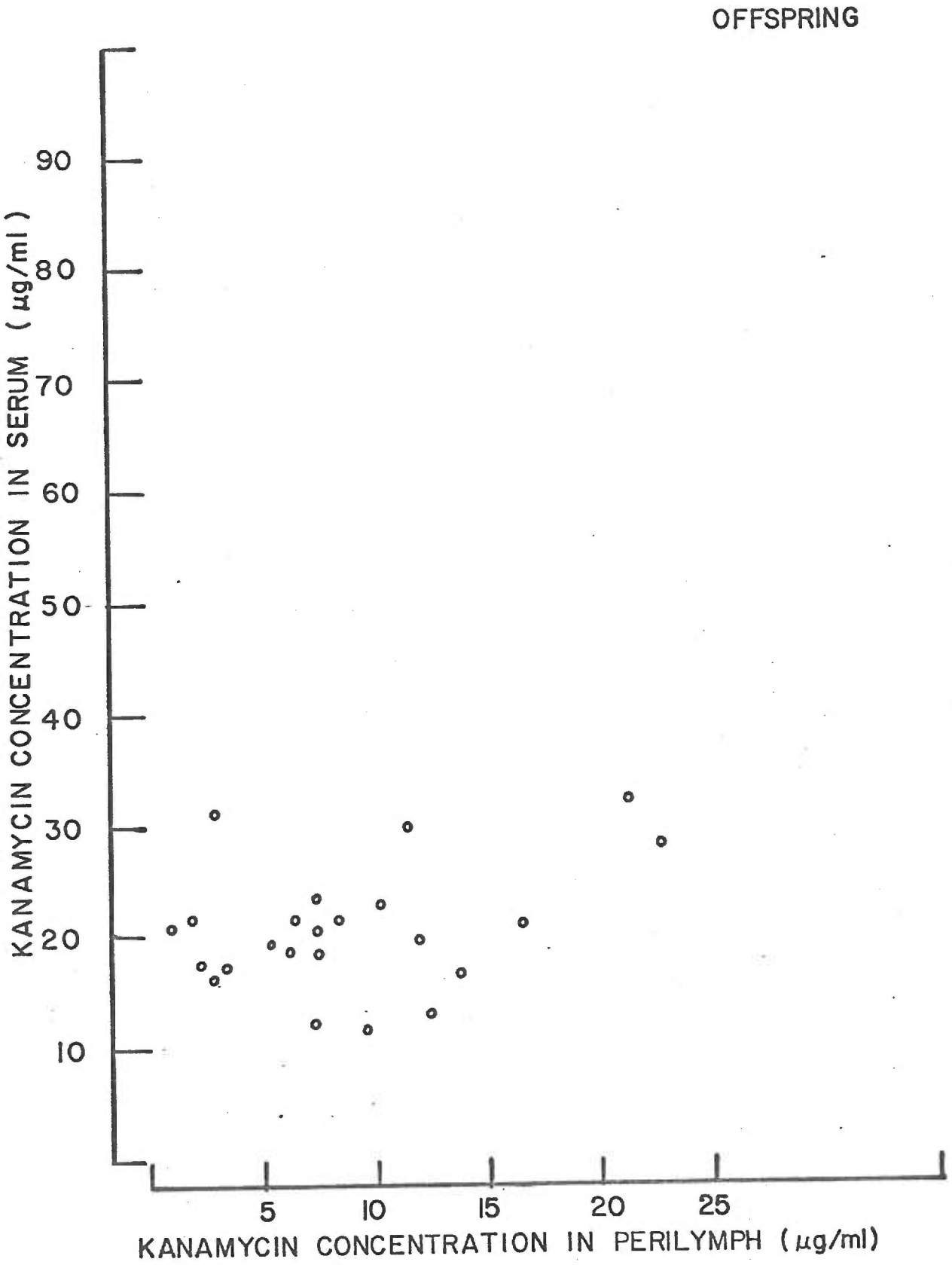

MOTHER
KANAMYCIN CONCENTRATION IN SERUM (μg/ml)
1000
900
800
700
600
500
400
300
200
100
10
20
30
40
50
60
70
KANAMYCIN CONCENTRATION IN PERILYMPH (μg/ml)

Figure 9. Fetal serum concentrations of kanamycin versus fetal perilymph concentrations of kanamycin. A low correlation coefficient (+.3) indicates that a strong relationship does not exsist between fetal kanamycin levels in serum and perilymph.

Figure 9


OFFSPRING
KANAMYCIN CONCENTRATION IN SERUM (μg/ml)
90
80
70
60
50
40
30
20
10
5
10
15
20
25
KANAMYCIN CONCENTRATION IN PERILYMPH (μg/ml)

Discussion

This experiment clearly demonstrates that the fetus is exposed to lower concentrations of kanamycin than the mother. The present experiment indicates that fetal serum contains about 4% of the concentration of kanamycin present in the maternal serum. This agrees with Andersen and Pedersen (1971) who determined fetal guinea pig blood concentrations of kanamycin were only about 7% of maternal blood concentrations.

Average maternal peak concentrations of kanamycin in serum agree in part with other studies. Stupp, et al, (1967) injected guinea pigs with a single dose of 250 mg/kg of kanamycin. They recorded an average peak concentration in serum of 510 micrograms of kanamycin per milliliter. The mean peak concentration for all eight adults in the present experiment was 578.7 micrograms kanamycin per milliliter. In contrast, Toyoda and Tachibana (1978) injected adult guinea pigs with a single dose of 400 mg/kg of kanamycin and measured average peak whole blood levels of 361 micrograms per milliliter. In this case a doubling of the kanamycin dose produced a peak blood level 38% below the present experiment. This difference between Stupp, et al,(1967), the present experiment and that of Toyoda & Tachibana (1978) is disturbing. One explanation might be that this study and Stupp, et al,(1967) used serum as the fluid assayed. Toyoda and Tachibana (1978) used whole blood. If we assume that kanamycin does not enter red blood cells those studies using serum and plasma would discard the volume of the intracellular water of the red blood cells. In

assays using whole blood this volume of intracellar water would be mixed into the assay medium. Red blood cells represent about 45% of the volume of human blood (Berkow, 1977) therefore whole blood kanamycin values should be about half of those of serum levels. A doubling of Toyota & Tachibana's whole blood values would put their data within the expected value of serum kanamycin based on this experiment and Stupp, et al (1967).

Surprisingly, in the present study fetal perilymph levels of kanamycin are about one third of those of maternal perilymph. This is much higher than would be predicted on the basis of the ratios between maternal and fetal serum concentrations. The maternal perilymph levels of kanamycin in both groups (25.8 and 28.3 micrograms per ml) correspond to data reported by Stupp, et al (1967). Stupp, et al injected adult guinea pigs with a single dose of 250 mg/kg of kanamycin and measured about 17 micrograms per milliliter of kanamycin in their perilymph. Injecting twice the dose of kanamycin (400 mg/kg) of the present experiment Toyoda and Tachibana (1978) measured a peak perilymph concentration of 41 micrograms per milliliter of kanamycin in the guinea pig--a little less than twice the maternal perilymph level measured in this experiment.

Both Stupp, et al (1967) and Toyoda and Tachibana (1978) report accummulation in the perilymph of adult guinea pigs. Stupp, et al measured a 59% increase in perilymph kanamycin concentration after a dose schedule of 250 mg/kg daily for 10 days. Toyoda and Tachibana noted an approximately 62% increase in kanamycin con-

centration in the perilymph of scala vestibuli for adult guinea pigs injected daily for 10 days. However, the peak kanamycin concentration was only increased 7% in scala tympani. Toyoda and Tachibana noted the peak concentration for scala tympani occurred earlier with chronic injections. Within the time period that measures were made in the present experiment Toyoda and Tachibana's study showed a statistically significant difference between chronic and single injected guinea pigs. The present study did not discover any evidence of accummulation in the perilymph of chronically injected guinea pigs.

The fetal and maternal kanamycin concentration data provide evidence that perhaps the fetus is operating on a lower portion of the log dose response curve than the mother. The placental structures probably are most responsible for the low kanamycin concentrations. Most kanamycin present in the maternal serum is not able to cross the membrane of the placenta. Kanamycin which crosses into the fetal circulation diffuses back into the maternal circulation as the maternal kanamycin concentration in blood decreases. Levels of kanamycin in amnionic fluid indicate that a second means of elimination exsists for the fetus. This route is through the fetal kidney. McCarthy and Saunders (1975) review some of the theories concerning the production and fate of amnionic fluid. In late gestation it is thought that the fetus contributes a large share of fluid to the amnionic fluid through urine. Boylan, Colbourn and McCance (1958) demonstrated that fetal guinea pigs of this age have functional kidneys. This urine

can carry drugs from the fetal circulation into amnionic fluid. Kauffman, Morris and Azarnoff (1975) measured high levels of gentamicin in the urine of fetal goats. In the present experiment an informal measurement of urine from a fetus whose mother received a single injection of kanamycin indicated the urine kanamycin concentration was in excess of 80 micrograms per milliliter. Table 7 lists fetal kanamycin serum levels and amnionic fluid kanamycin concentrations. Two groups can be identified. One group consists almost entirely of fetuses from multiple injection mothers. These fetuses have relatively high concentrations of kanamycin in amnionic fluid yet their blood levels of kanamycin are equivalent to the second group. The other group consists of fetuses from mothers injected once which contain little or no kanamycin in their amnionic fluid. Three guinea pig fetuses from mothers injected only once have amnionic fluid levels of kanamycin that place them within the range of those fetuses in the first group. The reason most once injected mother's fetuses had little or no kanamycin in their amnionic fluid was probably because they had not emptied their bladders of kanamycin-containing urine. Two of the three disparate fetuses from single injected mothers were sampled towards the end of the procedure and had time to empty their bladders. It is conceivable that the kidneys of the fetus act to lower plasma concentrations of aminoglycoside antibiotics by dumping aminoglycoside into the amnionic fluid surrounding the fetus.

Most authors believe that amnionic fluid is not a dead end

for drugs entering it. Little is known about the dynamics of the amnionic fluid but it is known that the fetus swallows and breathes it. Although the aminoglycoside antibiotics are not well absorbed acrossed the gastrointestinal tract in adults, little is known about their absorption within the gastrointestinal tract of the fetus. The aminoglycoside antibiotics are probably reabsorbed by the fetus, carried across the placenta and eliminated by the mother. This may lead to a much longer exposure of the fetus to the aminoglycoside antibiotic at a lower concentration.

Extending the length of exposure to the aminoglycoside antibiotics may increase their toxicity. Bennett, et al (1979) demonstrated nephrotoxicity to gentamicin could be dissociated from peak serum levels of the drug. They injected rats with 40 mg/kg per day gentamicin either in one whole dose, two half doses or three one third doses daily. After ten days the three times per day rats had the lowest serum concentrations of gentamicin but had significantly higher serum creatinine concentrations, indicating increased nephrotoxicity. Thus, dividing the dose of gentamicin produced lower peak serum concentrations but increased the nephrotoxicity. Thompson, Reiner and Bloxham (1977) showed similar nephrotoxicity in dogs to gentamicin and to tobramycin continiously infused or given in a single daily injection. Even if the amnionic fluid of the fetus is not implicated as a resevoir for the aminoglycoside antibiotics, we know that the fetus is exposed to lower levels of kanamycin than the mother. If the total daily dose given to the mother in experiment 1 was divided

into smaller more frequent doses the hair cells of the fetus would be exposed to lower peak concentrations of aminoglycoside antibiotic for longer total periods of time and fetal ototoxicity might be observed. The effects of lower peak concentrations with more frequent dosing on ototoxicity had not been explored prior to conducting experiment 3.

Experiment 3: Dissociation of peak serum levels of kanamycin from ototoxicity.

Experiment 3 was designed to determine if the ototoxic effect of kanamycin could be increased by extending the period of time the ototoxin was in contact with the hair cell while decreasing the peak level of kanamycin concentration to which the hair cells are exposed. Many researchers have believed that the important parameter for ototoxicity was the peak blood concentration of the aminoglycoside antibiotic. Stupp, et al (1967) suggested dividing the dose of kanamycin to reduce ototoxicity. This would be the wrong advice if peak serum concentrations of kanamycin and ototoxicity could be shown to be dissociated. Fee (1980) found no significant relationship between the serum concentration of tobramycin or gentamicin and human ototoxicity.

The present experiment was designed to determine if the ototoxic effects of kanamycin are dissociated from the peak serum levels of the drug.

Method

Subjects. Ninety-six young adult pigmented guinea pigs of the Topeka strain were used. Each was tested for normal hearing by eliciting the Preyer pinna reflex. At the begining of the

experiment all subjects weighed between 250 and 350 grams each. This weight range places these guinea pigs between 30 and 50 days of age, significantly younger that those mothers in the previous experiments. Both male and female guinea pigs were used in this experiment. The females were not pregnant. All animals were obtained from and were housed at the Kresge Hearing Research Laboratory.

Procedure. The guinea pigs were randomly assigned to one of four groups. Each group received a different dose level of kanamycin (0 mg/kg per day, 100 mg/kg per day, 200 mg/kg per day and 300 mg/kg per day). Each of the groups was further subdivided into three schedules of injection. One of the subgroups received a full dose once every twenty-four hours. One subgroup received a half-dose once every twelve hours. The third subgroup received a quarter-dose every six hours. In total there were twelve subgroups of eight guinea pigs each.

Guinea pigs were weighed and dosages calculated once every forty-eight hours.

Three different concentrations of kanamycin for injection were prepared--100 mg/ml, 200 mg/ml and 300 mg/ml. This was to control for any effect the volume of the injection may have and for ease of calculating the volume of the injection. The three concentrations were dilutions of the standard 333.3 mg/ml kanamycin solution (Kantrex, Bristol) by addition of physiological saline solution. The animals receiving a single injection every day were injected at 0800 local time. The animals receiving a

half injection twice daily were injected at 0800 and 2000 local time. Those receiving quarter injections four times per day were injected at 0200, 0800, 1400 and 2000 local time. Injections were round the clock for two weeks. The injections were given subcutaneously.

Four hours after the last of the injections, two guinea pigs from each of the twelve subgroups were sacrificed for blood and perilymph samples. Heart blood was sampled under Dial-urethane anesthesia. Perilymph samples were removed by suction into a weighed micropipet. The techniques used were those described in the previous experiment. Samples were assayed for kanamycin in the same manner as in experiment 2. A complete procedure for assaying body fluids for kanamycin concentrations is presented in Appendix A.

The remaining guinea pigs were given a 30 day rest period. This rest period permitted any cochlear pathology to stabilize and for the guinea pigs to recover from the stress of round the clock injections.

The electrophysiological procedure is described in Appendix B. Subjects were assessed in a random order. Each guinea pig was anesthetized with a 0.8 ml/kg injection of Dial-urethane. Fur was clipped from the guinea pig's neck and pinna. Breathing and body temperature were maintained as in the previous experiments.

The procedure for doing electrophysiology is covered in detail in Appendix B. Once the one microvolt isopotential function and the maximum outputs of ac cochlear potential at 1 kHz and 10

kHz were recorded for the left ear and the sound system was calibrated, the N_1 potential was measured for that ear. An explanation of what N_1 is and how it is measured is given in Appendix B.

Histological evaluation. Immediately after electrophysiological assessment was completed the inner ears of the guinea pig were removed for evaluation histologically. They were prepared in the manner described in Appendix B.

The right ear of every guinea pig was evaluated in the following manner. Representative sections of the organ of Corti were identified microscopically in the apex, turn 4, turn 3, turn 2 and base. In each of these regions individual outer hair cells can be identified in the organ of Corti. In addition, scars can be identified where hair cells have degenerated. A series of 80 hair cells and scars were counted for the innermost row of outer hair cells. The number of scars was tabulated for that row. Then a series was counted for the middle and the outermost rows of outer hair cells. The total number of scars in the three quantities was subtracted from 240 (3 rows X 80) and divided by 240 to arrive at the per cent of hair cells remaining in this cochlear region.

Data analysis. Kanamycin concentrations of perilymph and plasma samples were determined by a regression line derived in the manner described in Appendix A. Kanamycin levels of the samples were averaged for the two guinea pigs sacrificed in each of the twelve subgroups. Results of the assays of perilymph and the assays of the plasma were graphed. A three way analysis of

variance was conducted on the isopotential function data for all twelve subgroups. The stimulus frequency was treated as a repeated measure.

An average one microvolt isopotential function curve was derived for each of the twelve subgroups. The three isopotential curves for each of the three dose schedules were graphed by total daily dose. Thus, four total daily dose graphs (0, 100, 200 and 300 mg/kg per day) each contained three dose schedules (once, twice and four times per day).

In addition the maximum outputs were averaged for each of the twelve subgroups and were graphed. One graph was made for maximum outputs at 1 kHz and one graph was made for maximum outputs at 10 kHz.

The hair cell count data were averaged and graphed for each dose level. A three way analysis of variance was computed for the hair cell data. Region of the cochlea was treated as a repeated measure.

Results

Figure 10 is a graph of the average isopotential functions for the three groups who received saline injections. The line with filled circles indicates data measured for guinea pigs who received one full saline injection once per day, the open circle line represents guinea pigs who received a half injection twice per day, and the guinea pigs represented by the open squares received a quarter injection of saline four times per day. As one would expect, all points on the three curves lie within a few decibels of each other. These isopotential curves are equivalent to those

measured in normal untreated guinea pigs.

Figure 11 is a graph of the average isopotential functions of those guinea pigs who received 100 mg/kg per day. Again the solid circles represent isopotential functions measured from guinea pigs who received a whole dose once per day, the open circles those who received half a dose twice per day and the open squares those who received a quarter dose four times per day. The shaded area represents the area enclosed by the three saline curves in Figure 10. Again all three graphs are within a few decibels of each other. The 100 mg/kg per day graphs are all very close to the saline region. This would indicate little or no ototoxicity was measurable in those guinea pigs receiving 100 mg/kg per day.

Figure 12 is the average one microvolt average isopotential curves for those guinea pigs receiving a total of 200 mg/kg per day. Again the three dosage schedules are represented in the same manner as Figures 10 and 11. The shaded region again demarcates the control region. This graph demonstrates the effect on the isopotential function of cochlear ototoxicity. Those frequencies above 2 kHz in the guinea pigs who received 200 mg/kg per day required higher sound pressure levels to produce the one microvolt isopotential function than the guinea pigs in the control group. At 32 kHz about 20 dB more sound was required by the 200 mg/kg per day group. However, all of the dose schedule groups lie within a few dB of each other indicating no effect on the ototoxicity due to the dose schedule.

Figure 13 is derived in the same manner as the other three

figures except that it represents data collected from guinea pigs who received 300 mg kanamycin/kg per day. This graph indicates that ototoxicity was present at all frequencies tested since no points lie within the control region. Note again that all three curves overlap each other.

Table 9 presents the results of the analysis of variance conducted on the isopotential function data. It confirms the earlier analysis. A significant effect of total daily dose is noted. However, there is no significant effect of schedule nor is there a significant interaction effect of the total daily dose by schedule. As noted before in an earlier experiment there is a significant within subject main effect of stimulus frequency. This is probably because the isopotential function of the guinea pig is not flat across frequency. As would be expected with the aminoglycoside antibiotics there is a significant interaction effect of stimulus frequency by total daily dose. No interaction effect was noted for stimulus frequency by schedule nor for stimulus frequency by schedule by total daily dose.

Figure 14 represents the maximum outputs of the ac cochlear potential measured at 1 kHz grouped by total daily dose. These data confirm the isopotential function data just presented. The saline and 100 mg/kg per day maximum output data overlap each other. This indicates no detectable ototoxic damage for the 100 mg/kg per day guinea pigs whether the dose was divided into four injections, two injections or given as a single injection once per day. The 200 mg/kg per day guinea pigs average maximum output

Figure 10. The intensity of sound required to generate one microvolt of ac cochlear potential at 16 frequencies for those guinea pigs who received saline injections 1, 2 or 4 times per day for 2 weeks. Sound intensity increases the greater the distance above the origin. All schedules of injection produced equivalent results.

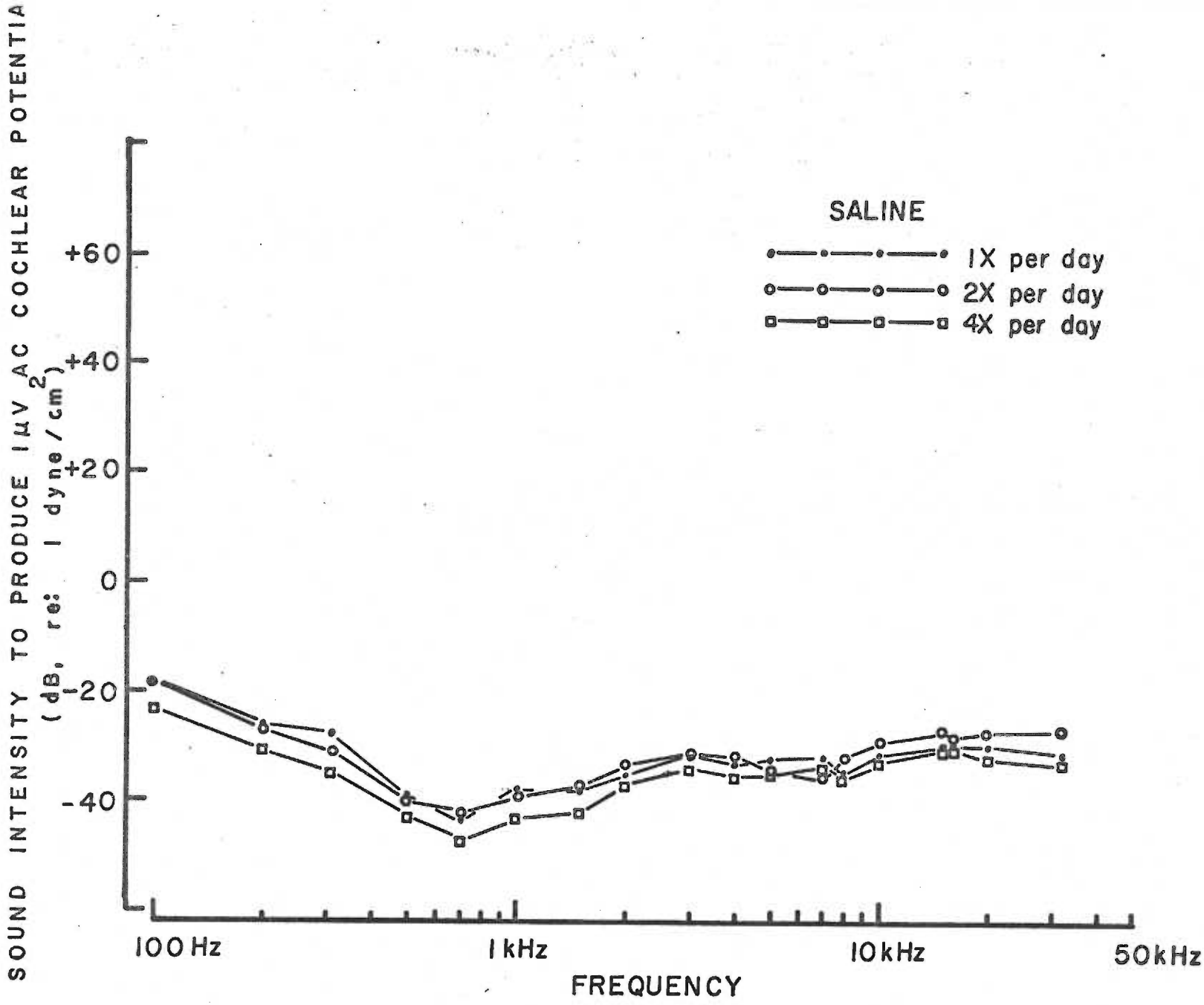

SALINE
1X per day
2X per day
4X per day
SOUND INTENSITY TO PRODUCE 1μV AC COCHLEAR POTENTIAL
(dB, re: 1 dyne/cm²)
+60
+40
+20
0
-20
-40
100 Hz
1 kHz
10 kHz
50 kHz
FREQUENCY

Figure 10

Figure 11. Sound intensity required to produce one microvolt of ac cochlear potential at 16 frequencies for guinea pigs injected with a total daily dose of 100 mg/kg of kanamycin given 1, 2 or 4 times per day. The shaded area indicates the saline control area. Note that the three dose regimens are all equal and also do not differ from the control region.

