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A Histopathological Assessment of the Effects of Streptozotocin-Induced Diabetes on the Ears of Chinese Hamsters

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**A HISTOPATHOLOGICAL ASSESSMENT OF THE EFFECTS OF
STREPTOZOTOCIN-INDUCED DIABETES ON THE EARS
OF CHINESE HAMSTERS**

by

Karen Ailsworth

**A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biomedical Sciences**

**Western Michigan University
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December, 1983**

A HISTOPATHOLOGICAL ASSESSMENT OF THE EFFECTS OF
STREPTOZOTOCIN-INDUCED DIABETES ON THE EARS
OF CHINESE HAMSTERS

Karen Ailsworth, M.S.

Western Michigan University, 1983

Research has produced evidence associating progressive, bilateral, sensorineural hearing loss with diabetes mellitus in human and animal subjects. The microangiopathy which is presumably the cause of hearing loss appears similar to the microangiopathy leading to retinal and renal atrophy.

In the present study the temporal bones of six streptozotocin-induced diabetic Chinese hamsters and six nondiabetic Chinese hamsters were histologically processed. Sections stained with hematoxylin and eosin were assessed for total auditory and vestibular otopathology and revealed no statistically significant differences between the two groups. Sections stained with periodic acid-Schiff's were evaluated for vascular basement membrane changes and revealed no statistically significant differences between groups. Streptozotocin-induced diabetes in the Chinese hamster may not be an adequate model for study of diabetes-induced otopathologies.

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CHAPTER I

INTRODUCTION

Diabetes mellitus is a complex metabolic disorder, with effects found in nearly every major physiological system. Acute diabetes may lead to hypersomolarity, peripheral circulatory failure, metabolic acidosis, and, in extreme cases, coma and death. The results of chronic diabetes include impaired microcirculation, most notably associated with retinal or glomerular damage; increased incidence of macrovascular pathology, usually in the form of atherosclerosis; disordered nerve function; and compromised immunological defense. In 1965, diabetes was the eighth leading cause of death in the United States (McDonald, 1970); by 1980, it ranked sixth (U.S. Department of Health and Human Services, 1982). As control of diabetes advances, diabetics live longer, but also suffer more often from long-term complications of the disease. It has been estimated that between 5% and 25% of the population of the western world will develop diabetes (Farquhar, 1972). Despite being a major world health problem, diabetes is a poorly understood disease.

As more is learned about diabetes, the criteria for defining the disease are in a state of flux. It is perhaps best defined as the "inability of glucose in physiological concentrations to penetrate certain types of cells" (Forsham, 1972, p. 5), particularly fat, muscle, and connective tissue cells. Farquhar (1972) postulates

that diabetes is not one disease but three: juvenile-onset, insulin-dependent diabetes, accounting for 3% of diabetic patients, adult-onset diabetes with symptoms, accounting for another 3% of diabetic patients, and latent or chemical diabetes, accounting for 94% of diabetics.

The etiology of diabetes is also complex. Evidence has shown hereditary, environmental, and viral factors each have contributed to the onset of diabetes. In animal subjects, up to four pairs of genes may be involved, whereas in humans the majority view is that the disease is transmitted by a single recessive gene (Rimoin, 1970). Environmental causes of diabetes include nutrition, physical activity, psychologic stress, chemicals, and hormonal imbalances (growth hormone, epinephrine, and progesterone are all insulin antagonists). Some researchers believe there is likely to be an autoimmune component involved in the etiology of diabetes. Betacytotrophic viruses, including Cocksackie B, cytomegalovirus, Epstein-Barr, and the mumps virus, may lead to diabetes. The appearance of diabetes in an individual may be a response to multiple factors acting in different proportions. Environmental and autoimmune factors are prominent among children and young adults, whereas nutritional factors and degrees of physical activity leading to obesity are closely linked to diabetes in adults (Tepperman, 1980).