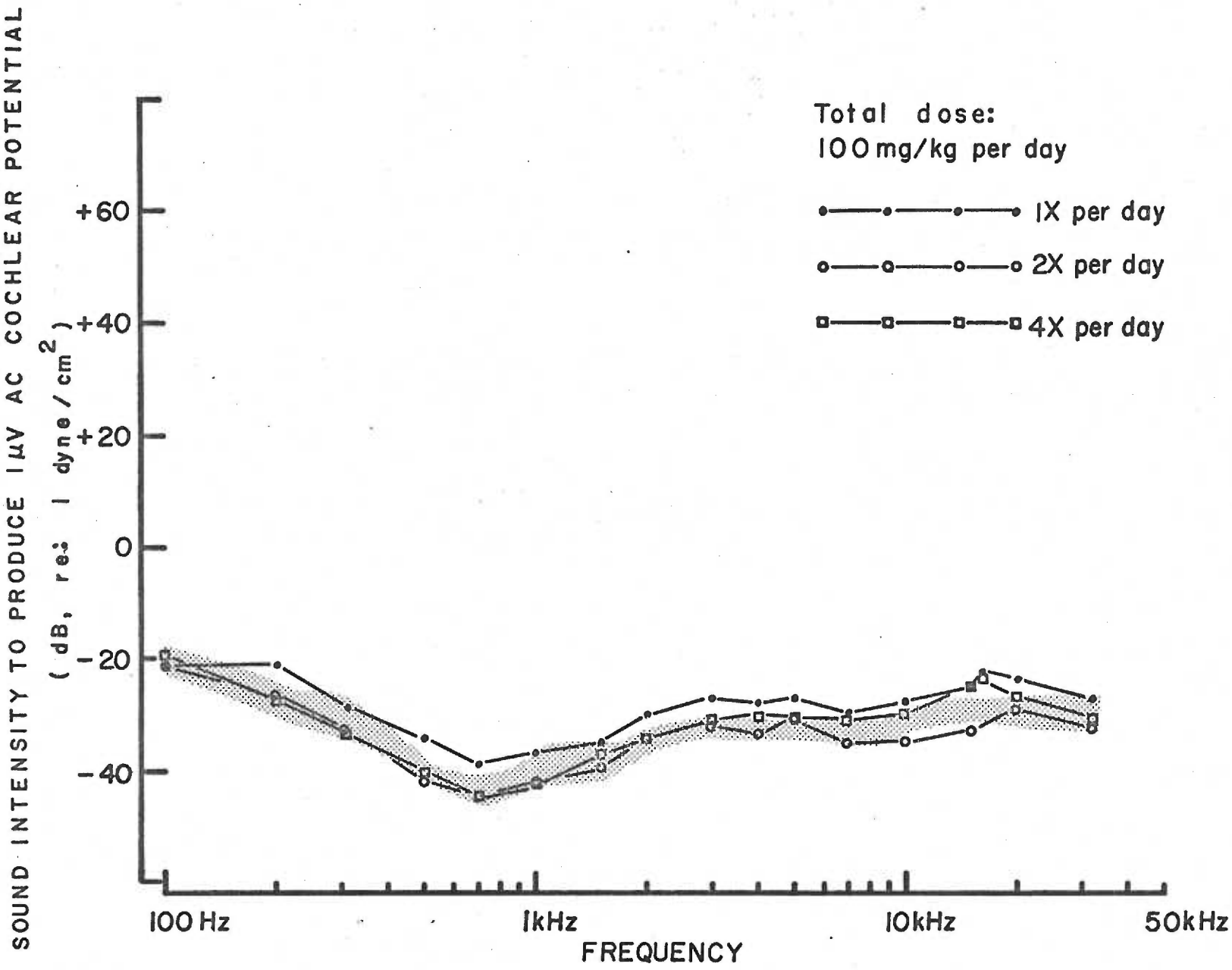

SOUND INTENSITY TO PRODUCE 1 μV AC COCHLEAR POTENTIAL
(dB, re: 1 dyne / cm2)
+60
+40
+20
0
-20
-40
100 Hz
1kHz
10kHz
50kHz
FREQUENCY
Total dose:
100 mg/kg per day
1X per day
2X per day
4X per day

FIGURE 11

Figure 12. Sound intensity required to produce one microvolt of ac cochlear potential at 16 frequencies These guinea pigs were injected with a total daily dose of 200 mg/kg divided into 1, 2 or 4 injections. Note that all three injection schedules are equivalent and that these data differ from the control region at the highest frequencies tested.

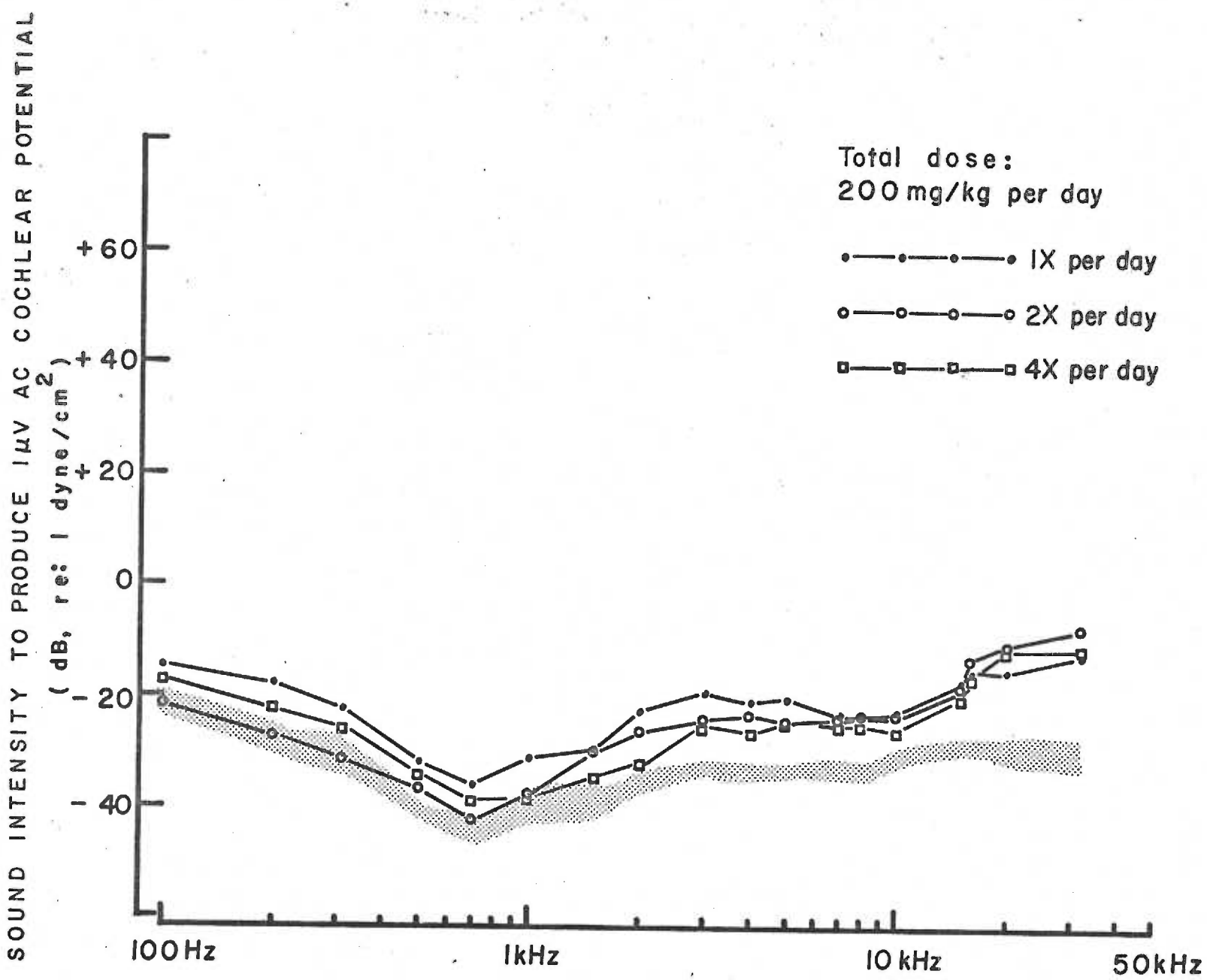

SOUND INTENSITY TO PRODUCE 1μV AC COCHLEAR POTENTIAL
(dB, re: 1 dyne/cm²)
+60
+40
+20
0
-20
-40
100Hz
1kHz
10kHz
50kHz
FREQUENCY
Total dose:
200 mg/kg per day
1X per day
2X per day
4X per day

FIGURE 12

Figure 13. Sound intensity required to produce one microvolt of ac cochlear potential at 16 frequencies. These guinea pigs were injected with a total daily dose of 300 mg/kg kanamycin either 1, 2 or 4 times per day. Note the extreme intensity of sound required at the highest frequencies to produce one microvolt. This loss of sensitivity has extended into the lower frequencies also. Again the three dose regimens produce results within a few decibels of each other.

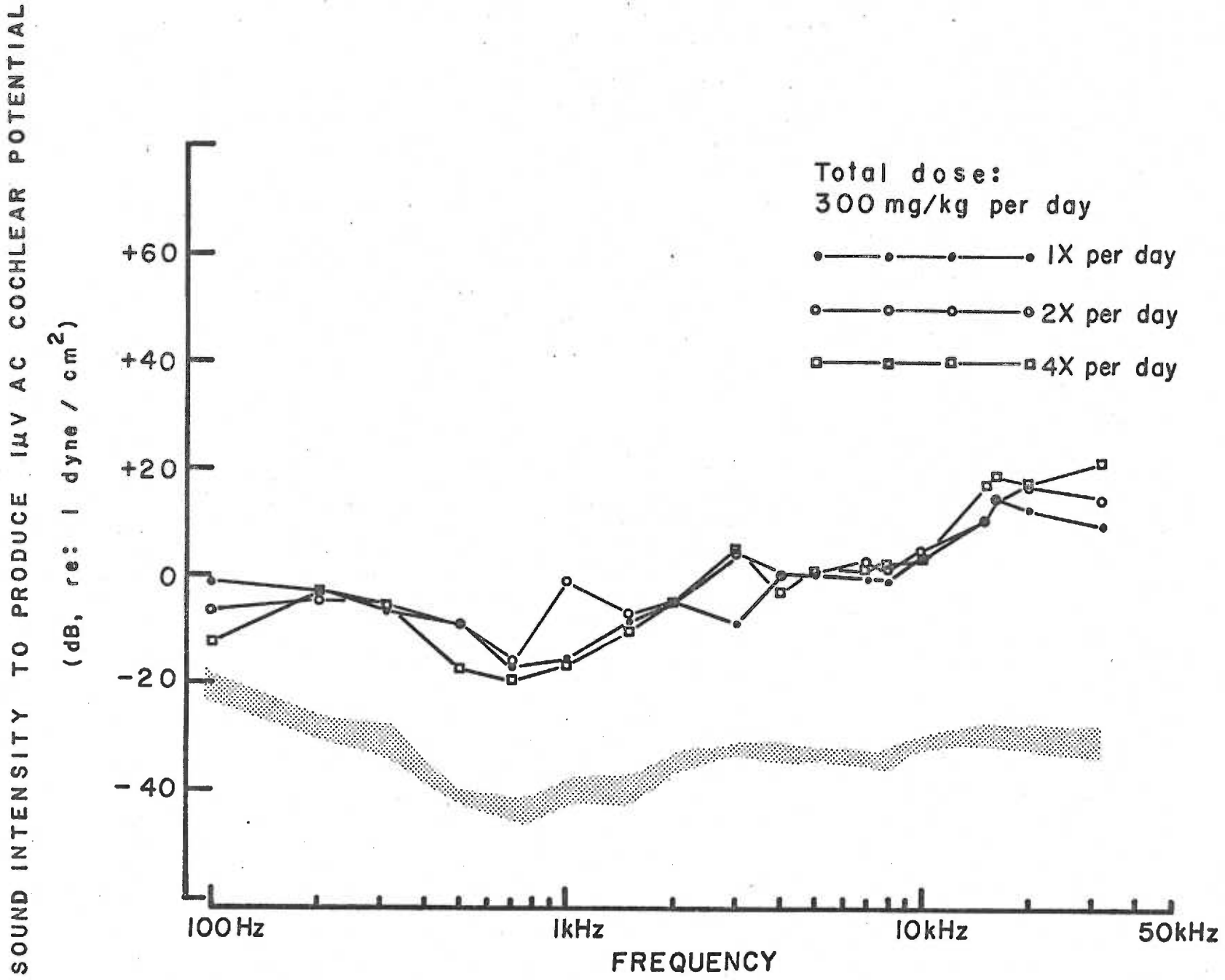

SOUND INTENSITY TO PRODUCE 1μV AC COCHLEAR POTENTIAL
(dB, re: 1 dyne / cm²)
+60
+40
+20
0
-20
-40
Total dose:
300 mg/kg per day
1X per day
2X per day
4X per day
100Hz
1kHz
10kHz
50kHz
FREQUENCY

FIGURE 13

Table 9

Analysis of Variance for Isopotential Functions of Guinea Pigs in Experiment 3

Summary Table

(3-way, repeated measures on stimulus frequency, equal N)

Source	Sums of Squares	df	Mean Squares	F	p
Between subjects					
Total Daily Dose	453395.03	3	151131.68	35.66	p<.01
Schedule	2537.48	2	1268.74	0.30	NS
Total Daily Dose X Schedule	3920.29	6	653.38	0.15	NS
Error	559481.31	132	4238.49		
Within subjects					
Stimulus Frequency	105809.92	17	6224.11	111.64	p<.01
Stim.Freq. X Tot.Daily Dose	30589.25	51	599.79	10.76	p<.01
Stim.Freq X Schedule	2712.52	34	79.78	1.43	NS
Stim.Freq X Schedule X Tot.Day Dose	5892.38	102	57.77	1.04	NS
Error	125108.44	2244			

Total Daily Dose=Total dose of all injections given per 24 hours

Schedule=Number of injections given per 24 hour period

Stimulus Frequency=The frequency of the sound stimulus (100-32,000 Hz)

NS=Not significant

data indicate a depression of about 500 microvolts with all injection schedules, compared to the saline maximum output data. The important point is that all three injection schedules produce maximum outputs that were essentially equal. This indicates that the level of ototoxicity was similar in all three groups. The 300 mg/kg per day group data showed the greatest depression of the maximum output of the ac cochlear potential. This depression was about 1200 microvolts below the saline maximum outputs and about 700 microvolts below the 200 mg/kg per day maximum outputs.

Figure 15 is a graph of the average maximum outputs for all groups at 10 kHz. Overall the 10 kHz maximum outputs were much lower than the 1 kHz outputs. This is normal. Again the saline and 100 mg/kg per day groups were closely interposed. The average maximum outputs of the three groups who received 200 mg/kg per day were less than the saline groups and the 300 mg/kg per day groups were the lowest of all. These data support the previous data.

Figures 16, 17, 18 and 19 contain graphs for the N_1 thresholds for the four dose levels. Rather than belabor the point please note that the N_1 data confirm that described previously. For any particular total daily dose no difference in N_1's were detected for the three different dosage schedules. However, differences were detected for different total daily dosages. The saline and 100 mg/kg per day groups had N_1 thresholds that were equivalent. The 200 mg/kg per day and 300 mg/kg per day groups required intermediate and high sound levels respectively to produce N_1 thresholds. This confirms the one

Figure 14. Maximum outputs of the ac cochlear potential at 1 kHz for guinea pigs receiving 0, 100, 200 or 300 mg/kg per day of kanamycin, 1, 2 or 4 times per day.

Figure 14

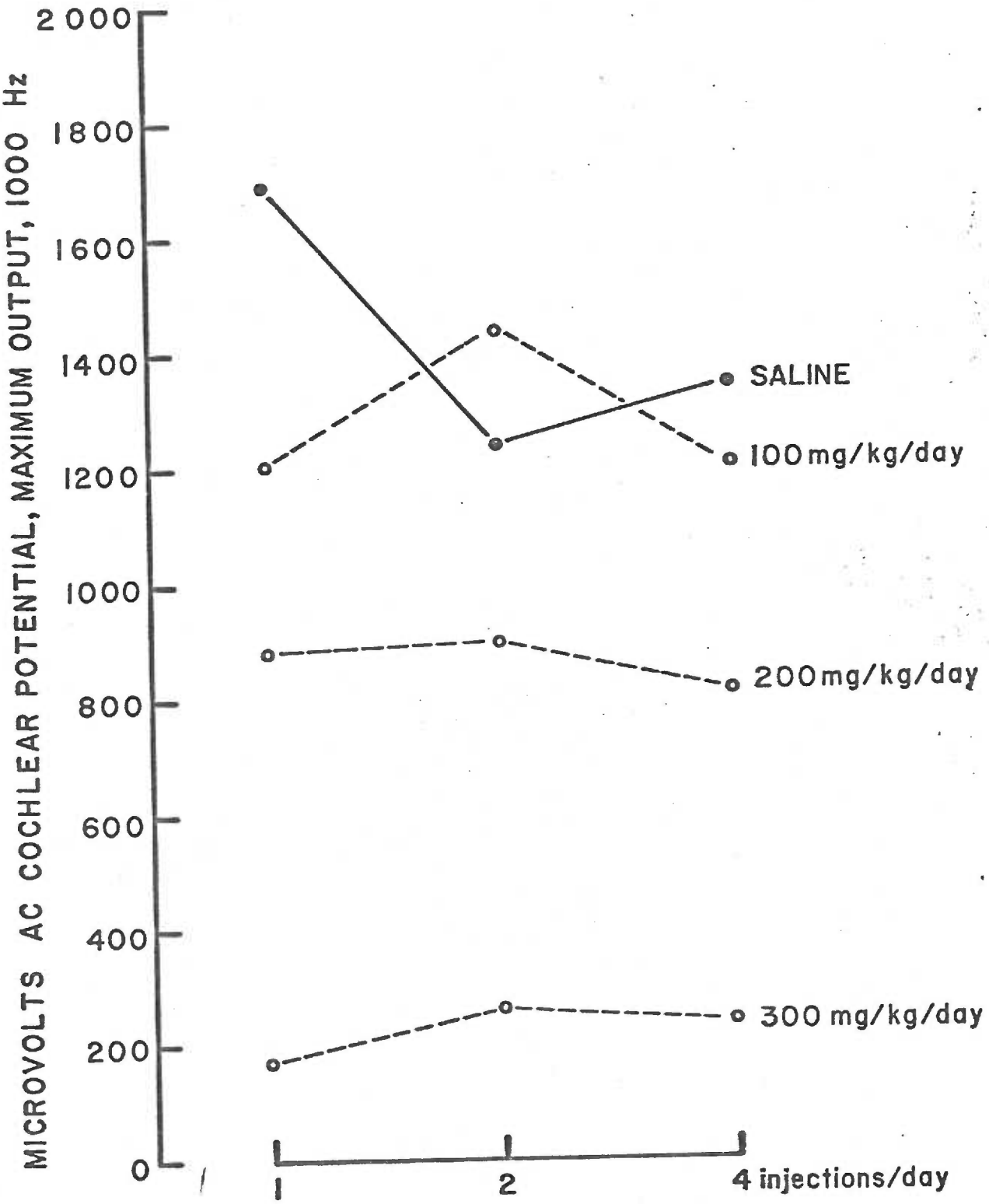

MICROVOLTS AC COCHLEAR POTENTIAL, MAXIMUM OUTPUT, 1000 Hz
2000
1800
1600
1400
1200
1000
800
600
400
200
0
SALINE
100mg/kg/day
200mg/kg/day
300 mg/kg/day
1
2
4 injections/day

Figure 15. Maximum outputs for the ac cochlear potential at 10 kHz for guinea pigs receiving 0, 100, 200 or 300 mg/kg per day of kanamycin in dose regimens of once, twice or four times per day.

Figure 15

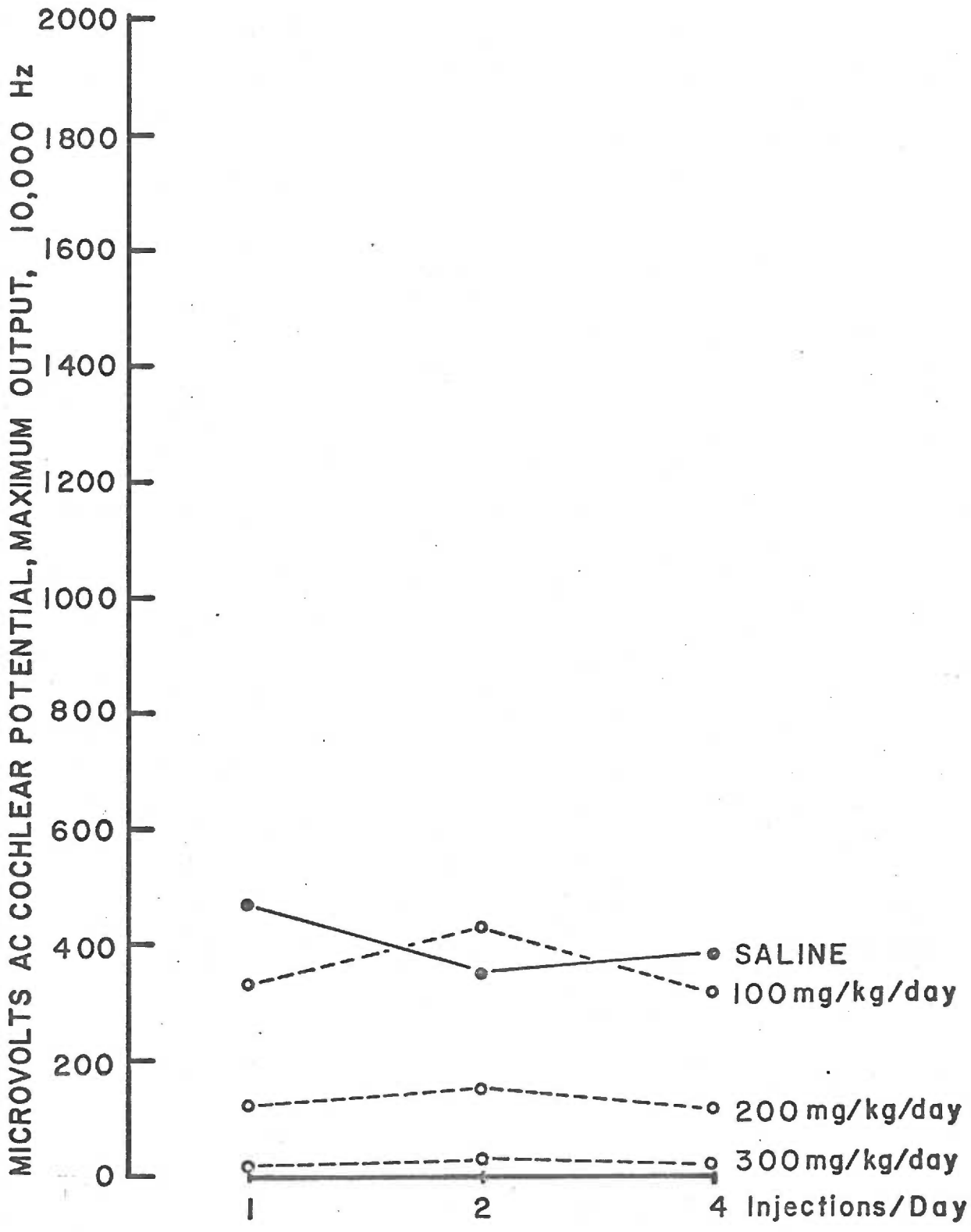

MICROVOLTS AC COCHLEAR POTENTIAL, MAXIMUM OUTPUT, 10,000 Hz
2000
1800
1600
1400
1200
1000
800
600
400
200
0
SALINE
100 mg/kg/day
200 mg/kg/day
300 mg/kg/day
1
2
4 Injections/Day

Figure 16. Sound intensity necessary to produce the threshold N_1 response at 2 kHz, 4 kHz, 8 kHz, 16 kHz, 20 kHz and 32 kHz. These guinea pigs received injections of saline 1, 2 or 4 times per day.

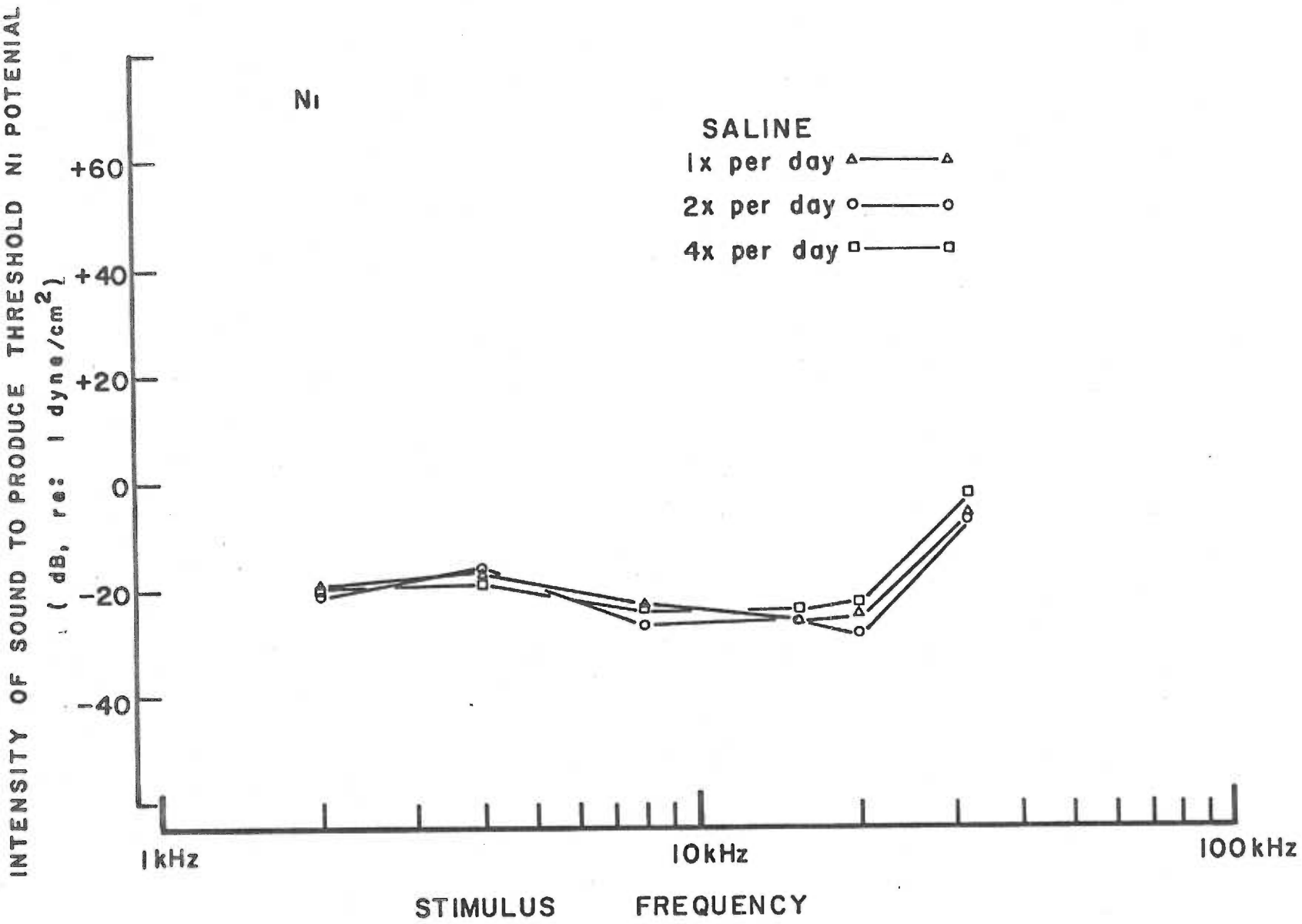

N1
SALINE
1x per day
2x per day
4x per day
INTENSITY OF SOUND TO PRODUCE THRESHOLD N1 POTENIAL
(dB, re: 1 dyne/cm2)
+60
+40
+20
0
-20
-40
1kHz
10kHz
100kHz
STIMULUS FREQUENCY

Figure 16

Figure 17. Sound intensity necessary to produce the threshold N_1 response at 6 frequencies for those guinea pigs receiving 100 mg/kg per day of kanamycin. The three dose regimens do not significantly differ.

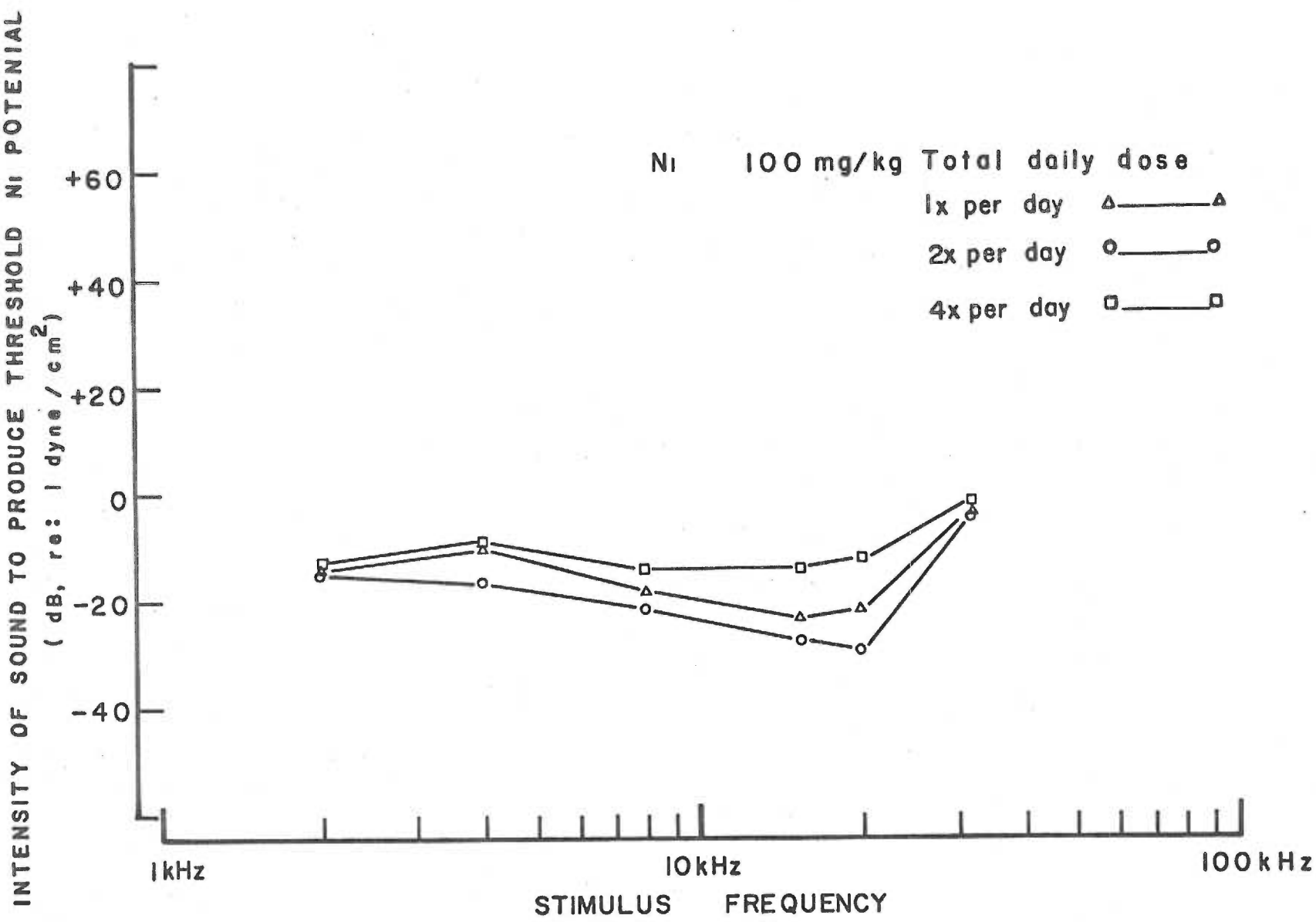

Ni 100 mg/kg Total daily dose
1x per day
2x per day
4x per day
INTENSITY OF SOUND TO PRODUCE THRESHOLD Ni POTENIAL
(dB, re: 1 dyne / cm2)
+60
+40
+20
0
-20
-40
1kHz
10kHz
100kHz
STIMULUS FREQUENCY

Figure 17

Figure 18. Sound intensity necessary to produce the threshold N_1 response at 6 frequencies for those guinea pigs receiving 200 mg/kg per day of kanamycin. Again the three dose regimens do not differ from each other.

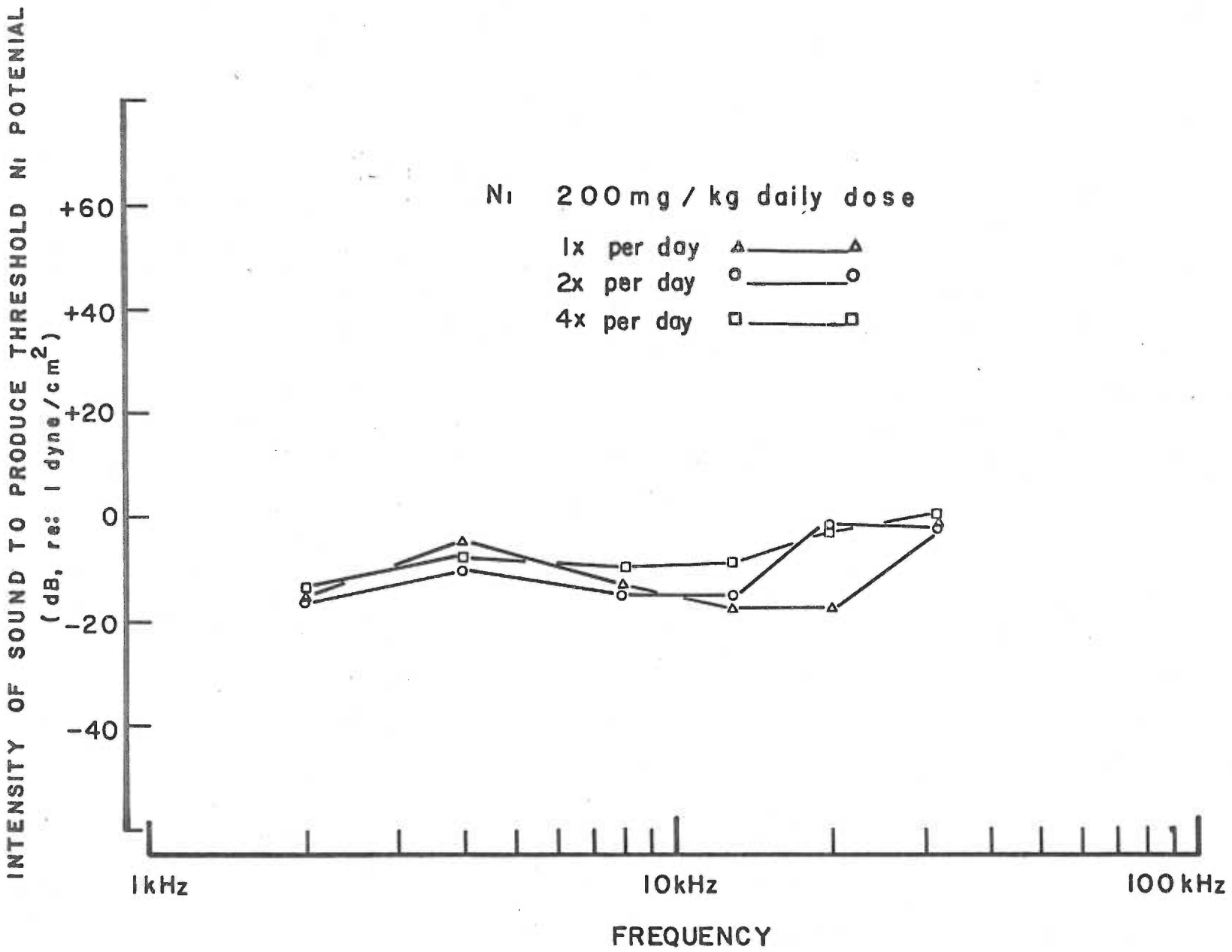

INTENSITY OF SOUND TO PRODUCE THRESHOLD Ni POTENIAL
(dB, re: 1 dyne/cm2)
+60
+40
+20
0
-20
-40
Ni 200mg / kg daily dose
1x per day
2x per day
4x per day
1kHz
10kHz
100kHz
FREQUENCY

Figure 18

Figure 19. Sound intensities at 6 frequencies required to produce threshold N_1 responses. These guinea pigs received 300 mg/kg per day of kanamycin 1, 2 or 4 times per day. The three dose schedules are not significantly different.

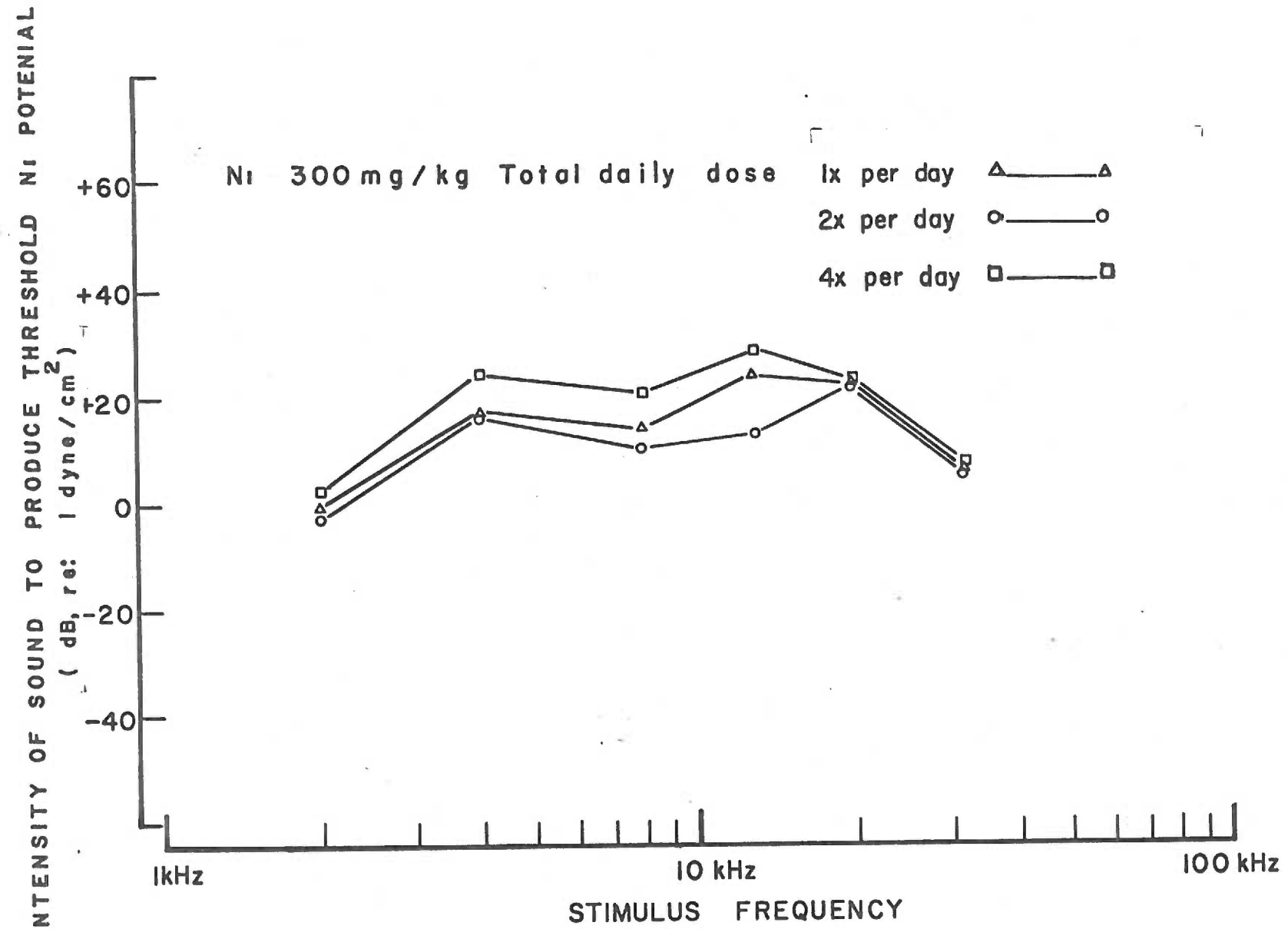

Ni 300 mg/kg Total daily dose
1x per day
2x per day
4x per day
INTENSITY OF SOUND TO PRODUCE THRESHOLD Ni POTENIAL
(dB, re: 1 dyne/cm²)
+60
+40
+20
0
-20
-40
1kHz
10 kHz
100 kHz
STIMULUS FREQUENCY

Figure 19

microvolt isopotential curve and maximum output data.

Plasma kanamycin concentration levels were found to vary with the absolute amount of kanamycin injected in a single injection. Thus when plasma levels were measured four hours post-injection the one time per day guinea pigs had a plasma concentration of kanamycin that was about four times as concentrated as the four times per day group. Figure 20 shows this relationship. Since each point contains only two animals there is high variability, but the overall trend can be observed.

Figure 21 shows that this relationship holds for perilymph concentrations of kanamycin, too. At four hours post-injection there appears to be a linear relationship between the absolute amount of kanamycin injected and the concentration of kanamycin in the perilymph. Again each point represents only two animals (four ears) so the variability is high. There does not appear to be any accumulation of kanamycin in the perilymph.

Data for outer hair cell counts of the guinea pig right ears are presented in Figures 22, 23, 24 and 25. The number of hair cells remaining in the saline groups are equal regardless of the injection schedule. The hair cell counts of the 100 mg/kg per day kanamycin groups are also equal regardless of the schedule of administration and are also equal to the control hair cell counts. The hair cell counts of the 200 mg/kg per day kanamycin groups begin to show a slight loss of hair cells in the whole cochlea but especially in the base and turn 2. All schedules of injection are basically equivalent in this group. A moderate loss of hair cells

Figure 20. Plasma concentrations of kanamycin in guinea pigs four hours after the final injection of the two week period. Each data point represents two guinea pigs.

Figure 20

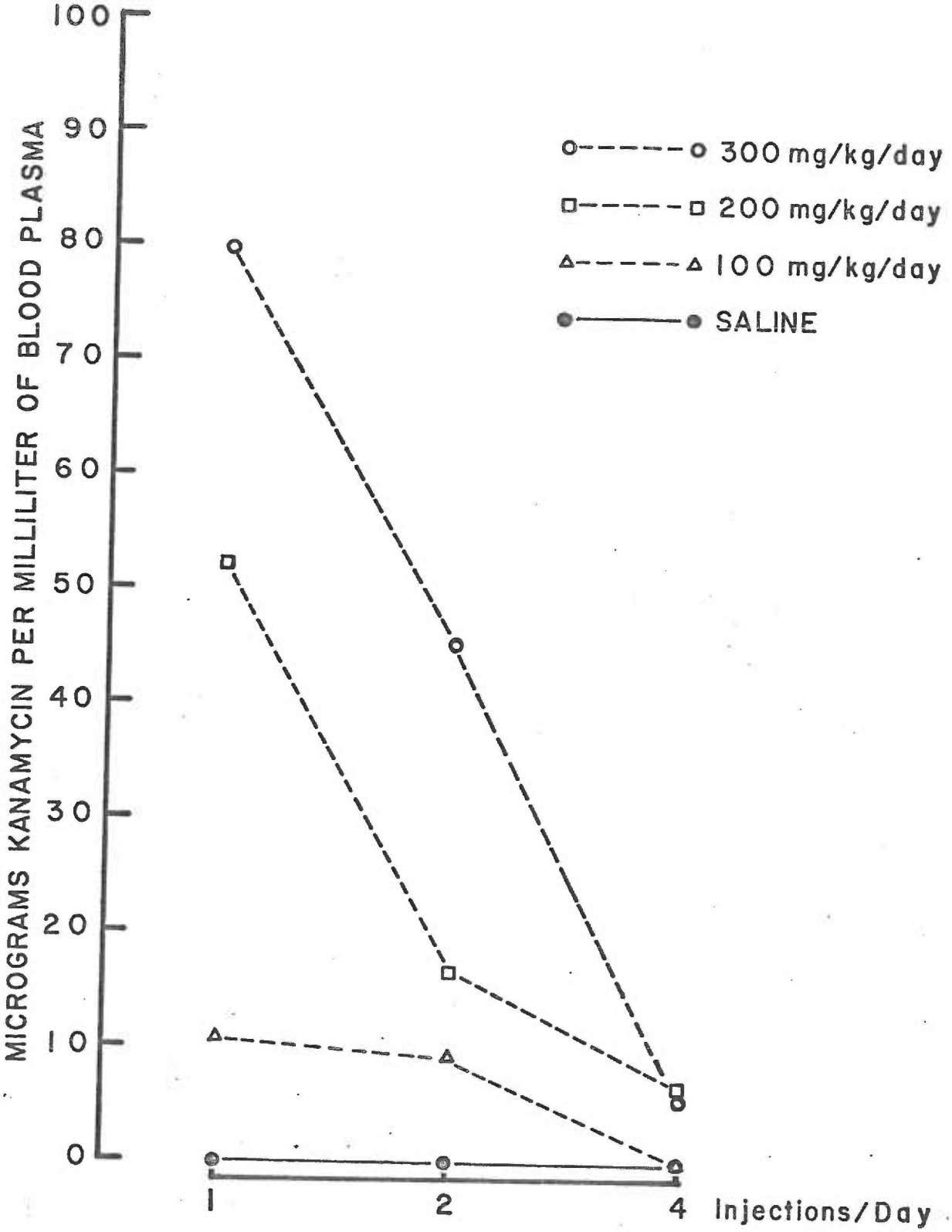
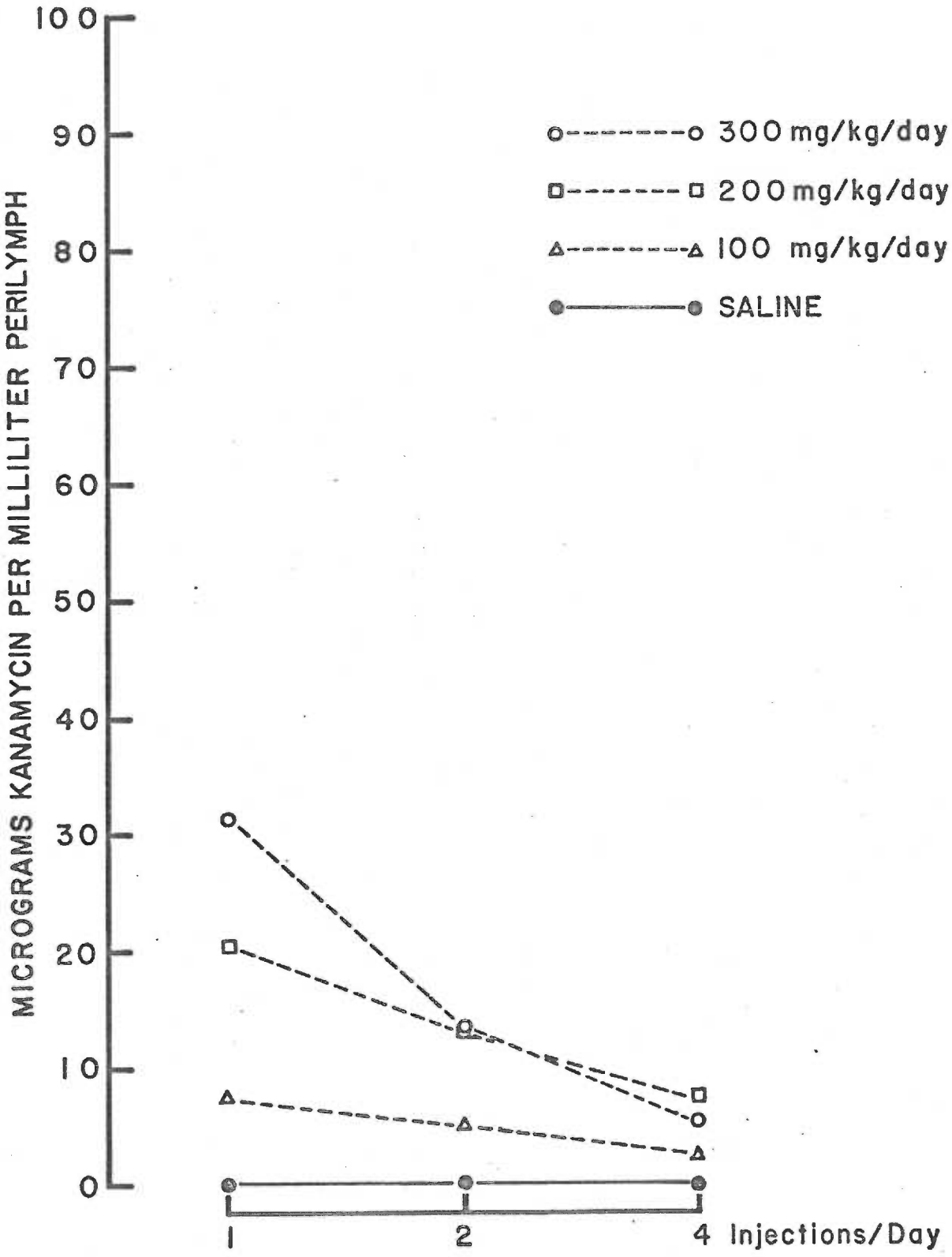

MICROGRAMS KANAMYCIN PER MILLILITER OF BLOOD PLASMA
100
90
80
70
60
50
40
30
20
10
0
300 mg/kg/day
200 mg/kg/day
100 mg/kg/day
SALINE
1
2
4
Injections/Day

Figure 21. Perilymph concentrations of kanamycin in guinea pigs four hours after the final injection of the two week period. Each data point represents 2 animals.