The principle metabolic action of insulin involves carbohydrate, protein and fat. Insulin deficiency has a general anabolic effect on these substances. Insufficient insulin inhibits hepatic and

peripheral uptake of glucose, and also causes indirect acceleration of gluconeogenesis. The combination results in hyperglycemia. If unchecked, extreme hyperglycemia may result in excessive sorbitol and glycoprotein production (Forsham, 1972), or ketoacidosis or nonketotic hyperosmolar coma. Diabetes entails disturbed protein metabolism as well. The uptake of branched chain amino acids is decreased, and consequently nitrogen repletion of muscle is decreased. Plasma alanine, an important glycogenic precursor, is also reduced. When hepatic uptake of alanine is increased, there is a concurrent increase in gluconeogenesis, which is a contributing factor to hyperglycemia (Felig, 1978). Finally, fat metabolism is disturbed. Mobilization of fatty acids from adipose cells increases, and plasma triglyceride levels rise. Fatty acids may be rapidly converted to ketones by the liver. Accumulation of these ketone bodies is a major cause of diabetic metabolic acidosis.

In addition to wide-ranging metabolic effects, diabetes may result in chronic complications. These may be classified as macrovascular disease, microvascular disease, or neuropathy. In addition, phagocytosis by leukocytes is often inhibited in the presence of the diabetic condition.

Diabetic macroangiopathy is usually the result of atherosclerosis or arteriolosclerosis. About 50% of all heart attacks occur in people with abnormal carbohydrate metabolism, and there is evidence that "silent coronaries" occur more frequently in diabetic patients than in non-diabetics. Diabetic macroangiopathy also is a factor in 75% of all cerebrovascular accidents. Diabetic

macroangiopathy may affect peripheral vessels, too, as seen in the five-fold higher incidence in diabetics of amputations for gangrene (Ellenberg, 1978).

Diabetic microvascular disease has been shown to manifest itself in the capillaries and small blood vessels of the glomeruli, retina, skin, stomach, liver, peripheral nervous system, intestines, muscle, and placenta (Osterby and Lundbaeck, 1970). Numerous protein fractions, glycoproteins, lipids, and lipoprotein changes in the blood have been analyzed, but evidence supports basement membrane thickening for the pathogenesis of diabetic microangiopathy.

Basement membranes are members of the collagen family of connective tissue. Because basement membranes are glycoprotein, they are periodic acid-Schiff (PAS) positive. Basement membranes are found sandwiched between endothelial and epithelial cells in glomerular capillary walls and underlying endothelial cells in all other capillaries. PAS staining has revealed glomerular basement membranes to be 2,000 to 4,000 Angstroms thick, although in other tissue basement membranes may be only several hundred Angstroms wide. This discrepancy in thickness has led some researchers to believe there are two types of basement membrane structure--basement membrane and basement membrane-like material--that merge at many points, and are indistinguishable using light-microscopic techniques.

Basement membranes are 93% protein and 7% carbohydrate. Half the carbohydrate portion consists of glucosylgalactose disaccharide

units linked to hydroxylysine residues.

Capillary basement membrane thickening prevalent in long-term diabetes has been hypothesized to be the consequence of glucose shunted by an unknown pathway from insulin-dependent to insulin-independent metabolic routes, i.e., to the sugar component of glyco-proteins. Beisswenger and Spiro (1970) suggest that high concentrations of glucose or of the metabolic derivatives of glucose could function in regulating both the hydroxylation and carbohydration of basement membranes. As a result, the overall rate of basement membrane synthesis is increased in the diabetic state due to greater availability of sugar nucleotides for attachment to the peptide chain.

Most studies have shown a positive correlation between duration or severity of insulin deficiency and capillary basement membrane thickness. Siperstein, et al. (1968) found evidence to support their hypothesis that capillary basement membrane thickness is a pathology which may be determined by a gene linked with the diabetic allele, and thus can be found in prediabetics. Most researchers agree that diabetic microangiopathy is irreversible and inevitable even with the best insulin control available today. Evidence has shown, however, that diabetics receiving several insulin injections per day have less severe microangiopathy than diabetics receiving one or no dose of insulin.

Probably the least examined long-range complication of diabetes is neuropathy. Diabetic nerve damage can occur in sensory,

motor, or autonomic neurons, and most commonly results in postural hypotension or disturbances of gastrointestinal or urinary bladder function. The cause of such neuropathy is unknown, but presumed to be a chronic metabolic disturbance in nerves exposed to high glucose concentrations over long periods of time.

Finally, researchers have observed that there can be impaired phagocytosis associated with diabetes. Under certain circumstances, such as uncontrolled ketoacidosis and, possibly, marked hyperglycemia, diabetics may have increased difficulty coping with infection. This may be due to the facilitation of growth of certain microorganisms because of the altered metabolic state. Alternatively, the greater frequency of infections may be the consequence of the failure of mobilized leukocytes to ingest organisms. There is no evidence of impairment of inflammatory response (Johnson, 1970).

CHAPTER II

THE PROBLEM

Diabetes-induced otopathologies could conceivably lead to sensory or vestibular malfunctions. Dublin (1976) estimates 50% of diabetics have some type of auditory dysfunction. Studies in the past century have associated slowly developing, bilateral, sensorineural hearing losses with long-term diabetes. Also, diabetes-linked hearing losses may appear in the form of sudden Meniere-like attacks with accompanying vestibular symptoms.

Numerous investigators have found a correlation between the long-term diabetic state and some form of macroangiopathy, microangiopathy, or neuropathy. Such pathologies include infarction of a major blood vessel in the brain stem (Kam-Hansen and Sorenson, 1978), narrowing of the capillaries within the stria vascularis (Jorgensen, 1961; Costa, 1967; Kovar, 1974; Oliveira, 1977), vasa nervorum of VII and VIII cranial nerves (Kovar, 1973), modiolus (Jorgensen, 1961; Costa, 1967), internal acoustic meatus (Jorgensen, 1961), and spiral ligament (Oliveira, 1977). Nerve pathologies observed are demyelination of the VIII cranial nerve, atrophy of the spiral ganglia (Makishima and Tanaka, 1971), a decrease in the number of Scarpa's ganglion cells (Naufal and Schuknecht, 1972), and necrosis in cochlear and vestibular nuclei (Kam-Hansen and Sorenson, 1978).

Most recently McIntire and Benitez (1981) and Oostveen and McIntire (1983) have completed studies relating the effects of

diabetes mellitus on the inner ear by using genetically diabetic Chinese hamsters as their subjects. McIntire and Benitez studied 17 hamsters and found no major nerve damage or basement membrane changes, but found three times the incidence of otopathology in the diabetic group compared to the controls. Oostveen and McIntire did a follow-up study using two lines of the genetically diabetic hamster and found significant differences in overall pathology and capillary basement membrane changes in the diabetics as compared to the normal hamsters.

The purpose of the present study is to research the effects of streptozotocin-induced diabetes on the inner and middle ears of the M-line of Chinese hamsters. Specifically, the present study includes assessment of the nerves, blood vessels, basement membranes, sensory epithelium, fluid cavities, and overall otopathology, using histological methods to assess serial sections of the inner and middle ears. Overall otopathology will be studied using the hematoxylin and eosin (H & E) method of staining, and periodic acid-Schiff's reagent will be used to obtain a detailed view of capillary basement membranes.

The spontaneously diabetic Chinese hamster has proven to be among the best models of human insulin-dependent, adult-onset, diabetes. In this study normal (M-line) Chinese hamsters will be rendered diabetic with streptozotocin, not only to observe the effects of diabetes on the inner and middle ears but to provide information for future comparison of chemical-induced diabetes with the spontaneously occurring (genetic) syndrome within Chinese hamsters.

Significance

With technologic advances which provide better control of the disease, diabetics are not only living longer but are enabled to reproduce, increasing the probability of an even higher percentage of diabetics in the future. So not only will there be more health care dollars spent in the future on the treatment of diabetes, but the proportion of funds spent on chronic complications of the disease will increase as the diabetic population ages. Better understanding of the pathophysiology of diabetes can only help to solve this widespread problem.

Long-term diabetes may result in damage to the inner ear. This study will provide some information about the otopathology of diabetes, particularly with respect to capillary basement membrane changes. In addition, chemically-induced diabetes is often more economically feasible than genetic diabetes to study. This experiment will add information as to whether chemically-induced and genetic diabetes are physiologically equivalent models.