Figure 21


MICROGRAMS KANAMYCIN PER MILLILITER PERILYMPH
100
90
80
70
60
50
40
30
20
10
0
300 mg/kg/day
200 mg/kg/day
100 mg/kg/day
SALINE
1
2
4 Injections/Day

in the base and turn 2 can be identified in the hair cell counts of the 300 mg/kg per day kanamycin groups. These hair cell counts also indicate a number of hair cells were destroyed in other regions of the cochlea. However, again all three dose schedules appear to have produced equal ototoxic damage. This analysis is confirmed by the analysis of variance carried out on the hair cell data (Table 10). The statistically significant main effects were total daily dose and the within subjects region of the cochlea. It is known that the normal apex is often missing hair cells. Perhaps this effect is similar to the frequency effect noted earlier. The statistically significant interaction effect was total daily dose by cochlear region--as would be expected knowing the effects of the aminoglycoside antibiotics begin in the base.

Discussion

This experiment clearly indicates that peak kanamcyin concentration levels in plasma and perilymph are not related to ototoxicity. Although peak levels of kanamycin in the four times per day groups were roughly one fourth of the one times per day groups, they had equal levels of ototoxicity measured electrophysiologically and histologically. This indicates perhaps that the area under the pharmacokinetic curve may be more important for predicting chronic ototoxicity than the absolute peak concentrations of kanamycin in plasma or perilymph. If peak concentrations of kanamycin in plasma and perilymph were related to ototoxicity the expectation would be that those guinea pigs receiving two and four injections per day would exhibit less oto-

Table 10

Analysis of Variance for Right Ear Hair Cell Data

of Guinea Pigs in Experiment 3

Summary table

(3-way, repeated measures on cochlear region, equal N)

Source	Sums of Squares	df	Mean Squares	F	p
Between subjects					
Total Daily Dose	191645.67	3	63881.89	22.58	p< 01
Schedule	2978.57	2	1489 28	0.53	NS
Total Day Dose X Schedule	3806 90	6	634.48	0.22	NS
Error	169770.67	60	2829.51		
Within subjects					
Cochlear region	3439.19	4	859.80	7.56	p<.01
Dose X region	17923.16	12	1493.60	13.28	p<.01
Schedule X region	667 60	8	83.60	0.74	NS
Region X dose X schedule	1196.23	24	49.84	0.44	NS
Error	26987.69	240	112.45		

NS=Not significant

Figure 22. Hair cell counts for the right ears of guinea pigs who were injected with saline daily for two weeks. Note that there was no difference in the hair cells present for the three dose schedules: once, twice or four times per day.

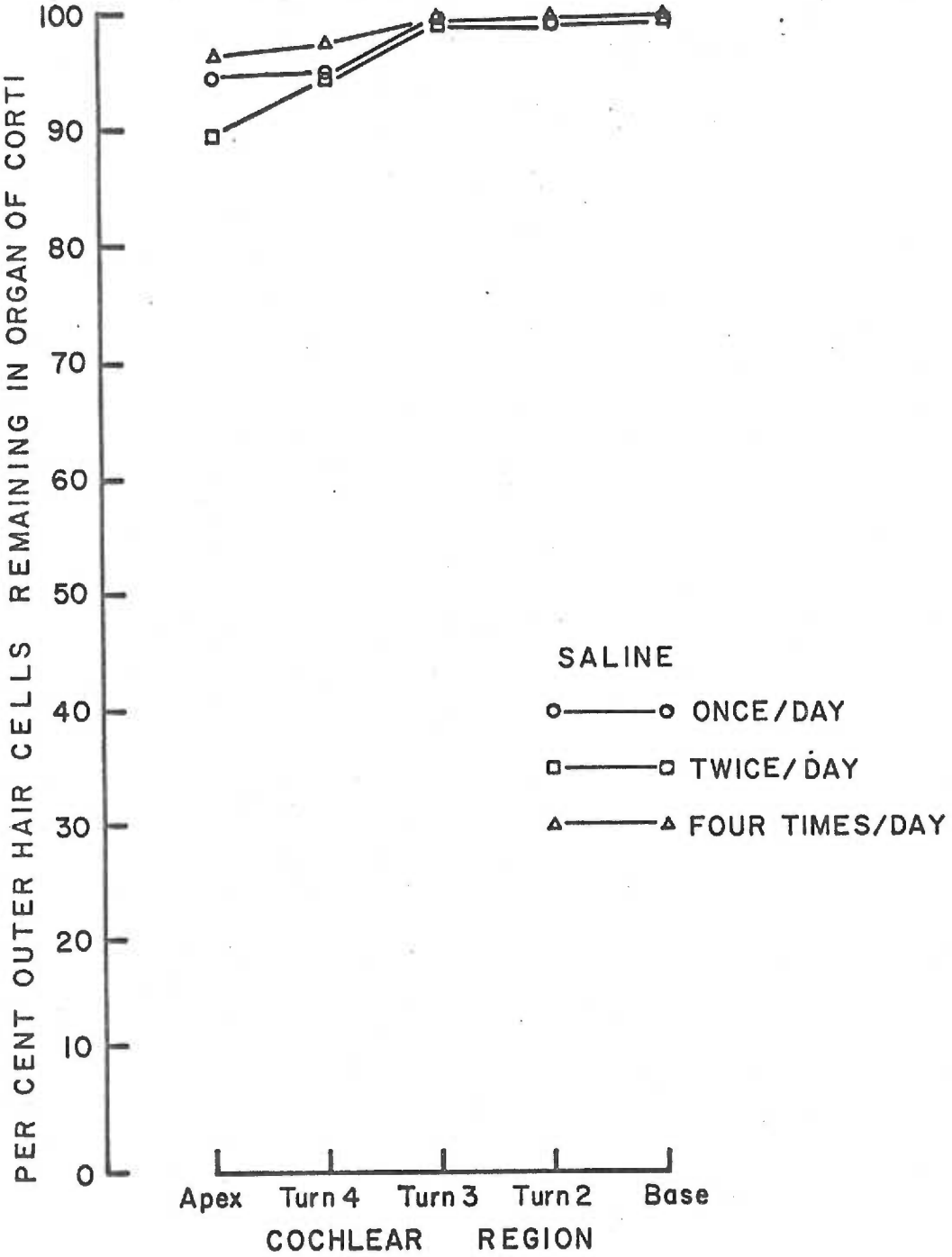

PER CENT OUTER HAIR CELLS REMAINING IN ORGAN OF CORTI
100
90
80
70
60
50
40
30
20
10
0
SALINE
ONCE/DAY
TWICE/DAY
FOUR TIMES/DAY
Apex
Turn 4
Turn 3
Turn 2
Base
COCHLEAR REGION

Figure 22

Figure 23. Right ear hair cell counts for guinea pigs who recieved daily injections of 100 mg/kg kanamycin for two weeks. Note the lack of difference between animals injected one, two or four times per day. Note the similarity of this graph and the control group.

Figure 23

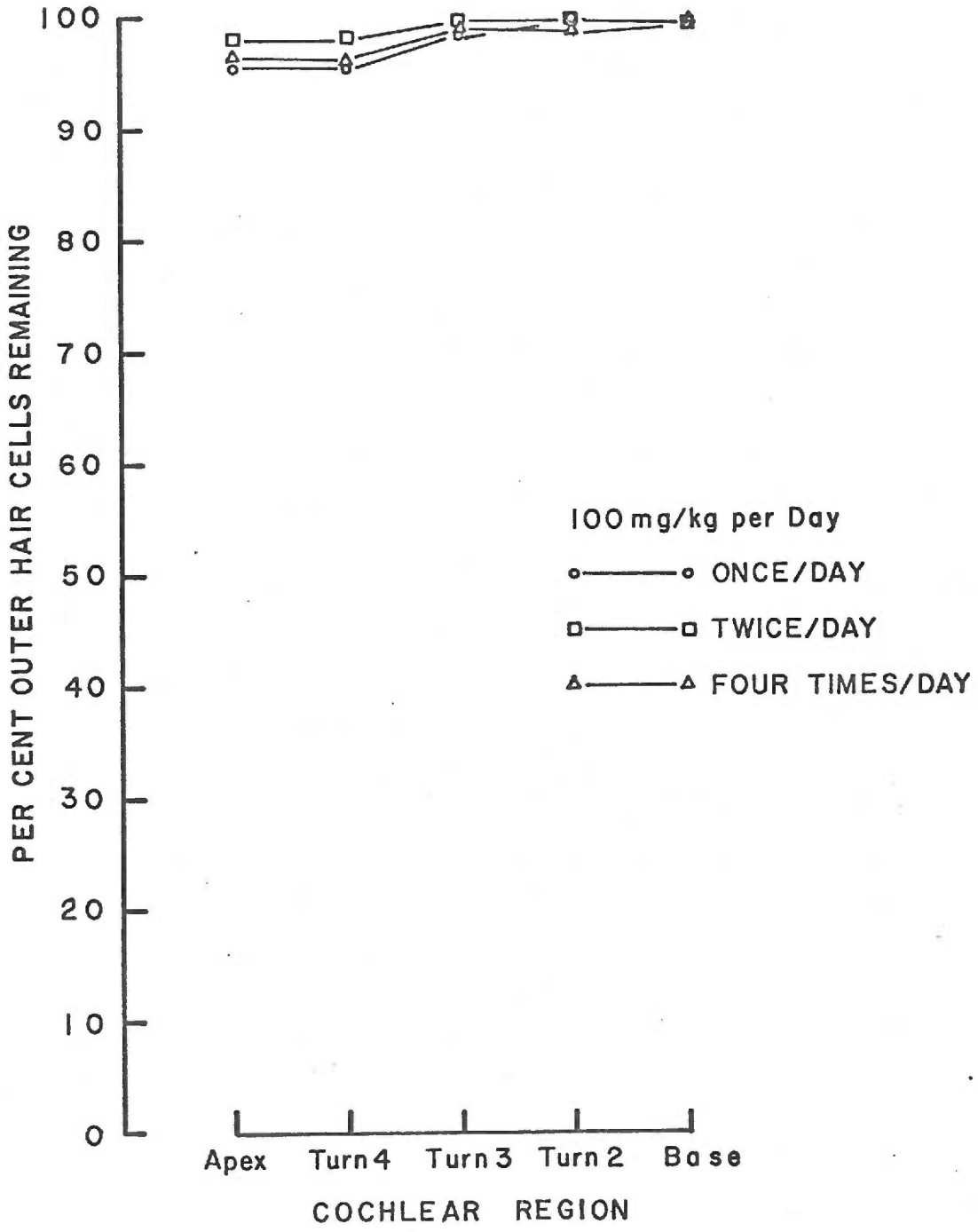

100
90
80
70
60
50
40
30
20
10
0
PER CENT OUTER HAIR CELLS REMAINING
100 mg/kg per Day
ONCE/DAY
TWICE/DAY
FOUR TIMES/DAY
Apex
Turn 4
Turn 3
Turn 2
Base
COCHLEAR REGION

Figure 24. Percentage of outer hair cells remaining in the right ears of guinea pigs injected daily with a total daily dose of 200 mg/kg. Note that these curves do differ from the previous two graphs. Although there are slight differences between the once, twice and four times per day groups the differences are probably not significant.

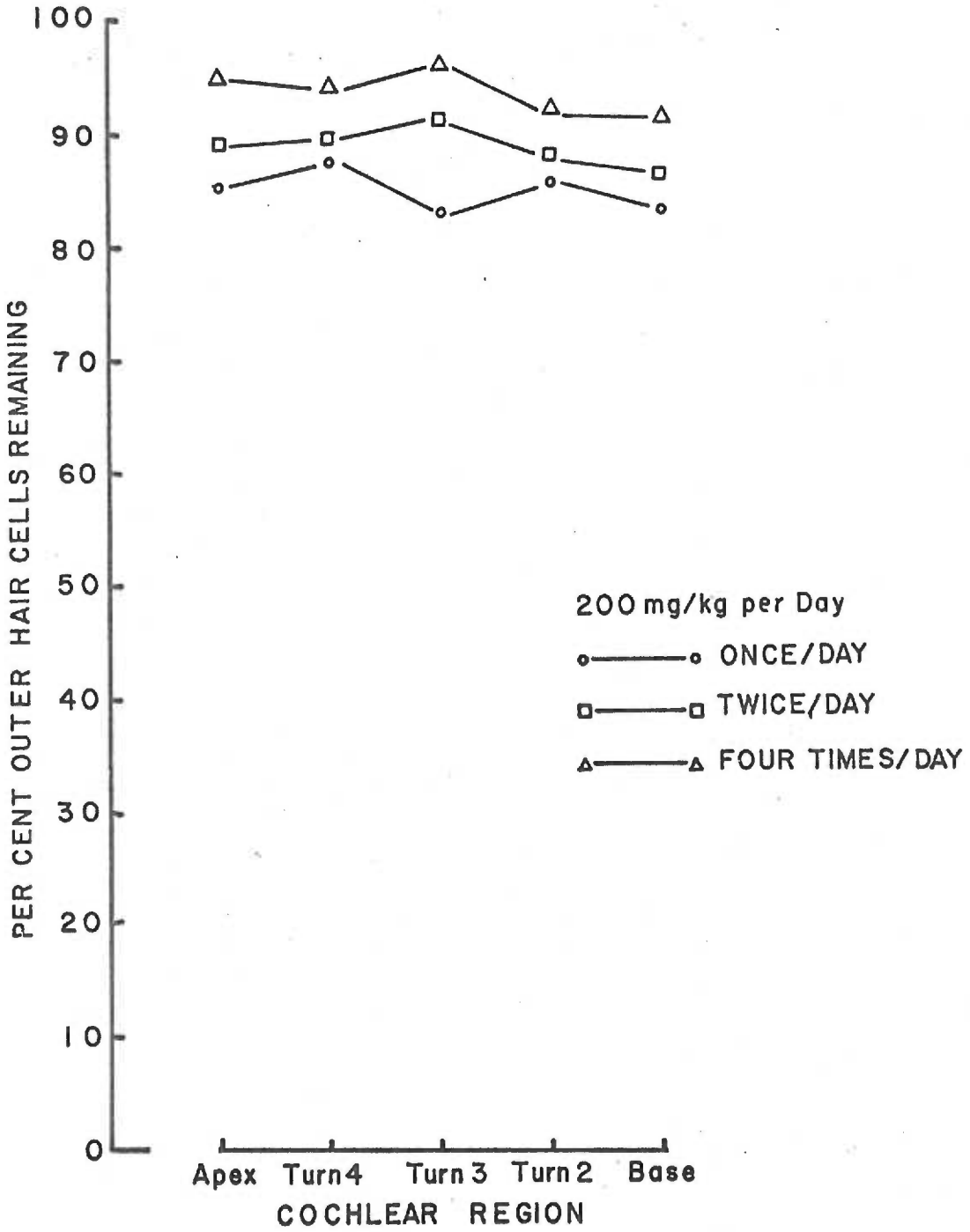

100
90
80
70
60
50
40
30
20
10
0
PER CENT OUTER HAIR CELLS REMAINING
200 mg/kg per Day
ONCE/DAY
TWICE/DAY
FOUR TIMES/DAY
Apex
Turn 4
Turn 3
Turn 2
Base
COCHLEAR REGION

Figure 24

Figure 25. Percentage of outer hair cells present in the right ears of guinea pigs who had been injected with 300 mg/kg per day for 14 days. Note the extensive damage in the base which extends back towards the apex. The three dose schedules are not significantly different.

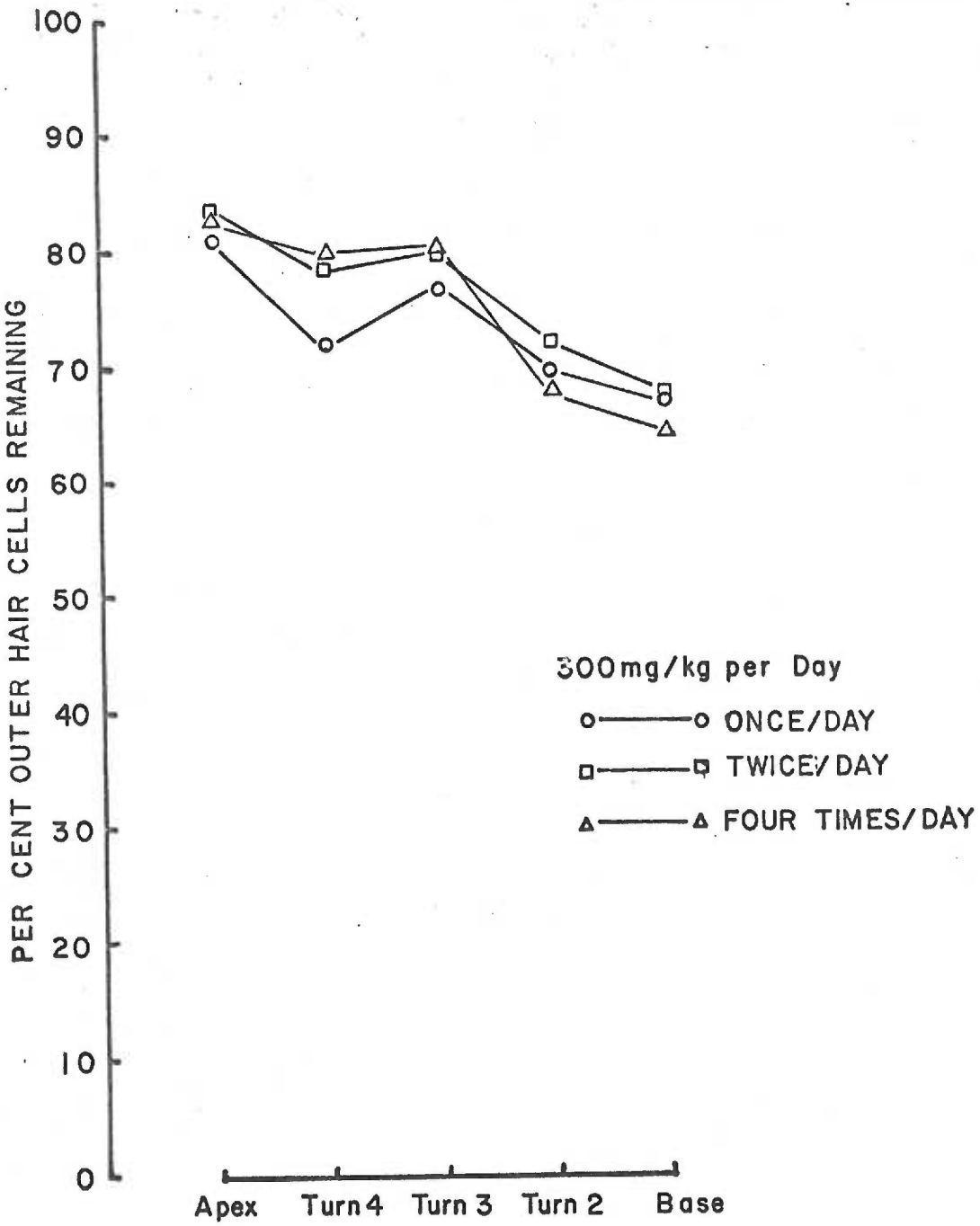

100
90
80
70
60
50
40
30
20
10
0
PER CENT OUTER HAIR CELLS REMAINING
300mg/kg per Day
ONCE/DAY
TWICE/DAY
FOUR TIMES/DAY
Apex
Turn 4
Turn 3
Turn 2
Base
COCHLEAR REGION

Figure 25

toxicity to the same total daily dosage than the one time per day guinea pigs. The expectation would be that the four time per day guinea pigs would have the least amount of ototoxicity of all.

However, based on the above data increasing the length of exposure of the guinea pig to the aminoglycoside antibiotic may counteract the lowered peak concentrations to produce equivalent ototoxicity. This increases the area under the pharmacokinetic curve. Actually the area under the pharmacokinetic curve can be increased in two ways. First, removing the avenue of elimination, by compromising the kidneys the drug would not be eliminated as efficiently. The time required to eliminate one half of the aminoglycoside antibiotic ($t_{1/2}$) would be extended and the area under the pharmacokinetic curve would be increased. The second way in which the area under the pharmacokinetic curve could be increased is through reabsorption from a drug "sink." This sink could be located anywhere within the body. In the fetus this sink may be the fluid surrounding the fetus. This fluid is theoretically "outside" of the fetus. When the fetus empties its bladder into the amnionic sac the aminoglycoside antibiotic becomes sequestered. Little is known about the fate of the aminoglycoside but it is probably reabsorbed by the fetus after being swallowed and inspired into the lungs. Since the aminoglycoside has not been modified by either the mother or the fetus, reabsorption of the aminoglycoside from the amnionic fluid through the fetal gut is equivalent to continuously injecting a small amount of drug into the fetus. This would increase the area under the

pharmacokinetic curve.

Note that in Figure 21 and in Table 5 that some fetal perilymph concentrations of kanamycin approach the levels noted in guinea pigs injected four times per day with the two greater doses of kanamycin in this experiment. One might predict ototoxicity in the fetus on this basis. However, the perilymphs for these two experiments were sampled at two different times--four hours in the adult case and two hours in the case of the fetuses. The actual adult peak level might be higher that which was measured. Also, and probably more important, the adults were exposed to this perilymph concentration of kanamycin four times in a twenty four hour period while the hair cells of the fetuses were only exposed to these perilymphatic levels once in twenty four hours. The last experiment in this study indicated the importance of the time parameter in determining ototoxicity.

General Discussion

Introductory comments indicate the long term effects of infant deafness. Hearing is the mechanism by which children learn about themselves, their world and other people. The deaf child is severely handicapped. Therefore, it is important to prevent iatrogenic congenital deafness whenever possible.

One purported cause of iatrogenic deafness is prenatal exposure to the aminoglycoside antibiotics. These drugs have been documented to produce deafness in human adults and children when administered post-natally. Little is known about their ototoxic effects when administered prenatally. Even less is known about

the comparative ototoxic effects of the aminoglycoside antibiotics administered during pregnancy. Human case studies generally measure only ototoxicity in the offspring. Those which do report maternal ototoxicity do not usually correlate maternal and fetal ototoxicity. Even in the case of experimental fetal ototoxicity little is said about the effects of the aminoglycoside antibiotics on the hearing of the mother. This study was an initial effort to compare the ototoxic effects of aminoglycoside antibiotics on fetuses and their mothers. It was also an initial effort to determine why those differences occurred.

Generally the present study found no fetal ototoxicity when measured electrophysiologically. Mothers injected with 200 mg/kg per day kanamycin for two weeks required increased sound levels to produce one microvolt of ac cochlear potential at frequencies above 2 kHz. This was not the case for the offspring who had been *in utero* during the injections. The maximum outputs for mothers injected during pregnancy with kanamycin were less than those mothers injected with saline during pregnancy. The offspring of mothers injected with kanamycin during pregnancy produced electrophysiological results equivalent to those offspring whose mothers were injected during pregnancy with saline.

There are many reasons fetuses would demonstrate less ototoxicity to kanamycin exposure than the adult. First, the fetus may be less sensitive to the ototoxic effect of the aminoglycoside antibiotic than the adult. This hypothesis suggests that two separate, parallel log dose-response curves exist-- one for the

adults, the other for the fetuses. As development progresses the dose response curve for the fetus moves left to join with that of the adult. Figure 26 schematically represents this hypothesis. Perhaps structural or functional differences could underlie such a difference. The only way to test this hypothesis would be to expose both mother and fetus to the same dose of aminoglycoside antibiotic. Because of the inability of the aminoglycosides to cross the placental membranes, obtaining levels of antibiotic in the fetus equivalent to those in the mother might prove technically difficult.

The second possibility is that the mother and fetus share the same dose response curve. However, since the fetus receives such a small amount of kanamycin in its perilymph and blood it is on the lower subthreshold end of the log dose-response curve. The mother has higher levels of kanamycin in her perilymph and blood and therefore operates higher on the dose-response curve. The mother operates high enough on the curve to display ototoxicity. This hypothesis is schematically represented in Figure 26. Although this study cannot conclusively support either one of the two theories, levels of kanamycin measured in the fetus and mother would indicate that the differences in kanamycin levels between the mother and fetus plays some role in the ototoxicity differences observed.

Other researchers (Uziel, et al, 1977) giving higher doses over shorter periods of time or giving the same or lower doses (Akiyoshi, et al, 1977; Mesollelo, 1963) over longer periods of

Figure 26. Two possible hypotheses for the sensitivity of fetal and maternal ears to the ototoxic effects of aminoglycoside antibiotics

A. Separate dose-response curves exsist for the fetuses and adults. As the fetus develops, its dose-response curve shifts to the left until it matches that of the adult.

B Mother and fetus share on the same dose-response curve. However, the fetus receives such a low dose of ototoxic drug that any damage is undetectable.

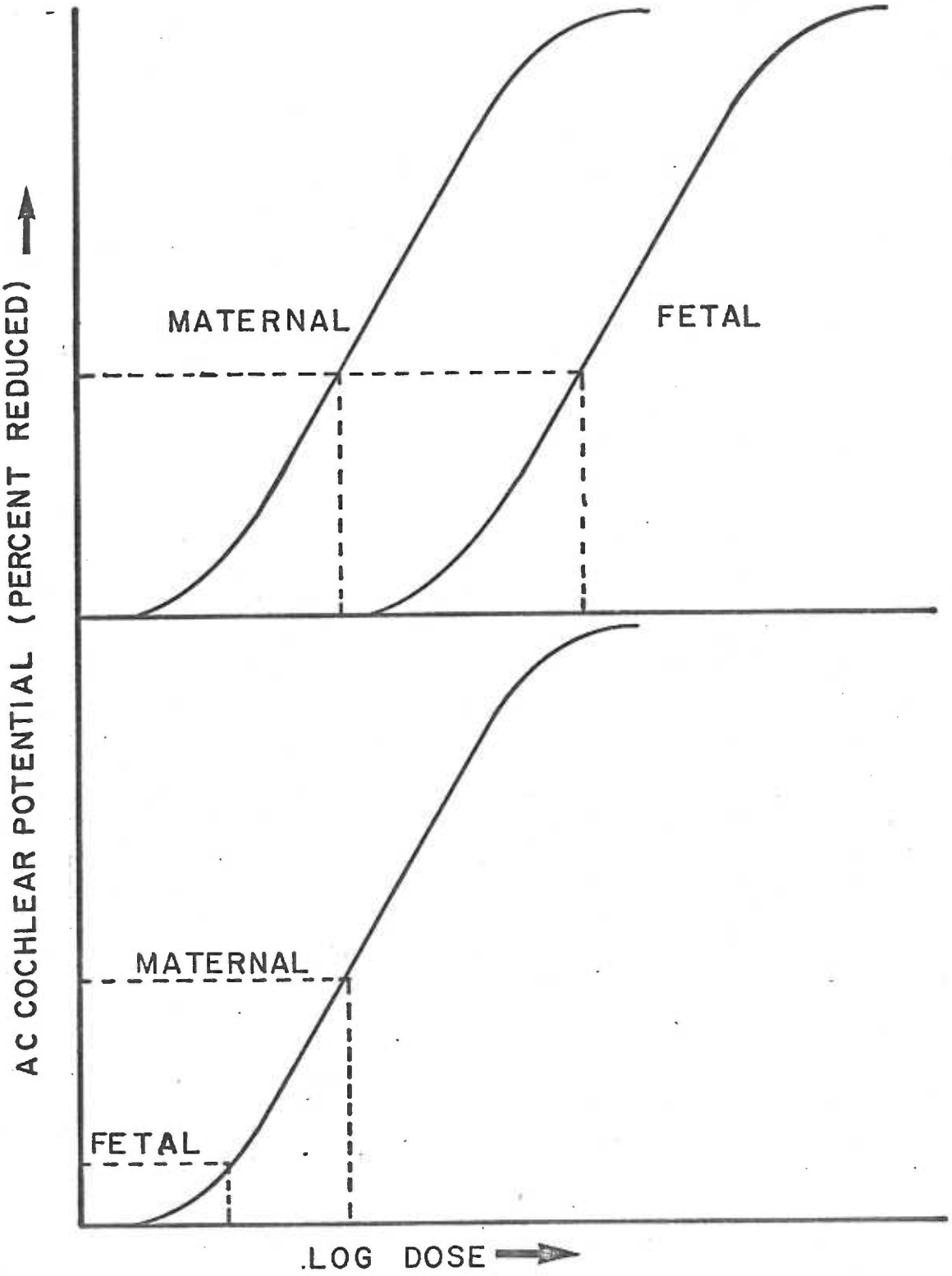

AC COCHLEAR POTENTIAL (PERCENT REDUCED)
MATERNAL
FETAL
MATERNAL
FETAL
LOG DOSE

Figure 26

time during pregnancy did detect ototoxicity in the resulting offspring. These data would appear to lend support to a hypothesis of differential drug exposure of mother and fetus. (As an aside, an attempt was made by this researcher to raise the concentration of kanamycin in the fetus by increasing the dose. One pregnant mother was injected with 300 mg/kg per day of kanamycin and two pregnant mothers with 400 mg/kg per day of kanamycin for a planned two weeks. The two mothers injected with 400 mg/kg of kanamycin died late into the injection series and the mother being injected with 300 mg/kg per day aborted her fetuses).

On the other hand there are some data in rats that indicate there may be a "critical period" for increased ototoxicity around the time of the onset of auditory functioning. Newborn rats have been referred to as "free living fetuses." At birth they are still functioning at an early stage of development in many aspects including the eye and ear. The ear does not begin to function until the eleventh day after birth and becomes completely developed on about the eighteenth day after birth (Pujol, Carlier and Lenior, 1980). Osako, et al (1979) were able to demonstrate that rat pups injected from day 11 to 20 after birth with 400 mg/kg or 200 mg/kg per day with kanamycin showed greater ototoxicity than those pups injected either from day 1 to 10 or for any other consecutive ten day period after 20 days of age. They used the amplitude and latency of the brainstem evoked response and auditory evoked cortical response as their measures. They used various frequencies and intensities of sound stimuli. Although

some methodological questions can be raised, other investigators appear to have replicated these results. Marot, et al (1980) injected rat pups from birth to day 8 with 400 mg/kg per day of kanamycin or from day 8 to day 16 after birth with the same drug and dose. The day 8 to 16 group demonstrated greater ototoxicity than the day 0 to 8 group. Carlier and Pujol (1980) injected three groups of rat pups with 200 mg/kg per day of amikacin for 10 days. The first group was injected from the day of birth, the second group was injected from day 10 and the third group was injected from day 30. Carlier and Pujol were able to measure greater ototoxicity in the group injected from day 10 to 20 after birth. They then injected another series of rat pups with 50 mg/kg per day of amikacin for ten day periods. These were days 10 to 20, 15 to 25, 20 to 30 and 30 to 40. Only those rat pups injected 10 to 20 days after birth and those injected 15 to 25 days after birth showed a change in threshold for N_1. Since these three studies were conducted independently it would indicate that these measures are reliable. The question becomes is this "critical period" a universal mammalian phenomenon or is it specific only to rats. Uziel, et al (1977) presented some evidence that the later in pregnancy guinea pigs are injected with kanamycin the greater the N_1 potential depression in the fetuses post-natally. This would coincide with the onset of auditory functioning in the fetal guinea pig. If there is a "critical period" for ototoxicity in the guinea pig it is possible that kanamycin in this experiment was injected too early in the

pregnancy and missed it. Presently there is little evidence that a "critical period" exists for any animal other than the rat. Is this "critical period" for ototoxicity in the rat an auditory system effect or an effect of some other body system? A hypothesis might be proposed whereby the kidneys of the rat are affecting kanamycin pharmacokinetics and producing increased ototoxicity. Figure 27 illustrates one possible hypothesis. This hypothesis assumes that the hair cells of the rat cochlea are immune to the ototoxic effects of kanamycin until they have become functional. Prior to day 10 the ear would be protected from high concentrations of kanamycin because the hair cells are not susceptible for what ever reason. The kidney increases its glomerular filtration rate from early after birth until past day 20. Thus the ear is protected to some extent after day 20 because of the increased effeciency of the kidney. But there is a period between day 10 and 20 where the kidney is not functioning well enough to rid the body of kanamycin nor are the hair cells immune to the toxicity of kanamycin since they are now functional. This would be interpreted as a "critical period." It is essential that a researcher in the future replicate these rat pup experiments but measure levels of kanamycin in the blood and perilymph of the rat pup. Only then will this mystery of a post-organogenesis "critical period" be solved. Also, these experiments should be replicated in a second species. Stephens (1972) has carefully detailed the development of the hamster making it an ideal choice for replication. The hamster auditory system also does not deve-

Figure 27. Hypothetical model to explain "critical period" to ototoxic effects of kanamycin in the rat. The ear may be immune to the effects of kanamycin before it begins to function, around day 10. The kidney does not efficiently eliminate kanamycin until about day 20. Kanamycin injections between days 10 and 20 will have longer half lives ($t_{1/2}$) than the same injections given later, exposing the hair cells longer to kanamycin. Those doses given between day 1 and 10 produce high levels of kanamycin with long half lives but the hair cells are immune to the ototoxic effects of kanamycin.

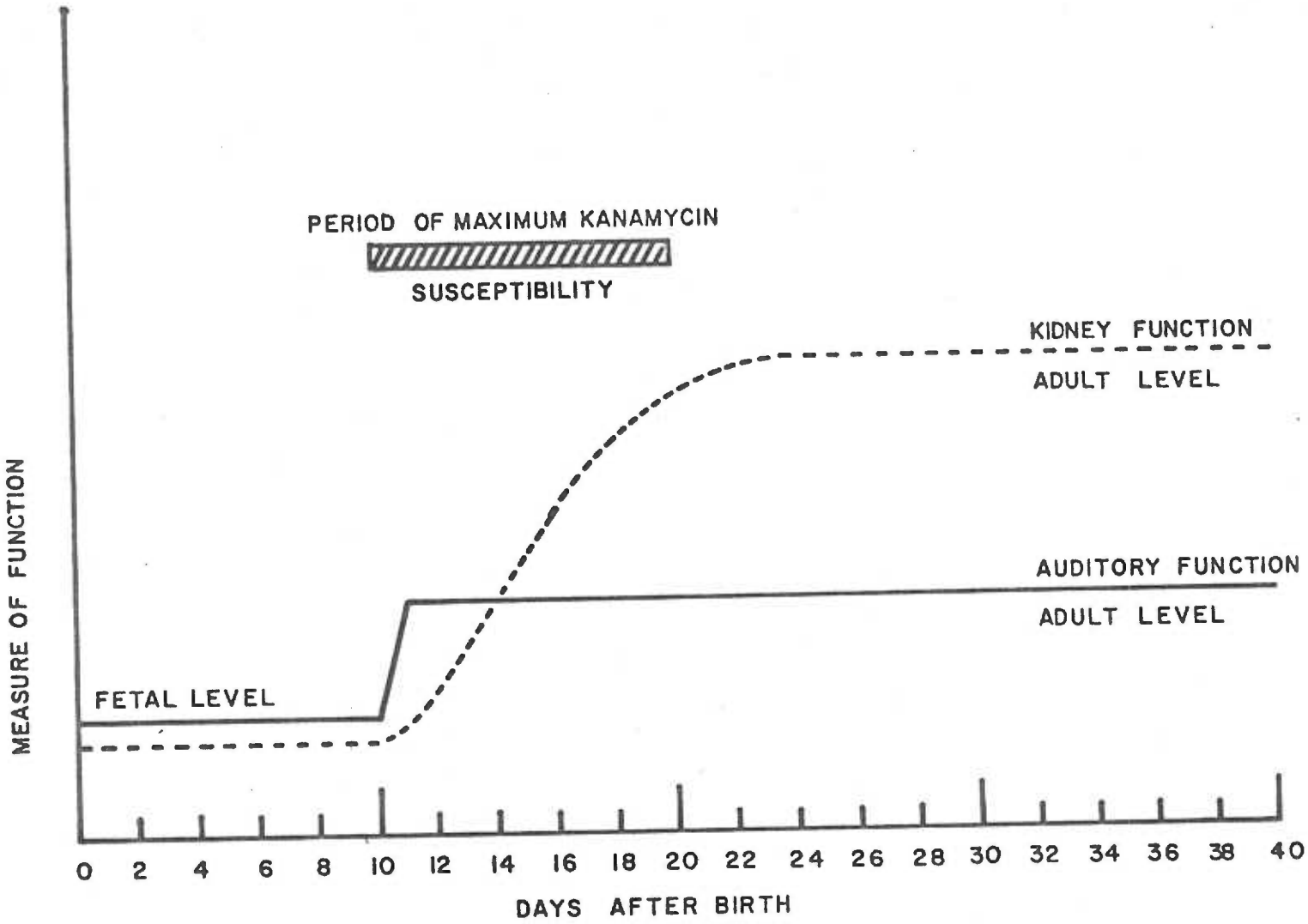

PERIOD OF MAXIMUM KANAMYCIN
SUSCEPTIBILITY
KIDNEY FUNCTION
ADULT LEVEL
AUDITORY FUNCTION
ADULT LEVEL
FETAL LEVEL
MEASURE OF FUNCTION
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
DAYS AFTER BIRTH

Figure 27

lop fully until after birth.

The guinea pig fetus differs from the rat pup described above in that it has two routes of elimination for the aminoglycoside antibiotics--the placenta and the kidneys. It is known that the nephrons of the fetal guinea pig are operating during the period of exposure of this study. Glomerular filtration continues to develop until 49 days after birth in the guinea pig (Spitzer and Edelman, 1971, Spitzer and Brandis, 1974). The mother has only one avenue for eliminating an aminoglycoside antibiotic--her kidneys. The maternal kidneys are also the ultimate path for elimination of fetal kanamycin.

This indicates that the amnionic fluid may be an important depot for the fetus for at least temporarily eliminating aminoglycoside antibiotics. Little is known about the dynamics of the amnionic fluid. If it has the important role that this study hints at, research should be conducted to measure pharmacokinetics of the aminoglycoside antibiotics in it. Can aminoglycoside antibiotics be reabsorbed by the fetus from the amnionic fluid? Can the fetus absorb enough aminoglycoside antibiotic from the amnionic fluid to produce ototoxicity? Could amniocentesis provide important information about the exposure of the fetus to ototoxic compounds in humans? These questions open up whole new avenues of study.

The problem of amnionic fluid serving as a pharmokinetic depot for drug is not unique. Some of the most highly lipid soluable barbiturates have their action modified by the

sequestering action of body depot fat. Thus, thiopental a "short acting" barbiturate can become a "long acting" barbitutate if the body's fat stores become saturated with the drug. The fat becomes a depot for thiopental. The amnionic fluid could be analogous for the aminoglycosides.

If the fetus can reabsorb the aminoglycoside antibiotics from amnionic fluid the fetus would be exposed to a long-lasting, low level of drug. The final experiment of this study indicates that ototoxicity of kanamycin can be dissociated from the peak plasma and perilymph levels. This is important for the fetus because although it only receives a tiny amount of aminoglycoside antibiotic with each injection, every aminoglycoside antibiotic given within a twenty four hour period adds to the ototoxic potential. Perhaps the integrated area under the pharmacokinetic curve is the important factor for determining ototoxicity. A study should be conducted to integrate the area of the pharmacokinetic curve and correlate this with ototoxicity.

Another avenue for experimentation should be organ of Corti tissue binding studies for kanamycin. The data in experiment 3 can be interpreted to indicate that aminoglycoside receptors on the organ of Corti bind molecules of kanamycin for long periods of time. Over the course of 24 hours as many aminoglycoside receptors become bound with quarter doses of kanamycin injected every six hours as become bound with a single full dose of kanamycin injected once per day. Only through tissue binding experiments could this hypothesis be examined. There are some data from

tissue binding studies in kidney tissue to support this hypothesis (Kardos, Eichholz and Schaffner, 1981). This might also provide a technique for predicting ototoxicity for newly developed aminoglycosides. Those aminoglycosides which bind tightly for long periods of time might be more ototoxic than those which bind less tightly for shorter periods.

The demonstration of peak kanamycin concentration dissociated from ototoxicity has clinical significance. Based on these data it would not appear to be efficacious to divide an aminoglycoside antibiotic dose in order to prevent ototoxicity. It would be advisable to use the largest dose tolerated by the patient, given over longer time intervals. This would have the effect of killing a larger proportion of the infectious bacterial population but producing the same amount of cochlear ototoxicity. Physicians do not save ears by dividing the dose into two, four or more smaller doses. Since bacterial resistance to the aminoglycoside antibiotics is conferred in a single, large step (Goldstein, et al, 1974), use of low doses of kanamycin would remove the drug sensitive organisms and leave the resistant organisms to grow--producing a drug resistant infection. Drug resistance can be minimized by using larger doses.

A caution should be included about extrapolating from one species to another. The half-life of kanamycin in humans is different from its half-life in guinea pigs. It is also well known that the size of dose necessary to produce ototoxicity in a guinea pig would be excessive if given to a human being. Of course these

experiments cannot be conducted on human beings. It is known that although these interspecies comparisons cannot be made on a quantitative level, the qualitative relationships do hold between the mammalian species

In conclusion, there is still much to learn about fetal ototoxicity. It is a frustrating and complex field of study.

Appendix A

A Radioenzymatic Assay for Kanamycin

The following appendix will consist of three parts. The first part will provide a brief introduction to the theory behind the assay, the second part will be a cookbook approach to conducting the assay and the third part will review some of the possible sources of artifact.

Background

Streptomycin, the first aminoglycoside antibiotic, was discovered in the early 1940's. It was first used clinically in 1945. Many new aminoglycoside antibiotics were discovered and introduced into clinical use over the next thirty years. After a time many of these antibiotics began to lose their effectiveness against gram negative bacteria they once easily destroyed.

In the 1960's studies were conducted in bacterial resistance. It was found that basically three forms of bacterial resistance could be identified. First, it became clear that the aminoglycoside antibiotics' site of action was the 30s subunit of the bacterial ribosome. The aminoglycoside antibiotic interacted with the ribosome to cause misreading of the bacterial RNA and misassembly of bacterial enzymes (Davies and Davis, 1968). Without enzymes to power the cellular physiology the bacterium dies. The first method of resistance is for the bacterial ribosome to be modified so that the aminoglycoside antibiotic no longer interacts with it. This is accomplished through genetic mutation. The second method of resistance involves changes in the

cell wall transport mechanism which transports the aminoglycoside into the bacterium. The aminoglycoside antibiotics are not effective against bacteria which do not actively transport them acrossed the bacterial cell wall. This change in transport mechanism involves genetic mutation. The third method of bacterial resistance involves the production of enzymes which modify the antibiotic to prevent an interaction with the ribosome. These enzymes are produced from strands of non-chromosomal RNA or DNA transferred between Gram negative bacteria of the same or different species. These enzymes are called R-factor enzymes--"R-factor" stands for resistance factor. R-factor enzymes use commonly available molecular substrates and covalently bond them to the active portions of the aminoglycoside antibiotic molecule. Commonly used molecules are acetyl CoA and adenosine triphosphate (ATP). These are enzymes they are usually specific for one or two aminoglycoside antibiotics.

About ten years ago it was determined that the R-factor enzymes present in resistant bacteria could be utilized to assay concentrations of aminoglycoside antibiotics in body fluids (Case and Mezei, 1978; Davies, Brzezinska and Benvenste, 1971; Smith, Van Otto and Smith, 1972; Williams and Northrop, 1976). Today radioenzymatic assays based on R-factor enzymes can be conducted for most of the aminoglycoside antibiotics. They are relatively fast, inexpensive, safe and capable of assaying very small volumes of fluid. The assay used in the present study was a radioenzymatic assay.

The Assay

The assay consists of six parts--the enzyme, citrate buffer, phosphate buffer, acetyl CoA, ^{14}C-acetyl CoA and the sample to be assayed. In addition a phosphocellulose filter paper disc is important to the assay. Specifics of preparing the reagents used in this assay will be included at the end of this section.

The R-factor enzyme used in this assay was derived from kanamycin resistant Escherichia coli (E.coli), strain W677. The R-factor enzyme is kanamycin-6'-acetyltransferase. As indicated by the name this enzyme transfers the acetyl group of acetyl CoA to the 6' position of the kanamycin molecule. If one were to incorporate a radioactive isotope of carbon into the acetyl CoA molecule the acetyl CoA could be used to label the kanamycin molecule. This is the basis of this assay.

The kanamycin-6'-acetyltransferase can be isolated in any moderately equipped laboratory. A much more efficient method is to purchase this enzyme from a commercial supplier. The enzyme used in this experiment was purchased from P.L. Biochemicals, Inc., 1037 West McKinley Ave., Milwaukee, WI 53205 (catalog number 0844). Another commercial supplier is Calbiochem. The enzyme was stored frozen.

Each assay was mixed in the following proportions:

Citrate buffer	30 microliters
Enzyme (P.L. Biochemicals, #0844)	5 microliters
Phosphate Buffer	25 microliters
Acetyl CoA	13 microliters
^{14}C-Acetyl CoA	2 microliters
Total volume of assay mixture	75 microliters
All standards and samples are ----------	20 microliters
Total Final Volume of assay	95 microliters

These reagent volumes were multiplied by the number of assays to be performed and the reagents were throughly mixed to insure uniformity. All reagents and mixtures were iced. Seventy-five microliters of assay mix were dispensed into numbered 12 mm diameter styrene test tubes. Twenty microliters of each of the kanamycin standard solutions were dispensed into numbered tubes containing assay mixture. These standards were run in duplicate. Twenty microliters of each sample was dispensed into a numbered assay tube. Perilymph kanamycin standards were made from chromatographically pure (94%) kanamycin sulfate (Boehringer-Mannheim Biochemicals, Indianapolis, Cat. No 106-805). Serum standards were made from the standard solution of kanamycin sulfate used for injection (Kantrex, Bristol Laboratories, 333 mg/ml). The standard solutions were assayed against one another and found to be equal to one another In the case of perilymph samples enough phosphate buffer was added to the perilymph sample to bring its final volume up to 20 microliters. All assay tubes were thoroughly mixed to insure homogenity of the sample and assay mixture.

All assay tubes were incubated at 37° C for 30 minutes in an oscillating water bath shaker After 30 minutes the assay tubes were placed back on ice.

Fifty microliter aliquotes of assay mixture were absorbed onto 15 mm numbered discs of phosphocellulose paper (Whatman P81) each of which was suspended from a stainless steel common pin. Phosphocellulose paper ionically bonds the aminoglycoside

antibiotics. All of the discs and pins were dropped into approximately 500 ml of glass distilled water 70-80° C for 30-60 seconds. This water was decanted and the beaker refilled with a 10° C rinse of glass distilled water for 30-60 seconds. The discs were rinsed another three times in 10° C distilled water. Agitation of the discs is very important during the rinsing process, so water was added with a dumping motion. Discs were removed from the final rinse and resuspended on their common pins. It is important that the discs are completely dry. The discs are either dried with an electric blower or allowed to air dry over night.

Radioactivity of each paper disc was determined by liquid scintillation counting. ^{14}Carbon emits beta particles as a consequence of nuclear breakdown. These beta particles impinge upon molecules of PPO dissolved in the surrounding solution. The PPO molecule emits a photon for every beta particle impinging upon it. A second compound in the solution, POPOP, absorbs the photon emitted by PPO and in return emits a known number of photons, thus "amplifying" the number of photons emitted by a single nuclear decay.

Each disc was placed into a 20 ml glass vial and covered with 10 ml of liquid scintillation counting mixture. The vials were loaded into a Beckman LS-3133P Liquid Scintillation Counter. Each vial was automatically counted for 20 minutes or until an error limit of 1.5% was obtained.

The counts-per-minute data obtained from the liquid scintillation counter were converted into micrograms of kanamycin per ml of body fluid in the following manner The standard con-

centrations of kanamycin were entered into a linear regression program along with the corresponding counts-per-minute. From these data a slope and intercept for a line was derived. This equation took the following form:

Concentration of
kanamycin, mg/kg = (Y intercept, cpm) + (slope·sample cpm)

In the case where the volume of the sample was less than 20 microliters in volume the counts per minute were multiplied first by the ratio of 20 microliters to the actual number of microliters used. Thus the counts per minute measured for a 5 microliter sample would be multiplied by 20/5=4 before being entered into the above equasion.

Reagents

The following are formulation instructions for the reagents used in this experiment All reagents can be made up in large quantities and the unused portions frozen

Citrate Buffer

Stock solution A 0.1 M citric acid
21.01 grams Citric Acid in 1000 ml water
Stock Solution B 0.1 M sodium citrate
29.41 grams $C_6H_5O_7Na_3 \cdot 2H_2O$ in 1000 ml water

Add 13.7 ml stock solution A to 36.3 ml stock soulution B and dilute to 100 ml with water. Final pH = 5.6. To 100 ml of citrate buffer add:
0.0514 gram of DTT (Cleland's reagent, Dithiothreitol, Calbiochem, A Grade) and 0.2033 gm $MgCl_2 \cdot 6H_2O$
This buffer can be frozen and stored indefinatly at 0°C

Phosphate Buffer (.099 M phosphate)
In to one liter of water add 0.645 gm of KH_2PO_4 and 25.38 gm of $Na_2HPO_4 \cdot 7H_2O$. Final pH = 8 - 8.3. This solution need not be frozen.

Acetyl Coenzyme A

Place 0.0033 gm of acetyl CoA in a 10 ml volumetric flask and add water to 10 ml mark. Final concentration is equal to 1.85 nmole/5 ml.

^{14}C-Acetyl Coenzyme A

Dilute acetyl CoA to 20 microCi per milliliter. Specific activity equal to 57 mCi/mole. Suppliers of (1-14C) Acetyl Coenzyme A include New England Nuclear (cat. no. NEC-313) and Amersham.