CHAPTER III

LITERATURE REVIEW

Diabetes mellitus is among the diseases known longest to man, having been clinically described by Celsus circa 10 A.D. By 1000 A.D. its complications as a degenerative disease were noted, and in 1886 the importance of the pancreas to diabetic etiology was recognized when Minkowski and Von Mering produced diabetes in a dog by removing its pancreas. In the following century, research on diabetes accelerated; Banting and Best, building upon the work of earlier investigators, isolated insulin in 1921; Embden, Meyerhof, Cori, Krebs, and others, established various metabolic pathways since 1925; the structure of insulin was elucidated in 1955 by Sanger; and by 1967 Meienhofer was able to chemically synthesize the hormone.

With regular administration of insulin, the immediate complications of diabetes were sharply reduced. Death from diabetic coma is now uncommon, as are many of the infections such as tuberculosis which often accompany the diabetic state. The life span of a diabetic has been prolonged significantly. However, not all the difficulties associated with diabetes have been solved, as has been made apparent by the appearance of the long-term complications of vascular disease and neuropathy which are now the major clinical challenges when treating diabetes.

Macrovascular complications most often manifest themselves as atherosclerotic disease of the blood vessels of the brain, heart,

kidney, or extremities. Atherogenesis may occur in the absence of metabolic disfunction, but diabetes appears to aggravate the production of atherosclerotic plaques. Atherosclerosis most likely is the result of endothelial injury within the vessel walls, which leads to increased endothelium permeability. Platelet adhesion is the immediate consequence of endothelial injury, and the platelet adhesion acts as a mitogenic stimulator leading to smooth muscle cell proliferation and lipid accumulation. Diabetes may contribute to atherogenesis in three ways. First, if a diabetic state is created by an excess of growth hormone, smooth muscle cell proliferation will be accelerated by the growth hormone. Second, diabetes may promote thromboxane synthesis, which has a stimulatory effect on platelet adhesion and mitogen release (Waitzman et al., 1977). Finally, the metabolic disorder in diabetes causes an upset in the lipoprotein balance. With diabetes there are relatively more low density lipoproteins (LDL) which accumulate in vessel walls, and there are also fewer of the high density lipoproteins which attenuate the effects of LDL (Colwell, 1977).

Microangiopathy is a second type of vascular complication commonly associated with long-term diabetes, and is most commonly the cause of renal insufficiency when found in the glomeruli and blindness when located in the retina. Capillary basement membrane thickening (CBMT) is accepted as the most conspicuous cause of microangiopathy (capillary occlusion), but the cause of CBMT is still under investigation. Spiro (1963) hypothesized that in the diabetic state glucose is shunted from insulin-dependent pathways

to an insulin-independent metabolic route, e.g., from glycogen synthesis or energy yielding pathways towards the sugar component of glycoproteins, which form 7% of the proteins in collagen found in basement membranes (Beisswenger and Spiro, 1970).

Beisswenger and Spiro (1970) found that diabetics have significantly lower amounts of lysine residues and equivalently greater amounts of hydroxylysine residues and hydroxylysine residues linked to disaccharides. They suggested that the increased substitution of carbohydrates from insulin-independent metabolic routes on hydroxylysine residues leads to fewer residues available for cross-linking and condensation of the basement membranes, which could ultimately account for the increased permeability found in the capillaries of many diabetics.

There are two major theories for CBMT etiology:

- 1) CBMT is the result of carbohydrate intolerance.
- 2) CBMT is the result of genetic determinants independent of factors responsible for carbohydrate intolerance, but associated with carbohydrate intolerance. Kilo, et al. (1972) found that muscle CBMT in humans was proportional to age of the patient, the number of other complications (90% had retinopathy or nephropathy), and duration of diabetes (93% had the disease 20 years or longer). Daysog, et al. (1961) also found established renal glomerular and vascular lesions were unaffected by severity or clinical control of the disease. Engerman (1977) found alloxan diabetic dogs with poorly controlled diabetes had more microvascular retinal lesions than did dogs with well-controlled diabetes. One eye removed from

these dogs several years before sacrifice was in better vascular condition than the other removed at death, regardless of degree of control of diabetes. Vascular abnormalities were less severe in those dogs with well controlled diabetes. This evidence lends further support to the idea that vascular abnormalities are due to insulin deficiency rather than postulated hereditary effects associated with diabetes, and the degree of microangiopathy is related to the duration of the disease.