Liquid Scintillation Counting Mix

To a standard 8 pt bottle of scintillation grade toluene add

15.12 gm PPO (2,5-Diphenyloxazole)
0.378 gm POPOP (1,4-bis[2 (5-phenyloxazlyl)]benzene)
Allow to stand overnight at room temperature.
Store mixture in a dark bottle.

Assay Artifact

As with any assay procedure this assay has certain limitations. Whenever the researcher challenges these limitations the chances of artifact being introduced increase. In addition methodology can introduce artifact. In order to understand these problems better a discussion of some of the known steps where artifact can enter will be reviewed.

1. Obtaining the sample. The sample must be clean and free of contaminating fluids. Thus perilymph samples must be free of blood, cerebrospinal fluid, middle ear fluid, etc. and blood samples must be free of amnionic fluid, urine, etc. Micropipets, syringes and needles should be used only once and then discarded to prevent aminoglycoside antibiotic contamination.

The animal should not have received any other aminoglycoside antibiotic. Kanamycin-6'-acetyltransferase will label other aminoglycoside antibiotics such as gentamicin. Any other aminoglycoside antibiotic present will interfere with the ability of the assay to quantify kanamycin.

2. Quantifying the volume of the sample. Once the perilymph sample is obtained it should be weighed as soon as possible. This is important because, for example, a five microliter sample can evaporate a substantial portion of its volume even though only a small surface area is exposed to the air Also care must be taken to prevent changes in the tare weight of the micropipet. The micropipets should only be handled with clean vinyl gloves and care taken to prevent micropipet contact with moist tissue or fur.

3. Transfer and storage of samples. Perilymph samples are recovered by blowing the sample into a premeasured volume of buffer. Care must be taken to avoid blowing any saliva into the assay tube. In expelling the sample care should also be taken to avoid splashing buffer onto the sides of the test tube. This could produce increased evaporation. The styrene test tubes are closed with Parafilm. The Parafilm seal should be air tight The Parafilm can be cut by the edge of the test tube, so each Parafilm seal should be inspected.

4. Sample size. There are two common areas of artifact for this assay: a sample volume which is too small, common with perilymph samples, and a sample in which the kanamycin is too concentrated, common with blood samples.

There are at least four reasons small sample volumes cause inaccuracies. First, a small sample cannot be weighed as accurately as a large sample. It therefore cannot be quantified as well as a large sample. Second, the concentration of kanamycin in the sample becomes highly diluted with small samples. For example, a

0.5 microliter sample is diluted 179 times by the added buffer while a 5.0 microliter sample is diluted only 17 times. Third, a highly diluted sample is not counted as well on the scintillation counter. Decays due to the radiolabel may be obscured in the background activity. Fourth, the calculator program which is used to determine the concentration of kanamycin present multiplies the measured counts per minute of any sample under 20 microliters by a ratio of 20 divided by the actual sample volume. The measured counts per minute of a large sample is only multiplied by 2, 3, or 4 times. But in the example of a 0.5 microliter sample the counts per minute are multiplied 40 times. This can have a serious effect on the variability for these small volumes.

In order to determine the smallest perilymph sample volume which was still usable, an experiment was conducted.

Method

Two stock solutions of kanamycin base, one containing 5.0 micrograms per milliliter and the other containing 10.0 micrograms per milliliter of water, were prepared.

A calibrated micropipeter (Pipetman) was used to deliver 0.5, 1.0, 1.5, 2.0, 5.0, 10 0, 12.0, 15.0 and 20.0 microliter samples of each of the two stock solutions into numbered styrene sample tubes. An ascending series of standards was pipeted followed by a descending series for each solution. Enough phosphate buffer was added to bring all samples to 20 microliters total volume. This was equivalent to the regular perilymph procedure. Samples were assayed in the manner described above and the total micrograms of

kanamycin base per milliliter of water was derived by the standard linear regression calculator program

In addition another set of standard samples were used. These samples were assayed under a procedure that more closely approximated the normal perilymph procedure. First, a set of glass micropipets were individually tared. Then, using mouth suction, a sample of the standard solution of unknown volume was drawn into the pipet. The pipet was reweighed and the sample blown into a styrene test tube. A volume of phosphate buffer was added to make the total volume of the sample 20 microliters. Again samples were assayed and the concentration of kanamycin was derived in the standard manner.

Results and Discussion

Figures 28, 29 and 30 indicate that samples smaller than 2 microliters were not accurately assayed. The differences between the derived level of kanamycin and the known level of kanamycin were anywhere from 10 to 110%. The amount of variability did not appear to be dependent upon the micropipeter or analytical balance since both procedures appeared to produce about the same amount of variability.

5. Sample concentration. Blood samples will often contain many hundreds of micrograms of kanamycin per milliliter of fluid. This assay is not able to cope with extremely concentrated samples. When the sample volumes are large enough (1 ml or more) to conduct multiple assays, some of the sample can be diluted to bring the kanamycin concentration to within the range useable by the assay.

Figure 28. Calculated concentration of a 5 microgram per milliliter stock kanamycin solution. Sample volume was varied between 0.5 and 20 microliters. Note how the volume of less than 2 microliters leads to increased variability.

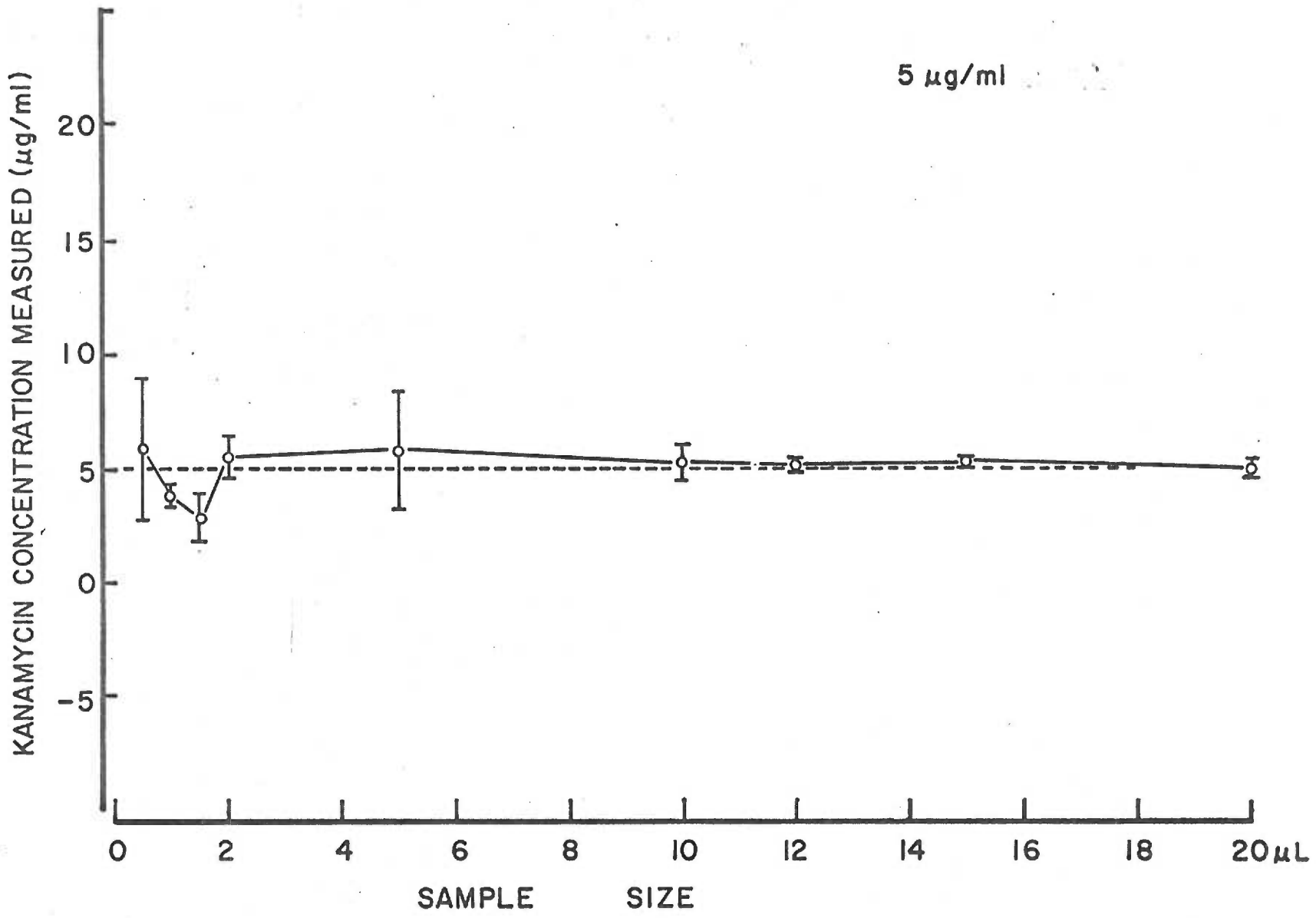

5 µg/ml
KANAMYCIN CONCENTRATION MEASURED (µg/ml)
20
15
10
5
0
-5
0
2
4
6
8
10
12
14
16
18
20µL
SAMPLE SIZE

Figure 28

Figure 29. Calculated kanamycin concentration for a stock 10 microgram per millilter kanamycin solution. Note extreme variability below 2 microliters. Vertical bars are ±1 standard deviation.

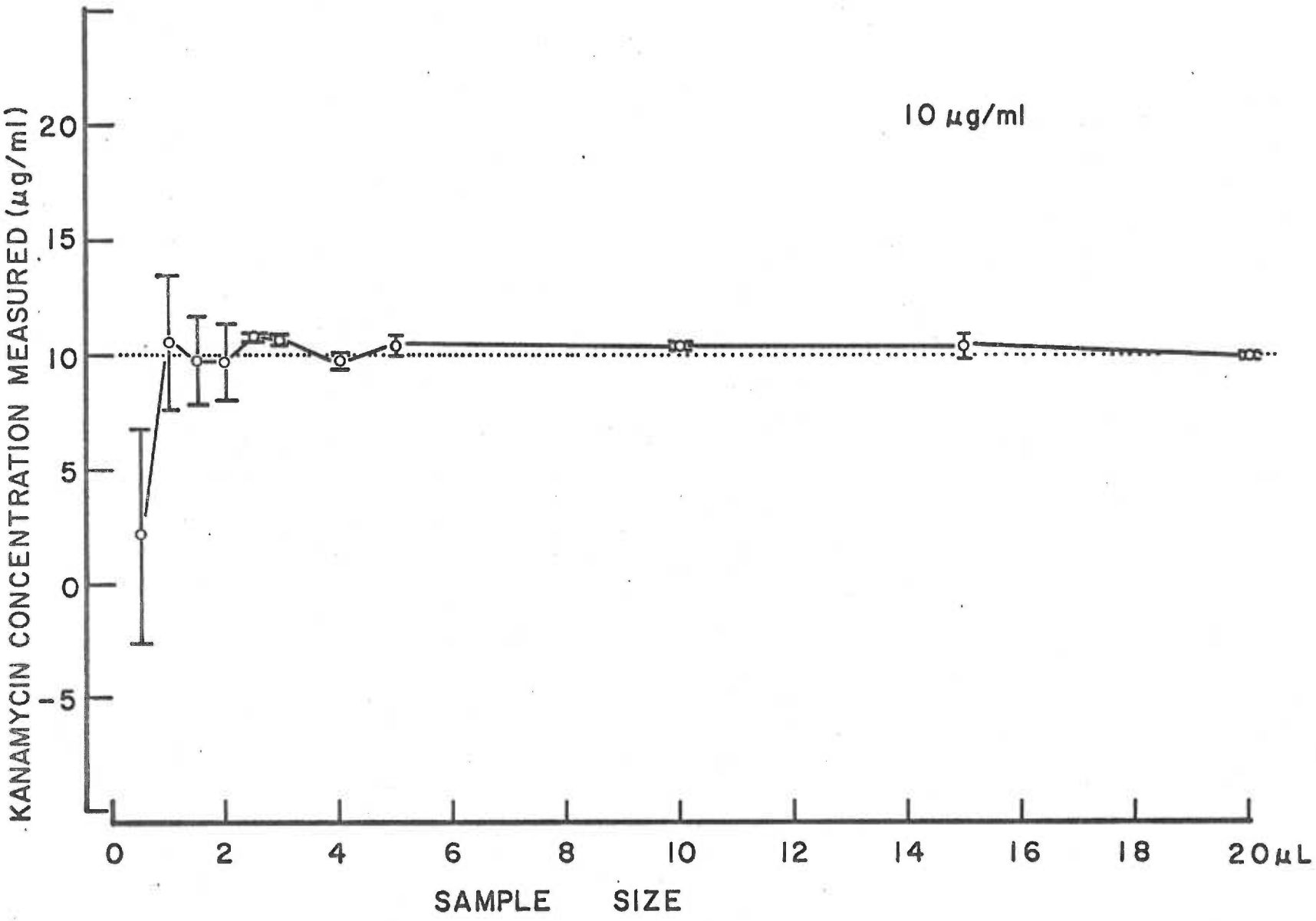

10 µg/ml
KANAMYCIN CONCENTRATION MEASURED (µg/ml)
20
15
10
5
0
-5
0
2
4
6
8
10
12
14
16
18
20µL
SAMPLE SIZE

Figure 29

Figure 30. Concentrations of a stock kanamycin concentration measured when the volume varies. This experiment used a 5 microgram per milliliter solution of kanamycin loaded into glass micropipets. The pipets were weighed, samples taken and reweighed. The solution was then assayed in the same manner as a perilymph sample. Note the trend which is similar to the previous two graphs--extreme variability below 2 microliters which increases as the sample decreases in volume.

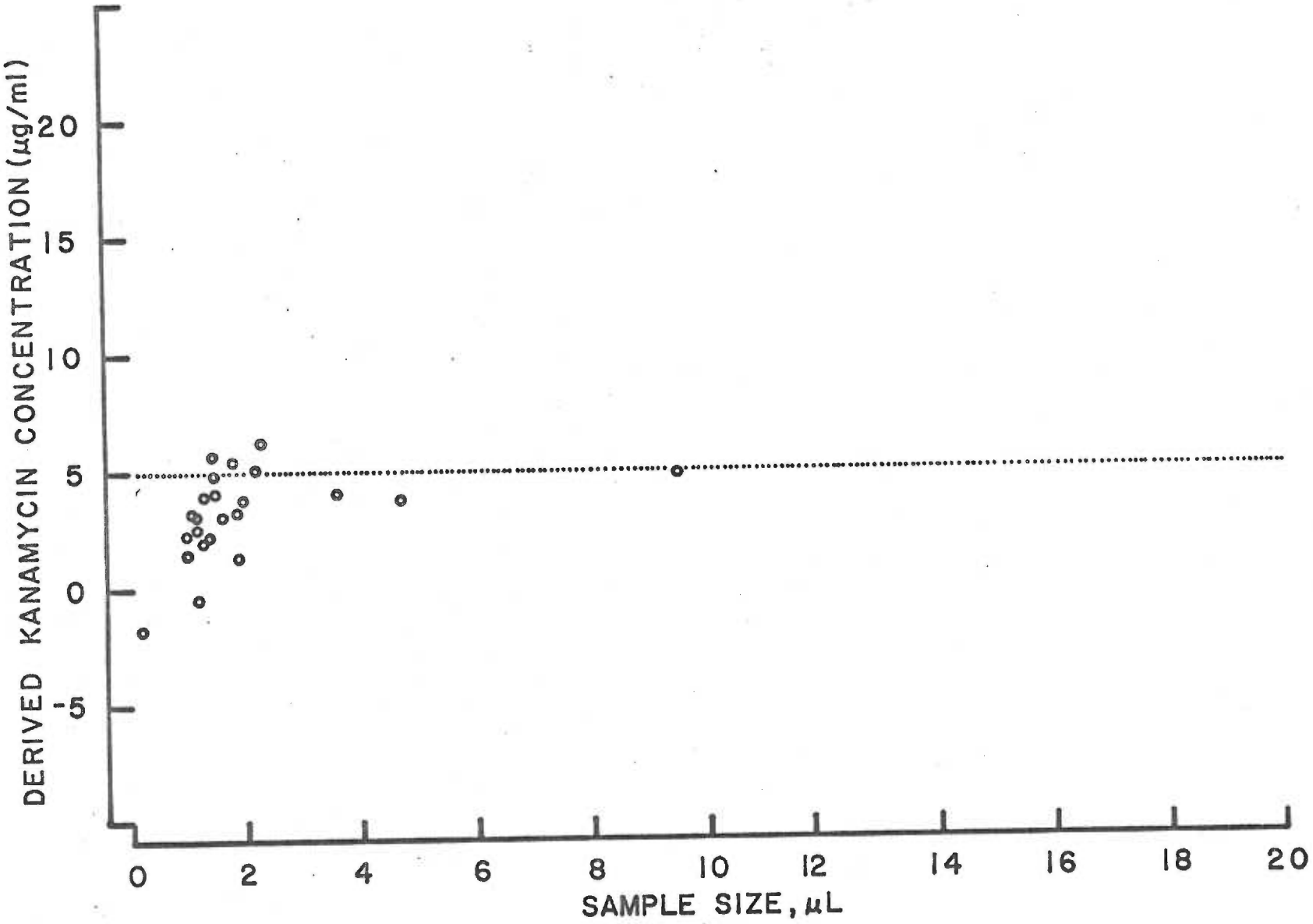

DERIVED KANAMYCIN CONCENTRATION (µg/ml)
20
15
10
5
0
-5
0
2
4
6
8
10
12
14
16
18
20
SAMPLE SIZE, µL

Figure 30

The precise reason for high kanamycin concentrations in the sample interacting with the assay is unknown. Perhaps the kanamycin interacts with the enzyme to modify its binding charteristics for its substrates Another possibility is since each assay has only a limited quantity of radiolabelled acetyl CoA perhaps the radiolabel is exhausted before all kanamycin molecules are labelled.

In this experiment a sample was determined to be diluted enough when one further dilution produced a decrease in the number of decays per minute recorded by the scintillation counter. In addition, the decays per minute had to be within the linear region of the standard curve.

6. Sample quenching. The beta particles emitted by the ^{14}C-acetyl CoA can be stopped before exciting a molecule of PPO by many substances that may be present on the phosphocellulose paper disc or in the scintillation fluid. This process is called quenching. Since water can quench beta particles it is essential that the phosphocellulose discs be thoroughly dried before being counted in the scintillation counter. Usually an overnight drying is sufficient.

Conclusion

Described above is a relatively straightforward assay procedure for measuring kanamycin in body fluids. Its advantages are it requires only microliter size samples, its relative speed and its relative simplicity. Its disadvantages are the narrow range of kanamycin concentrations which are able to be assayed (0.5 to 50

micrograms per milliliter) and the inaccuracies in assaying very small sample sizes (less than 3 microliters). Human hazards exist in handling the radiolabelled acetyl CoA and toluene.

By understanding the areas where the assay is weak, data generated can be scrutinized to determine its validity. For example, data for any perilymph sample in this study of less than 2.0 microliters in volume were discarded. Maternal serum samples were diluted with normal plasma in a descending concentration series (maternal:normal, 20:0, 10:10, 5:15, 2:18, 1:19 and 0.5 to 19.5) until the decays per minute measured on the scintillation counter dropped to one half of those of the previous dilution.

Appendix B

The Adult Mammalian Ear

The descriptions of the mamalian ear presented in the main portion of this paper are mainly those of the developing fetal ear. Generally, it was assumed the reader has had a basic knowledge of the adult ear This section was included for those individuals who would like to review the structure and function of the adult mammalian ear Many fine textbooks exsist on the human and mammalian ear and this author urges the interested reader to consult those textbooks for further information. Geldard (1972) and Gulick (1971) are especially worthy of consideration.

The ear is an organ system which converts the condensations and raferactions of air (sound) first into mechanical movement, then hydraulic movement and finally into neural impulses. The ear is still a puzzle but many principles of its operation are understood.

The ear is divided anatomically into three sections--the external, middle and inner ear The first part is the most visible. The external ear consists of the pinna--basically non-functional in humans--and the external auditory canal. The external auditory canal ends with the thin tympanic membrane. The external auditory canal contains hairs and produces wax to prevent entry of foreign matter, insects, etc.

The tympanic membrane closes the auditory canal proximally, separating it from the middle ear. The tympanic membrane is a thin, tough membrane which vibrates in response to sound.

The middle ear, the second part, is an air filled space open to the atmosphere only by the eustachian tube. Within the middle ear are the three smallest bones of the body--the ossicles. The ossicles transform the motions of the tympanic membrane into motions in the fluid of the inner ear. This is accomplished through leverage. The head of the malleus is attached to the large tympanic membrane and forms the long arm of the lever. The incus is attached to the pivot of the malleus by ligaments and forms the short arm of the lever. This difference in lever length multiplies the force presented to the tympanic membrane. The fulcrum for this lever is the pivot of the malleus and incus. The load for the incus is the head of the stapes. The stapes acts like a piston for its footplate pushes on the oval window of the cochlea. Sound energy impinges upon the relatively large tympanic membrane, this force is multiplied by the lever ratio of the malleus-incus lever system and is transferred to the small oval window membrane. Thus the ossicles act as an impedence matching transformer, matching the charteristics of air to the charteristics of liquid. In addition a muscle attaches to the malleus and one attaches to the stapes. These muscles act to decrease the efficiency of the ossicular chain to protect the inner ear from noise damage. Liquids cannot be compressed. Since the inner ear is enclosed in bone, a place where pressure can be relieved is necessary. This takes the form of the round window.

The inner ear, the third part of the ear, contains the elements which convert the vibrations of sound into the neural

impulses the brain can understand The inner ear, or cochlea, resembles a snail shell. It has 2½ turns in the human but 4 turns in the guinea pig The number of turns differs from species to species. However, all mammalian cochleae have virtually the same microscopic structure. The cross sectional space within the cochlea is divided longitudinally into three compartments, or scalae, by two membranes. The middle space, or scala media, is separated from the scala vestibuli by Reissner's membrane and from the scala tympani by the basilar membrane. Scala vestibuli and tympani are joined togeather only at the apex by a hole called the helicotrema. Scala vestibuli and tympani are filled with a fluid called perilymph. Perilymph has a high sodium concentration and a relatively low potassium concentration, similar to extracellular fluid. Scala media is filled with endolymph which has a low concentration of sodium and a high concentration of potassium, like intracellular fluid. These fluids are important because the sound receptors are bathed in them. The sound receptors, or hair cells, are believed to have their tops bathed in endolymph and the rest of their cell bodies bathed in perilymph. This difference in ionic concentration appears to be important for generation of electrical potentials in the cochlea.

The hair cells are resident within the organ of Corti. The organ of Corti is attached to the scala media side of the basilar membrane and is present the entire length of the basilar membrane. The hair cells are arranged into four longitudinal rows the entire length of the organ of Corti. There is one row of inner hair

cells, call inner because of their position close to the axis of the cochlea. There are three rows of outer hair cells. The inner and outer hair cells differ at least morphologically and probably functionally (Dallos, 1981). Inner hair cells are flask shaped and have a straight row of hairs apically which resemble a paint brush. The outer hair cells are cylindrical in shape and have apical hairs arranged in the shape of a "W". Inner and outer hair cells differ in their susceptability to ototoxic drugs (Hawkins, 1976).

The basilar membrane seems to be the "filter" which allows the ear to dissect complex sounds into simple sounds. The membrane differs in width, thickness and stiffness from the base to the apex of the cochlea. Because of these physical differences from the base to apex the basilar membrane responds uniquely to sound. The maximum displacement for low frequency sounds is localized towards the apex and for high frequency sounds is localized toward the base. Since the organ of Corti is tightly attached to the basilar membrane the site of maximum displacement will be the place where hair cells are maximally stimulated. The hairs of the outer hair cells are inserted into a gelatinous membrane called the tectorial membrane which causes the hairs to be exposed to a shearing force. This shearing force is the effective stimulus for the hair cell. It is believed a neurotransmitter is released by the hair cells but the neurotransmitter's identity remains unknown. The outer hair cells generate an electrical response commonly known as the cochlear microphonic, but more correctly as the ac cochlear potential. The intensity of the ac cochlear

potential at any one frequency is correlated with the number of outer hair cells present at that portion of the basilar membrane and the intensity of sound presented. The nerve fibers of the auditory nerve synapse upon the hair cells. However, ten times more nerve fibers synapse upon the inner hair cells than on the outer hair cells. This is not intuitively obvious since there are three outer hair cells for every inner hair cell in the normal organ of Corti.

As described in the main part of the study the aminoglycoside antibiotics damage the outer hair cells. Destruction of the outer hair cells begins with the most basal and inner most row of outer hair cells. Thus the individual undergoing aminoglycoside antibiotic treatment will lose the ability to hear high frequency sounds first. With continued aminoglycoside antibiotic treatment the hearing loss will affect lower and lower sound reception until most of the hearing has been damaged. Because few of the sounds important to us, e g. speech and music, include the highest frequencies, the loss of hearing in those frequencies is not usually noticed. It is only when the hearing loss impinges upon the speech frequencies that the patient may complain.

The ear is a complex organ system. Because of its position within the densest bone of the body--the petrus portion of the temporal bone--little is known about the functioning human inner ear. In order to make physiological determinations of the functioning ear experimental animals are used of which guinea pigs, cats and chinchillas are the most widely used. These animals

are ideal, for their cochleae are not embedded in bone like those of humans and primates. A bulla, or bony "bubble", an extention of the middle ear space, almost totally surrounds the inner ear of these animals. It is possible to enter the middle ear spaces of these animals while leaving the tympanic membrane intact and the ear functional. The researcher can then easily place electrodes within or on the cochlea, infuse drugs into the cochlea or remove cochlear fluids. These operations are very difficult in human beings.

Recording the ac cochlear potential

Wever and Bray (1930) discovered the ac cochlear potential. The ac cochlear potential is a receptor potential which is an electrical analog of the sound impinging upon the ear. This potential is usually recorded from the round window membrane of the cochlea. The ac cochlear potential is thought to be generated by the hair cells of the intact cochlea (Dallos,1981; Vernon and Meikle, 1974).

The ac cochlear potential, in addition to being an electrical analog of the sound stimulus and believed to be somehow involved in the production of the nerve action potential, can be used to determine the pathology of the outer hair cells of the cochlea. Hawkins and Laurie (1952) first described the effects of streptomycin and dihydrostreptomycin intoxication as decreasing the magnitude of the ac cochlear potential of the cat. Since that time others have confirmed that the change observed in the ac cochlear potential is correlated with destruction of outer hair

cells of the organ of Corti (e.g. Brummett, Fox, Bendrick and Himes, 1978; Ostyn and Tyberghein, 1968; Ward and Fernandez, 1968). Probably more important Walloch and Taylor-Spikes (1978) have indicated that the ac cochlear potential is a relatively good predictor of behaviorally measured auditory thresholds in the guinea pig. This indicates that the ac cochlear potential is a valid measure of cochlear function.

The following is the procedure used to measure the ac cochlear potenial, calibrate the sound system and comfirm no artifact is interfering with the recordings. Immediately after this section the procedure for preparing the ears for histological evaluation will be reviewed.

Surgical procedure. The guinea pig was weighed and anesthetized by an intraperitoneal injection of Dial with urethane (100 mg/kg allobarbital, 400 mg/kg urethane). Fur was clipped from around the pinna and neck of the guinea pig. An incision was made on the neck and the trachea was blunt dissected from the overlying muscles. A cut was made between tracheal cartilage rings and a tracheal catheter inserted. The guinea pig was respirated through the catheter by a Harvard Apparatus small animal respirator. A rectal thermistor probe was inserted. This sensor provided a measure of body temperature to a transistorized controller (EKEG Electronics Co. Ltd, Vancouver, B C., Model TCU 691) which operated a heating pad upon which the guinea pig lay. Body temperature was maintained at 38° C ($\pm$ 0.2° C).

The surgical technique for recording the ac cochlear poten-

tial was a modification of that described by Vernon and Meikle (1974). The left pinna was removed and the muscle overlying the auditory bulla was reflected back. The point of a scapel was used to open a hole in the bulla. The hole was enlarged with a hemostat or forceps. A brass speculum was positioned in the external auditory meatus and a patent pathway for sound confirmed by observation through a stereomicroscope. A one millimeter calibrated probe tube attached to a half inch B & K microphone was placed into a hole in the ear speculum. Using this arrangement sound measurements could be made just lateral to the tympanic membrane.

The active electrode for measuring the ac cochlear potential consisted of a 0.1 mm silver ball electrode attached to a micromanipulator. This electrode was placed upon the round window membrane of the cochlea visually with the aid of a stereomicroscope. The second active electrode consisted of an alligator clip which was attached to the cut skin around the ear. The common electrode was a hypodermic needle pushed through the skin of the rear foot.

Measuring the ac cochlear potential. The ac cochlear potential was differentially amplified one thousand times by a Keithley 103 preamplifier (lower filter setting of 10 Hz, upper filter setting of 100 kHz) and then measured by a General Radio 1900A wave analyzer (Figure 30b). A wave analyzer is basically a sensitive voltmeter with a very narrow input bandpass filter. The wave analyzer was operated with the frequency under test as the

center frequency and a bandpass of 3 Hz

Production and measurement of sound. The production and measurement of the sound stimulus in a reliable and reproducible manner is very important for measuring cochlear ototoxicity. The signal must be of low distortion and capable of high intensity.

The sound stimulus was generated in the following manner (Figure 31a). A sinusoidal electrical signal was generated by a General Radio oscillator and was passed through the filter section of the wave analyzer. This sets the wave analyzer's electronic filter section to the frequency of the electrical signal. The signal emerged from the output of the wave analyzer and was adjusted to one-half volt amplitude. The magnitude of the electrical signal was then modified by a General Radio decade attenuator. The decade attenuator is a precision resistor network capable of providing 0 to 110 decibels (dB) of attenuation in one dB steps. The attenuated signal was then amplified by a Phase Linear Model 700 power amplifier operated in the constant gain mode. Power amplifiers tend to add noise to the signal so the signal was further attenuated by a power attenuator (tail end attenuator) capable of handling the high wattage signal. This power attenuator was capable of attenuating the signal 0, 20, 40 or 60 dB. The sound stimulus was generated when this electrical signal was presented to the voice coil of a Western Electric 555 speaker. The speaker was mounted outside of the sound attenuating, shielded room. An eighteen inch length of bakelite tubing was attached to the front of the speaker and extended

Figure 31a. Schematic diagram of equipment used in this study to generate the ac cochlear potential. A sinusoidal electrical signal was generated by the General Radio 1162-A Coherant Decade Frequency Synthesizer. The electrical signal passed through the General Radio 1900-A Wave Analyzer which set the electronic filters of the wave analyzer to the signal's frequency. The amplitude of the output signal was monitored by a Simpson Model 49 Voltmeter. The Monsanto 100-A Frequency Counter served to monitor the frequency of the signal. The signal was attenuated by a General Radio 1450-TA Decade Attenuator. The attenuator controlled the amplitude of the electrical signal in 1 dB steps to a total 110 dB. The signal was amplified by a Phase Linear Model 700 Power Amplifier operating in the constant gain mode. A power attenuator provided a final amount of attenuation to remove noise from the signal. Finally the signal was applied to the voice coil of a Western Electric 555 Speaker which transduced the signal into sound. The Western Electric 555 Speaker requires a 7 volt power supply to power the internal electromagnet. The guinea pig was inside a sound attenuating, sheilded room.

Figure 31b. Schematic diagram of equipment used in this study to record the ac cochlear potential. A silver ball electrode rested upon the round window membrane of the guinea pig. The electrical signal from the electrode was differentially amplified 1000 times by a Keithley 103 Preamplifier. The signal was then measured by a General Radio 1900-A Wave Analyzer, operating with a 3 Hz bandwidth.

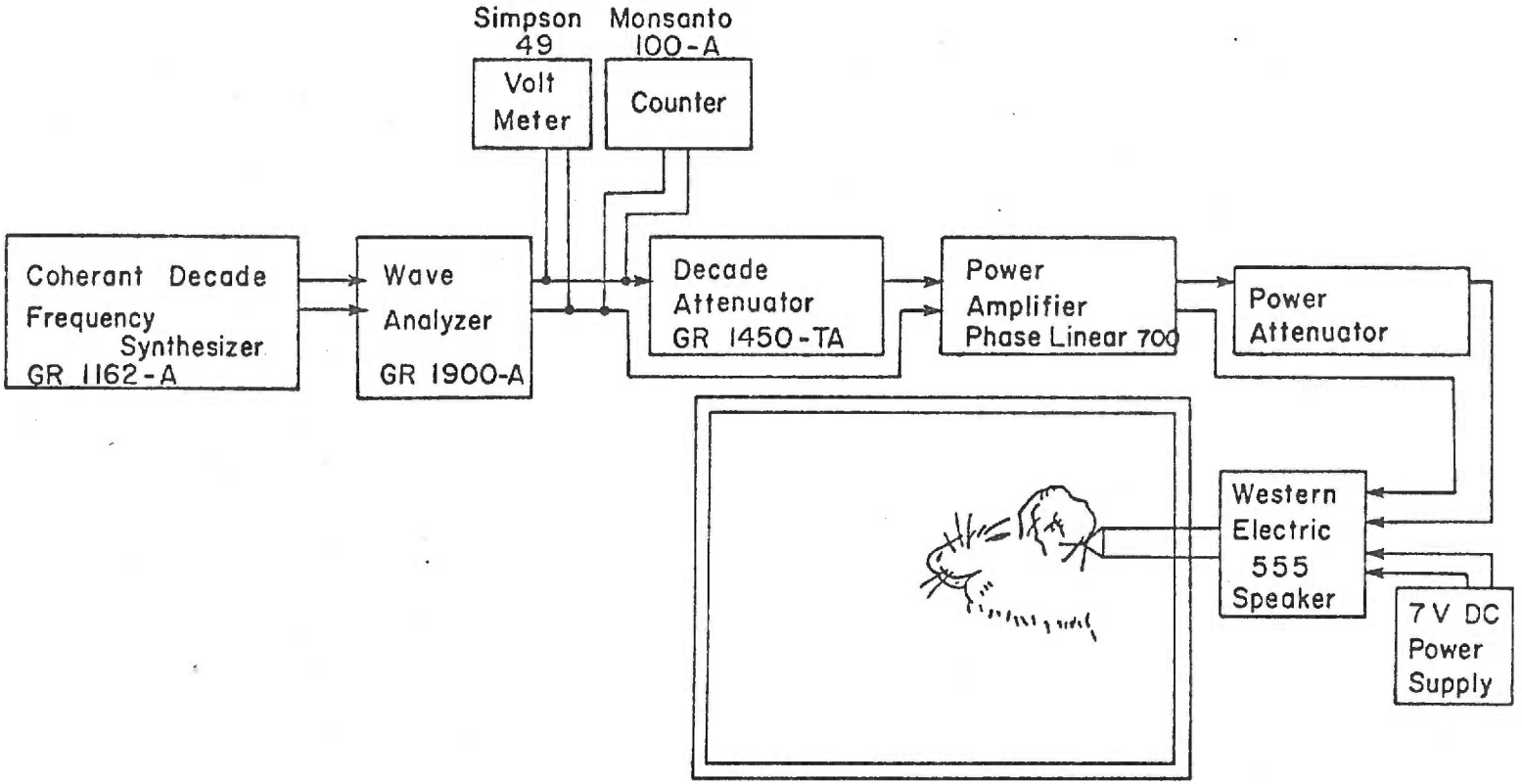

Simpson
49
Volt
Meter
Monsanto
100-A
Counter
Coherant Decade
Frequency
Synthesizer
GR 1162-A
Wave
Analyzer
GR 1900-A
Decade
Attenuator
GR 1450-TA
Power
Amplifier
Phase Linear 700
Power
Attenuator
Western
Electric
555
Speaker
7 V DC
Power
Supply

Figure 31a

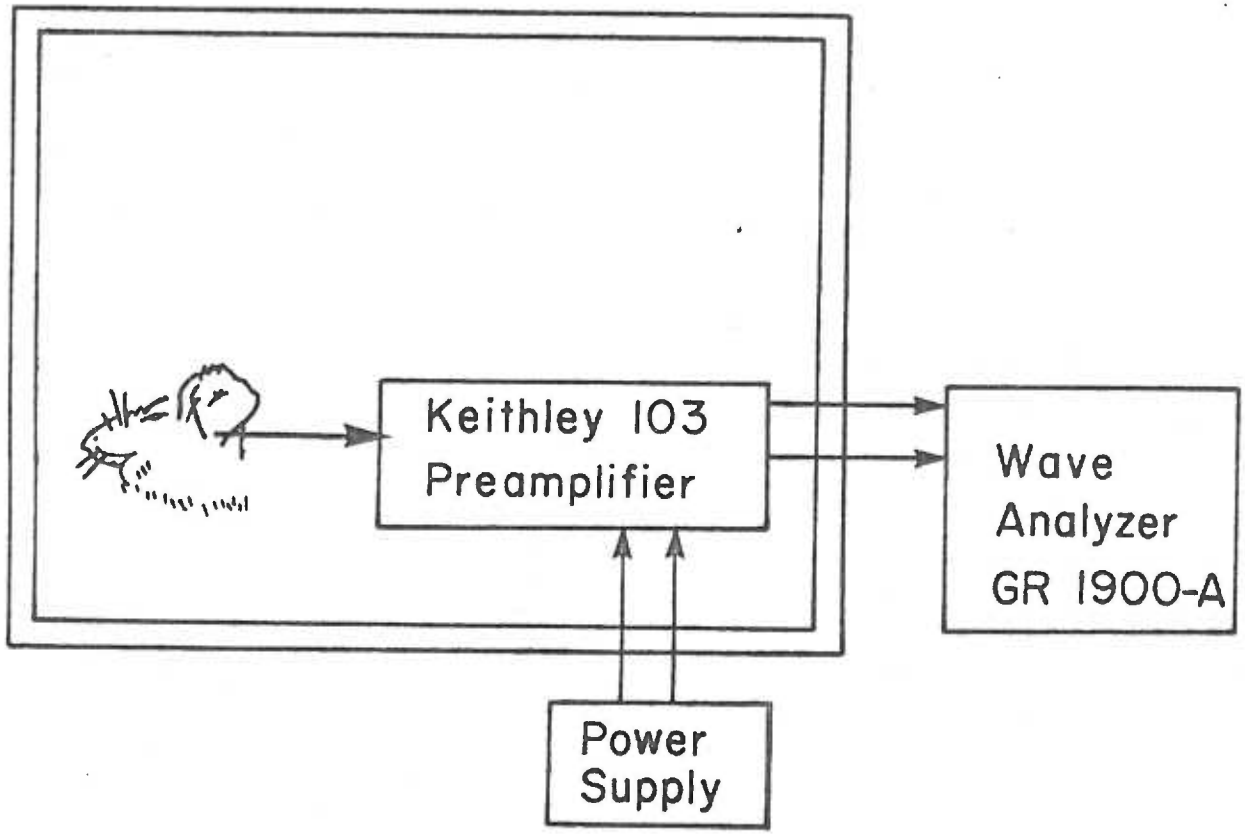

Keithley 103
Preamplifier
Wave
Analyzer
GR 1900-A
Power
Supply

Figure 31b

toward the animal. A short piece of surgical tubing was attached to the distal end of the bakelite tube and the tubing fit into the speculum mounted in the guinea pig's ear. This entire sound production system from the speaker diaphram to the tympanic membrane was sealed. Sealing included placing stopcock grease around the junction between the speculum and the external auditory meatus. A sealed sound system provides for better low frequency sound charteristics than an open system (Vernon and Meikle, 1974).

Sound was measured by using the calibrated probe tube-microphone combination. The opening of the probe tube was located just lateral to the tympanic membrane. The probe tube-microphone had been previously calibrated such that a one dyne/cm^2 sound pressure at the probe tip opening produced a known voltage output from the microphone for a known sound frequency. When it became time to calibrate the sound levels for each guinea pig ear, attenuation was removed from the sound system until the output voltage measured from the microphone equaled the known microphone output at one dyne/cm^2. The amount of attenuation left in the sound system at this point was the amount needed to generate one dyne per cm^2 of sound pressure at that particular frequency. By subtracting the number of decibels of attenuation remaining in the sound system for producing 1 microvolt of ac cochlear potential from the number of decibels of attenuation remaining in the sound system when the sound system is producing one dyne per cm^2, the actual sound pressure necessary to produce 1 microvolt of ac cochlear potential in dB's referenced to one dyne per cm^2 is obtained.

See Vernon, Katz and Meikle, 1976 for a discussion of sound measurement and the decibel system.

Artifact. Most modern speakers generate sound by producing a magnetic field. This magnetic field expands and contracts at the same frequency as the sound being produced. This varying magnetic field will induce voltages in any conductors within the field. These induced currents can be mistaken for the ac cochlear potential.

This artifact can be minimized by increasing the distance between the speaker and the electrode. This lowers the magnitude of the magnetic field. The eighteen inch distance represented by the bakelite tube leads to good separation at normal stimulus levels.

At extremely high sound pressure levels, however, the magnetic field may extend outward far enough to induce a current into the electrode. In order to demonstrate the potential measured at the round window membrane at high sound intensities was not artifactual in origin the bias current to the Western Electric 555 speaker field coil was interrupted. The current to the voice coil continued at the previous magnitude but no sound was produced. If the ac cochlear potential decreased significantly artifact could be ruled out. Generally, the apparatus used was artifact-free up to +40 dB or more (referenced to 1 dyne/cm^2).

Measures made. Two standard measures of ac cochlear potential were made in this experiment. The first was the one microvolt isopotential function. Sound intensity was increased until one

microvolt of ac cochlear potential was measured. This was done at 100 Hz, 200 Hz, 310 Hz, 500 Hz, 700 Hz, 1 kHz, 1.5 kHz, 2 kHz, 3 kHz, 4 kHz, 5 kHz, 7 kHz, 8 kHz, 10 kHz, 15 kHz, 16 kHz, 20 kHz and 32 kHz

The second measure made was the maximum output. The amount of sound needed to produce ten microvolts of ac cochlear potential at 1 kHz as determined. Then the sound intensity was increased in 5 dB steps until the ac cochlear potential no longer increased in amplitude but actually decreased. The peak amplitude was taken as the maximum output at 1 kHz for that ear. Then the amount of sound necessary to produce one microvolt of ac cochlear potential at 10 kHz was determined and the process was repeated for 10 kHz

The pathological ear lacking outer hair cells required more sound to produce the one microvolt ac cochlear potential. In addition the pathological ear has a maximum output which is severely decreased in amplitude.

Calibration procedure. Immediately after the two measures of ac cochlear potential had been completed for each ear the sound system was calibrated for that ear. Attenuation was removed from the sound system until the calibrated probe tube-microphone indicated that one dyne/cm^2 of sound pressure was being generated just lateral to the tympanic membrane. The attenuator settings necessary to produce one microvolt of ac cochlear potential were then subtracted from the attenuator settings at which one dyne/cm^2 was generated. The result is the actual sound pressure level in dB's referenced to one dyne/cm^2.

Recording the N_1

The other electrophysiological event recorded from the round window membrane of the cochlea is the gross nerve action potential or N_1. The N_1 is generated by the neurons of the VIIIth cranial nerve to a tone burst or click. A click or tone burst allows the neurons to fire action potentials synchronously. The N_1 derives its name from the fact that it is the first negative going wave recorded at the round window membrane after a tone burst. The N_1 was recorded from the same electrode positions as the ac cochlear potential and used the same Keithley preamplifier. The N_1 signal was displayed and measured on a Tektronics 564 storage oscilloscope (Figure 32b). The stimulus was produced from an electronic signal derived from a precision oscillator. The oscillator's signal was passed through the filter section of a General Radio wave analyzer and into a Coulbourne solid state switch. This switch modified the continuous tone into tone bursts. These tone bursts were 10 milliseconds in length and had onset and offset times of 100 microseconds. Coulbourne timing circuitry caused the stimulus to be presented at the rate of one tone burst per second. The intensity of the tone bursts was controlled by a General Radio decade attenuator. The electrical bursts were transduced into tone bursts by the piezoelectric crystal of a Speakerlabs PT 100 speaker. Again sound was generated in a closed sound system. Figures 32a and b are schematic representation of the sound generation and measurement system

Figure 32a. Schematic diagram of equipment used in this experiment to produce the N_1 potential. A General Radio 1162-A Coherant Decade Frequency Synthesizer produced a constant electrical sine wave signal. This signal was passed through the General Radio 1900-A Wave Analyzer. The output signal amplitude and frequency were monitored on a Simpson Model 49 Voltmeter and Monsanto 100-A Frequency Counter respectively. A Coulbourn S84-04 Electronic Switch and other solid state timing logic turn the continious signal into short tone bursts. A General Radio 1450-TA Decade Attenuator allowed for precise control of the amplitude of the electrical signal in 5 dB steps for a total of 110 dB attenuation. The electrical signal was then applied to the crystal of a Speakerlab PT25005 Piezoelectric Speaker. The guinea pig was enclosed in a sound attenuating, sheilded room.

Figure 32b. Schematic diagram of equipment used to record the N_1 potential. This potential was obtained from a silver ball electrode resting upon the round window membrane of the guinea pig. The potential was amplified 1000 times by a Keithley 103 Preamplifier. The resulting signal was then displayed on a Tektronics 564 Storage Oscilloscope with a 3A9 Module. The Tektronics oscilloscope sweep was triggered by a logic pulse which also triggered the Coulbourne switch.

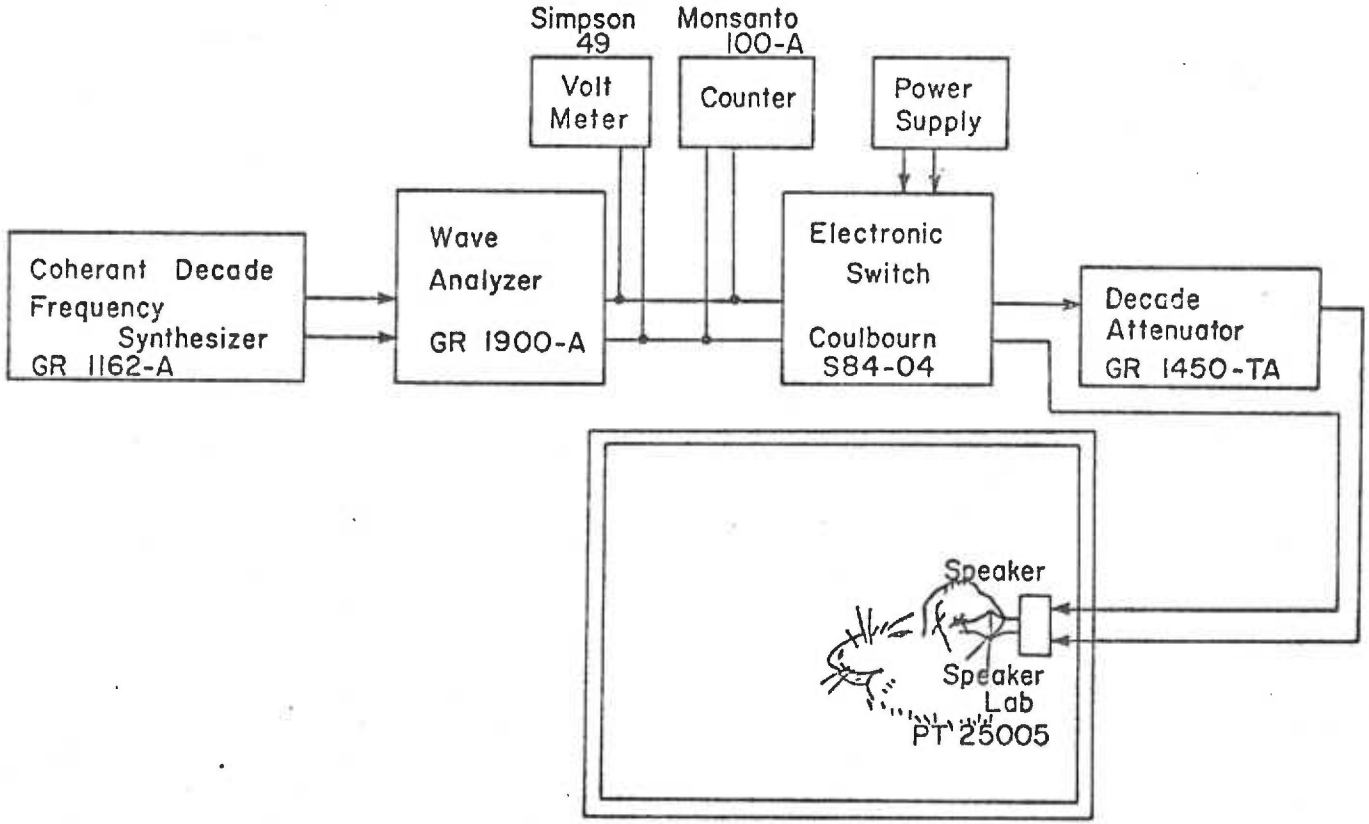

Simpson
49
Volt
Meter
Monsanto
100-A
Counter
Power
Supply
Coherant Decade
Frequency
Synthesizer
GR 1162-A
Wave
Analyzer
GR 1900-A
Electronic
Switch
Coulbourn
S84-04
Decade
Attenuator
GR 1450-TA
Speaker
Speaker
Lab
PT 25005

Figure 32[a]

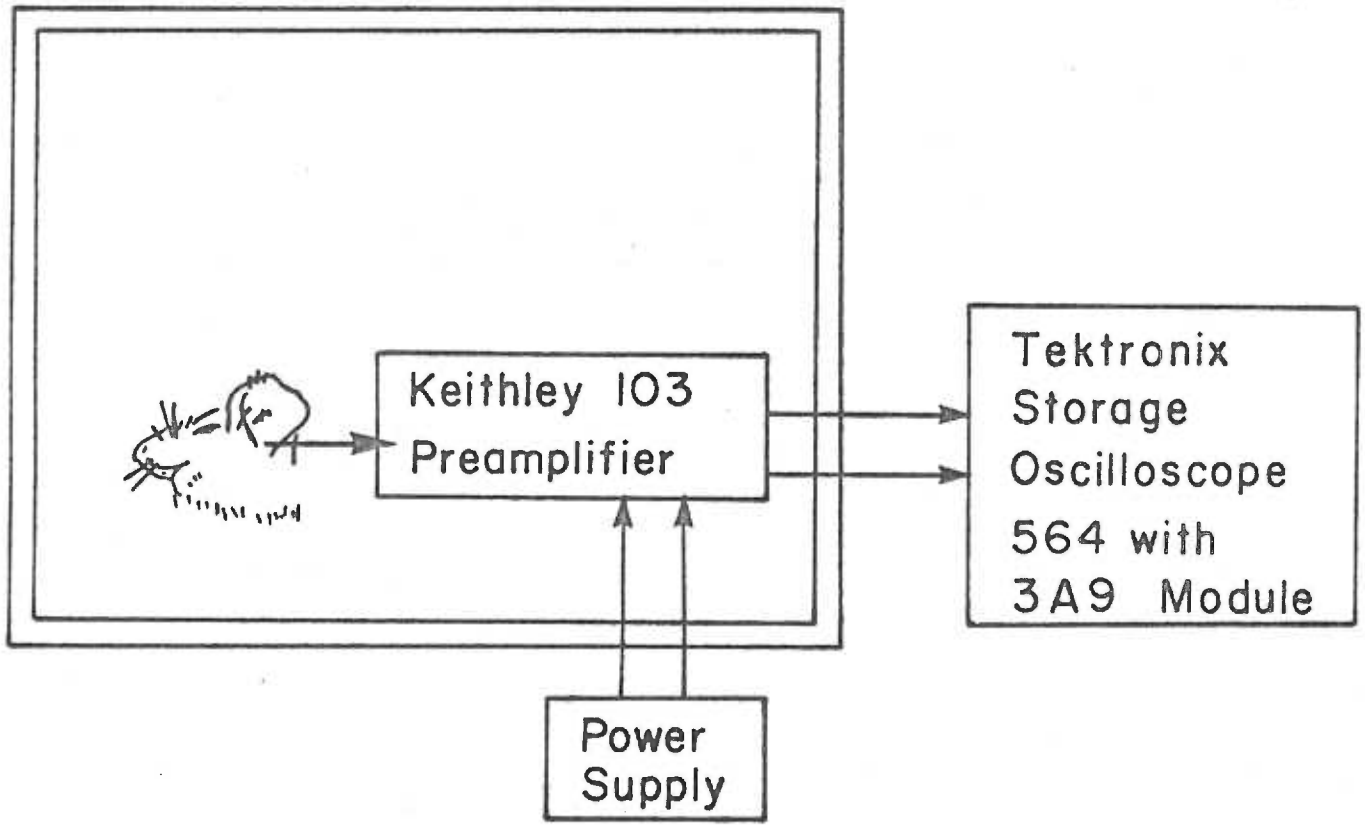

Keithley 103
Preamplifier
Tektronix
Storage
Oscilloscope
564 with
3A9 Module
Power
Supply

Figure 32b

for the N_1 potential.

Attenuation was removed in 5 dB steps until the N_1 response was just distinguishable from the overall electrical activity of the guinea pig. This level was taken as the threshold for the N_1 potential. Thresholds were determined at 2 kHz, 4 kHz, 8 kHz, 16 kHz, 20 kHz and 32 kHz.

The sound system for producing the N_1 potential was quantified by the calibrated B & K probe tube microphone. Rather than calibrating the system to tone bursts, the Coulbourne electronic switch was shorted to produce a continuous tone. This was necessary because the tone burst produced sympathetic resonances in the probe tube which would have led to spurious results.

The surface preparation technique

The following is the procedure used to prepare the inner ear of the guinea pig for histological evaluation. Immediately after electrophysiological measures were finished for both ears and both ears had been calibrated, the guinea pig ears were removed for anatomical measures. The guinea pig was decapitated and temporal bones with intact cochleae were broken away from the skull. Extraneous muscle tissue was removed and the bulla were opened to more clearly expose the cochleae. The stapes in each cochlea was pressed inward rupturing the oval window membrane. The round window membrane was ruptured. A Pasteur pipet was used to force Dalton's fixative containing 1% osmium through the cochleae. Perfusion was continued for five minutes. The cochleae were immersed in the fixative for two hours. The cochleae were then

dehydrated by serial perfusions of greater concentrations of ethanol (0%, 35%, 50% and 70%) in saline. The cochleae were stored in 70% ethanol solution until dissected.

Dissection consisted of removing bone from each cochlea until the basilar membrane-organ of Corti could be removed in one half turn pieces. These pieces were mounted on standard microscope slides with cover slips in glycerine. This is called the surface preparation technique.

The surface preparation technique has been described in greater detail by Johnsson and Hawkins (1976).

Appendix C

The Placenta

The fertilized egg of the reptile, fish, amphibian and bird must contain enough nourishment to sustain the developing embryo until it emerges and can function on its own. Also, the parent must either provide protection for the egg during incubation (such as birds do) or deposit a fantastically large number of eggs so some small number will survive to maturity on the basis of statistics (such as most of the fishes do).

The mammal has taken a different tack. The fertilized mammalian ovuum contains little nourishment. Nor do most mammals bear very large litters of young. The mammal is not tied to a nest during the development of the fetus as the bird is. The difference between the mammals and most other animals is the mammalian fetus develops within the safety of the maternal body. There it is kept at body temperature, with an almost unlimited supply of nutrients, free of predators. There are some serious problems with residing within the mother. How does the fetus obtain nourishment and oxygen and rid itself of poisonous carbon dioxide, urea and other biproducts? How does the fetus prevent the maternal immune system from attacking it?

These problems have been solved in the form of the placenta. The placenta is the maternal-fetal interface organ which allows for the transfer of oxygen, glucose, amino acids, vitamins, fats, water and electrolytes from the mother to the fetus and carbon dioxide, urea, water, electrolytes and other

waste products from the fetus to the mother.

In addition to its important role as an exchange organ the placenta is also an endocrine gland. It appears to be the source of hormones which suspend the estrus cycle after the corpus luteum degrades. If the estrus cycle continued during pregnancy the sloughing of the endometrium would remove the conceptus. The placenta may also produce chorionic gonadatropin--the hormone in urine whose presence is detected by standard "pregnancy tests."

The placenta is important because it is the primary route of fetal exposure to most drugs ingested by the mother. Since the placenta may consist of one, two or more membranes separating the maternal and fetal circulations it can have a profound effect on the kinetics of drugs entering or leaving the fetus.

With lipid soluable drugs the concentration of drug obtained in the fetal circulation may be almost as high as those in the mother. The infant delivered by cesarean section with the mother under general gas anesthetic will emerge asleep and lethargic, not at all like a normal infant.

Lipid insoluable, or water soluable, drugs may only cross the placental membranes with difficulty. The placenta may be responsible for much lower peak concentrations of drug in the fetus than in the mother. If the drug is a relatively large molecule, and is water soluable very little drug may enter the fetal circulation.

However, because the placenta poses a barrier for drugs entering the fetal circulation the placenta may also pose a

Even with these structural differences the guinea pig and human placenas are functionally very similar. Each sinus of the human placenta is organized functionally like the concentric guinea pig placenta.

In addition both placentas seem to exhibit similar effects on the kinetics of drugs. As stated in the main part of the paper many aminoglycoside antibiotics have been tested in pregnant women and pregnant guinea pigs. The ratios of drug concentrations in fetus and mother are similar in the two species.

The guinea pig would appear to be a useful model for human placental pharmacology. The only better model which is reasonably available would be the rhesus monkey. However, even this animal has drawbacks. The rhesus monkey has an auxillary placenta not found in the human. In conclusion, the placenta is an important adaptation by the mammal for increasing the probability of fetal survival. It can also provide access to the fetus for drugs taken by the mother. Only through animal models can we come to understand the factors affecting fetal and placental pharmacology.

References

Akiyoshi, M , Sato, K., Shoji, T. Sugahiro, K., Tajima, T., Koeda, T. Odaki, M. and Yokota, M Ototoxic effect of 3',4'-dideoxykanamycin on the inner ear in the intrauterine guinea pig embryos. Japanese Journal of Antibiotics, 27:735-745, 1974.

Akiyoshi, M , Yano, S., Tajima,T., Matsuzaki, M , Akutsu, S., Kishimoto, K., and Maeda, M Ototoxic effect of BB-K8 administered to pregnant guinea pigs on development of inner ear of intrauterine litters. Japanese Journal of Antibiotics, 30:185-196, 1977.

Alajmo, E. Ricerche sperimentali su di una possibile azione tossica della streptomicina sul sistema vestibolare durante la vita intrauterina. Bolletino Delle Malattie Dell Orecchio,Della Gola e Del Naso, 70:461-475,1952.

Altshuler, K.Z. The social and psychological development of the deaf child: Problems and treatment, in Fine, P.J.(ed) Deafness in Infancy and Early Childhood, Medcom Press: New York, 1974.

Andersen, J. and Pedersen, C. A method for determination of transplacental transmission of drugs--the destribution of kanamycin in pregnent guinea pigs. Acta Path. Microbiol. Scand. Section B, 79:204-208, 1971.

Anson, B J. and Donaldson, J.A Surgical Anatomy of the Temporal Bone, 3rd Edition, W.B. Saunders Co.:Philadelphia, 1981.

Appel, G.B. and Neu, H.C. The nephrotoxicity of antimicrobial agents, part 2. New England J. of Medicine, 296:722-728, 1977.

Bennett, W M. Plamp, C.E , Gilbert, D.N., Parker, R.A. and Porter, G.A. The influence of dosage regimen on experimental gentamicin nephrotoxicity: Dissociation of peak serum levels from renal failure. J. of Infectious Diseases, 140:576-580, 1979.

Berkow R.(ed) The Merck Manual of Diagnosis and Therapy, 13th Edition, Merck, Sharp and Dohme Research Laboratories:Rahway, NJ. 1977.

Bernard, B , Abate, M., Thielen, P., Attar, H., Ballard, C. and Wehrle, P. Maternal-fetal pharmacological activity of amikacin. Journal of Infections Diseases, 135:925-932, 1977a.

Bernard, B., Garcia-Cazares, S., Ballard, C., Thrupp, L., Mathies, A. and Wehrle, P. Tobramycin: maternal-fetal pharmacology. Antimicrobial Agents and Chemotherapy, 11:688-694, 1977b.

Bolletti, M., and Croatto, L. Sulla sordita da passagio transplacentare di streptomicina. Acta Paediat. Lat., 11:1-15, 1958.

Bordley,J.E., Brookhouser, P.E., Hardy, J. and Hardy, W.G. Observations on the effect of prenatal rubella on hearing. A preliminary report on the Baltimore epidemic of 1963-1965. in McConnell, F. and Ward, P. Deafness in Childhood, Vanderbilt University Press:Nashville, TN. 1967.

Boylan, J.W., Colbourn, E.P. and McCance, R.A. Renal function in the foetal and new-born guinea-pig. J. of Physiology (London), 141:323-331, 1958.

Brill, R.G. The superior IQ's of deaf children of deaf parents. in Fine, P.J.(ed), Deafness in Infancy and Early Childhood, Medcom Press:New York, 1974.

Brummett, R.E., Brown, R.T. and Himes, D.L. Quantitative relationships of the ototoxic interaction of kanamycin and ethacrynic acid. Archives of Otolaryngology, 105:240-246, 1979.

Brummett, R., Fox, K., Bendrick, T. and Himes, D. Ototoxicity of tobramycin, gentamicin, amikacin and sisomycin in the guinea pig. Journal of Antimicrobial Chemotherapy, 4(Suppl A):73-83,1978.

Brummett,R., Traynor, J., Brown, R. and Himes, D. Cochlear damage resulting from kanamycin and furosemide. Acta Otolaryngologica, 80:86-92, 1975.

Brummett, R., West, B., Traynor, J. and Manor, N. Ototoxic interaction between aminoglycoside antibiotics and diuretics. Toxicology and Applied Pharmacology, 29:45,1974.

Carlier, E. and Pujol, R. Supra-normal sensitivity to ototoxic antibiotics of the developing rat cochlea. Archives of Oto-Rhino-Laryngology, 226:129-133, 1980.

Case, R.V. and Mezei, L.M. An enzymatic radioassay for gentamicin. Clinical Chemistry, 24:2145-2150, 1978.

Chidynicki, S. Embryogenesis of the auditory part of the inner ear in the guinea pig. Acta Theriologica, 13:219-260, 1968.

Conway, N., and Birt, B. Streptomycin in pregnancy: effect on the foetal ear. British Medical Journal, 2:260-263, 1965.

Dallos, P. Cochlear physiology. Annual Review of Psychology, 32:153-190, 1981.

Davies, J. Brzezinska, M and Benveniste, R. R factors: Biochemical mechanisms of resistance to aminoglycoside antibiotics. Annals of the N.Y. Academy of Science, 182:226-233, 1971.

Davies, J. and Davis, B D. Misreading of ribonucleic acid code words induced by antibiotics: the effect of drug concentration. J. of Biological Chemistry, 243:3312-3316, 1968.

Ericson-Strandvic, B and Gyllensten, L. The central nervous system of foetal mice after administration of streptomycin. Acta Path. Microbiol. Scand. Section A, 59:292-300, 1963.

Fee, W E.,Jr. Aminoglycoside ototoxicity in the human. Laryngoscope, 90, Supp.24:1-19, 1980.

Geldard, F.A. The Human Senses, 2nd Edition. John Wiley and Sons:New York, 1972.

Goldstein, A , Aronow, L. and Kalman, S.M. Principles of Drug Action:The Basis of Pharmacology. Harper and Row:New York, 1968.

Goldstein, A , Aronow, L. and Kalman, S.M Principles of Drug Action:The Basis of Pharmacology, 2nd edition. John Wiley and Sons:New York, 1974.

Gregg, N.M. Congenital cataract following German measles in the mother. Transactions of the Opthalmological Society of Australia, 3:35-41, 1941.

Gregg, N.M. Further observations on congenital defects in infants following maternal rubella. Transactions of the Ophthalmological Society of Australia, 4:119-131, 1944.

Gulick, W L. Hearing: Physiology and Psychophysics. Oxford University Press:New York, 1971.

Hallgren, B Retintus pigmentosa combined with congenital deafness. Acta Psych. et Neur. Scand., Supp. 138:1-81,1959.

Hawkins, J.E. Drug ototoxicity, in Keidel, W.D. and Neff, W.D. (eds), Handbook of Sensory Physiology, Volume III, Auditory System: Clinical and Special Topics, Springer-Verlag:New York, 1976.

Hawkins, J.E and Johnsson, L.G. Microdissection and surface preparations of the inner ear, in Smith, C.A and Vernon, J.A (eds), Handbook of Auditory and Vestibular Research Methods, Charles Thomas:Springfield, IL. 1976.

Hawkins, J., and Laurie, M The ototoxicity of streptomycin. Annals of Otology, Rhinology and Laryngology, 61:789-809, 1952.

Javel, E. Neurophysiological correlates of auditory maturation. Annals of Otology, Rhinology and Laryngology, 89, part 2, Supp 74: 103-113, 1980.

Jones, H. Intrauterine ototoxicity. Journal of the National Medical Association, 65:201-203, 1973.

Kardos, N., Eichholz, A and Schaffner, C.P. Binding of aminoclylitol antibiotics to kidney and intestinal brush border membranes. J. of Antibiotics (Japan), 34:103-113, 1981.

Kauffman, R. Azaronoff, D. and Morris, J. Placental transfer and urinary excretion of gentamicin--comparison between an animal model and the human fetus. in Morselli, P., Garattini, S. and Sereni, F. (eds), Basic and Therapeutic Aspects of Perinatal Pharmacology, Raven Press:New York, 1975.

Kauffman, R., Morris, J. and Azarnoff, D. Placental transfer and fetal urinary excretion of gentamicin during constant rate maternal infusion. Pediatric Research, 9:104-107, 1975.

Kaufmann, P. and Davidoff, M. The guinea pig placenta. Advances in Anatomy, Embryology and Cell Biology, 53(2): 1-91, 1971.

Leroux, L. Existe-t-il une surdite congenital acquise due a la streptomycine? Ann. Oto-Laryng. (Paris), 67:194, 1950.

Marot, M , Uziel, A. and Romand, R. Ototoxicity of kanamycin in developing rats: Relationship with the onset of auditory function. Hearing Research, 2:111-113, 1980.

McCarthy, T. and Saunders, P. The origin and circulation of the amnionic fluid, in Fairweather, D.V.I. and Eskes, T.K.A.B. (eds), Amniotic Fluid--Research and Clinical Application, 2nd Edition, Exerpta Medica:London, 1978.

Mesollelo, C. Ricerche sperimentali sulla tossicita da kanamicina sull'orechio interno di cavia durante la vita intrauterine. Archivii Italiani di Laringologia, 71:37-37, 1963.

Nelson, N. and Forfar, J. Associations between drugs administered during pregnancy and congenital abnormalities of the fetus. British Medical Journal, 1:523-527, 1971.

Osako, S., Tokimoto, T. and Masuura, S. Effects of kanamycin on the auditory evoked responses during postnatal development of the hearing of the rat. Acta Otolaryngologica, 88:359-368, 1979.

Ostyn, F. and Tyberghein, J. Influence of some streptomyces antibiotics on the inner ear of the guinea pig--electrophysiological and histological study. Acta Oto-Larynogologica, Suppl. 234:1-91, 1968.

Pearson, A., Jacobson, A., Van Calcar, R. and Sauter, R. The Development of the Ear, American Academy of Opthalmology and Otolaryngology:Rochester, MN. 1967.

Pittinger, C. and Adamson, R. Antibiotic blockade of neuromuscular function. Annual Review of Pharmacology, 12:169-184, 1972.

Pridgen, J.E. Respiratory arrest thought to be due to intraperitoneal neomycin. Surgery, 40:571-574, 1956.

Prosen, C.A. and Stebbins, W.C. Ototoxicity, in Spencer, P.S. and Schaumburg, H.H. (eds), Experimental and Clinical Neurotoxicology, Williams and Wilcons:Baltimore, 1980.

Pujol, R., Carlier, E. and Lenoir, M. Otogenetic approach to inner and outer hair cell function. Hearing Research, 2:423-430, 1980.

Pujol, R. and Hilding, D. Anatomy and physiology of the onset of auditory function. Acta Otolaryngologica, 76:1-10, 1973.

Rawdon-Smith, A.F., Carmichael, L. and Wellman, B. Electrical responses from the cochlea of the fetal guinea pig. J. of Experimental Psychology, 23:531-535, 1938.

Rasmussen, F. The oto-toxic effect of streptomycin and dihydrostreptomycin on the foetus. Scand. Journ. of Resp. Dis., 50:61-67, 1969.

Riskaer, N., Christensen, E. and Hertz, H. The toxic effects of streptomycin and dihydrostreptomycin in pregnancy illustrated experimentally. Acta Tuberculosea Scandinavica, 27:211-216, 1952.

Robinson, G. and Cambon, K. Hearing loss in infants of mothers treated with streptomycin during pregnancy. New England Journal of Medicine, 271:949-951, 1964.

Rubel, E.W. Ontogeny of structure and function in the vertibrates auditory system, in Jacobson, M. (ed), Handbook of Sensory Physiology, Volume IX, Development of Sensory Systems, Springer-Verlag:New York, 1978.

Sakula, A. Streptomycin and the foetus. British Journal of TB and Diseases of the Chest, 48:69-72, 1954.

Schardein, J. Drugs as Teratogens. CRC Press:Cleveland, 1976.

Schein, J.D. and Delk, M T. Jr. The Deaf Population of the United States. National Association for the Deaf: Silver Spring, MD. 1974.

Scheinhorn, D. and Angelillo, V. Antituberculosis therapy in pregnancy--Risks to the fetus. Western Journal of Medicine, 127:195-198, 1977.

Schibetta, J.J. and Rosen, M.G. Response evoked by a sound in the fetal guinea pig. Obstetrics and Gynecology, 33:830-836, 1969.

Smith, C.A Anatomical correlates of deafness. J. of the Acoustical Society of America, 54:576-588, 1973.

Smith, D.H., Van Otto, B and Smith, A.L. A rapid chemical assay for gentamicin. New England J. of Medicine, 286:582-586, 1972.

Spitzer, A and Brandis, M. Functional and morphological maturation of the superficial nephrons: Relationship to total kidney function. J. of Clinical Investigation, 53:279-287, 1974.

Spitzer, A. and Edelmann, C.M.,Jr. Maturational changes in pressure gradiants for glomerular filtration. American J. of Physiology, 221:1431-1435, 1971.

Stephens, C.B. Development of the middle and inner ear in the golden hamster (Mesocricetus auratus). A detailed description to establish a norm for physiopathological study of congenital deafness. Acta Otolaryngologica, Supp. 296, 1972.

Stupp, H., Rauch, S., Sous, H., Brun, J.P. and Lagler, F. Kanamycin dosage and levels in the ear and other organs. Archives of Otolaryngology, 86:515-521, 1967.

Swan, C. Congenital malformatins in infants following maternal rubella during pregnancy: a review of investigations carried out in South Australia. Transactions of the Ophthalmological Society of Australia,4:132-141, 1944.

Thompson, W., Reiner, N. and Bloxham, D. Gentamicin and tobramycin nephrotoxicity in dogs given continious or once-daily intravenous injections. Assessment of Aminoglycoside Toxicity, A Symposium Sponsored by Eli Lilly and Company, Burgenstock, Switzerland, September 24-25, 1977.

Toyoda, Y. and Tachibana, M Tissue levels of kanamycin in correlation with oto- and nephrotoxicity. Acta Otolaryngologica, 86:9-14, 1978.

Uziel, A., Romand, R. and Gabrion, J. Intrauterine ototoxicity of Kanamycin in the guinea pig. From: Les Colloques de l'Institut National de la Sante et de la Recherche Medicale. Inner Ear Biology. Portman, M and Aran, J.M. (eds.), INSERM, 1977, 68:347-358.

Varpela, E., Hietalahti, J. and Aro, M J.T. Streptomycin and dihydrostreptomycin medication during pregnancy and their effect on the child's inner ear. Scandinavian J. of Respiratory Diseases, 50:101-109, 1969.

Vernon, J., Katz, B , and Meikle, M. Sound measurement and calibration of instruments, in Smith, C. and Vernon, J.(eds), Handbook of Auditory and Vestibular Research Methods, Thomas: Springfield, IL. 1976.

Vernon, J. and Meikle, M. Electrophysiology of the cochlea, in Thompson, R. (ed), Bioelectrical Recording Techniques, Part C, Receptor and Effector Processes, Academic Press: New York, 1974.

Walloch, R.A. and Taylor-Spikes, M. Auditory thresholds and cochlear microphonics from the same guinea pigs. J. of Auditory Research, 17:145-154, 1977.

Ward, P. and Fernandez, C. The ototoxicity of kanamycin in guinea pigs. Annals of Oto-Rhino-Laryngology, 70:132-142, 1961.

Watson, E.H. and Stow, R.M. Streptomycin therapy: Effects on the fetus. J. of the American Medical Assn., 137:1599-1600, 1948.

Weinstein, L. Antimicrobial agents: Streptomycin, gentamicin, and other aminoglycoside antibiotics, in Goodman, L.S. and Gilman, A. Macmillan:New York, 1975.

Weinstein, A., Gibbs, R. and Gallagher, M Placental transfer of clindamycin and gentamicin in term pregnancy. American J. of Obstetrics and Gynecology, 124:688-691, 1976.

West, B., Brummett, R. and Himes, D. Interaction of kanamycin and ethacrynic acid. Archives of Otolaryngology, 98:32-37, 1973.

Wever, E and Bray, C. Action currents in the auditory nerve in response to acoustic stimulation. Proceedings of the National Academy of Sciences, 16:344-350, 1930.

Williams, J.W. and Northrup, D.B Purification and properties of gentamicin acetyltransferase I. Biochemistry, 15:125-131, 1976.

Wilson, J.G. Environment and Birth Defects. Academic Press: New York, 1973

Woltz, J.H.E. and Wiley, M.M Transmission of streptomycin from maternal blood to the fetal circulation and the amnionic fluid. Proceedings of the Society for Experimental Biology and Medicine, 60:106-107, 1945.

Yoshioka, H., Monma, T. and Matsuda, S. Placental transfer of gentamicin. Pediatric Pharmacology and Therapeutics, 80:121-123, 1973.