There are those, however, who have produced evidence which fails to support this hypothesis. Vracko and Strandness (1967) found no quantitative differences in CBMT of abdominal wall skeletal muscle capillaries between diabetic and nondiabetic humans. Furthermore, Vracko's group used a less subjective type of measurement and a blind analysis, both methods not used in the aforementioned investigations. Vracko suggests that an anatomic difference in leg muscle capillaries rather than CBMT may make those capillaries more susceptible to the gangrene concomitant with the diabetic state.

Marks, et al. (1981) found that 37% of nondiabetic parents of children with Type I diabetes (juvenile onset, insulin dependent) had abnormally thickened basement membranes. This condition was not related to age, but was accompanied by an excess of DR4 antigen, leading the investigators to suggest CBMT may be hereditary. Friederici, et al. (1966) found no differences in the small blood vessels of the skin of diabetic and normal people. Siperstein, et al. (1973) studied muscle capillary basement membranes in

normal, diabetic, and prediabetic subjects (prediabetics being people whose parents are both diabetic but show no clinical signs themselves) and found CBMT to be unrelated to age, weight, severity, or duration of diabetes. Siperstein did, however, observe CBMT in 98% of diabetics, 8% of nondiabetics, and 50% of genetically prediabetics. Finally, he noted that subjects with severe hyperglycemia due to causes other than genetic diabetes infrequently showed CBMT. These observations lead Siperstein to conclude that CBMT is a vascular defect independent of the carbohydrate derangements of diabetes, and could possibly be an early sign of diabetes.

Diabetic neuropathies are perhaps the least adequately studied of the long-term complications of diabetes. The sites of nerve damage range from the extremities and joints to the gastrointestinal and genitourinary tracts and the autonomic nervous system. Pathologic changes have been observed in the anterior and posterior horns, posterior root ganglia, peripheral nerves, and end organs (Ellenberg, 1970). The genesis of diabetic neuropathy is still a matter of uncertainty. In fact, disordered nerve function has been observed to be unrelated to the control, duration, or severity of diabetes or presence of hyperglycemia.

Consequently, Ellenberg (1970) suggests that neuropathy may be concomitant with diabetes rather than resulting from the diabetic state. Also unknown is the cause of the neural lesions. Many investigators have produced evidence to support the idea of vascular insufficiency of the vasa nervorum. This may be the result of

capillary basement membrane thickening leading to complete closure of the blood vessels. Alternatively, results of other studies suggest metabolic influences to be the cause of nerve dysfunction in nerves exposed to high concentrations of glucose over long periods of time. These postulated metabolic influences are supported by studies showing improvement of nerve conduction velocity when insulin is administered (Ward, et al., 1971) and increased activity of the sorbitol pathway in diabetic neural tissue (Ellenberg, 1970), among others.

For nearly 100 years researchers have noted a relationship between diabetes and hearing disfunction. Such hearing loss may manifest either as a slowly progressive bilateral hearing loss of the perceptive type or as Meniere-like attacks, sudden hearing losses accompanied by vestibular symptoms. Less common are slowly progressive unilateral hearing losses.

There are two syndromes which have diabetes and hearing loss among their symptoms. Alport's syndrome includes the state of adult onset diabetes, progressive sensorineural hearing loss, and the presence of PAS positive granules in the kidney, along with atrophic glomeruli and capsular fibrosis. The etiology is unknown, but of possible significance is the fact that the kidney and cochlea have similar anatomical and ultrastructural features related to their role in fluid and electrolyte balance. Alstrom's syndrome, etiology also unknown, is associated with the presence of adult-onset diabetes, obesity, retinal degeneration, and sensorineural hearing

loss in the first decade of life which progressively grows more severe in the second and third decades. Bekesy and other audiometric tests suggest cochlear involvement.

Cases of Alport's and Alstrom's syndromes are rare; in order to learn more about the effects of diabetes on the inner ear, investigators have performed audiometric and vestibular tests on human diabetics, as well as studies of the temporal bones of human diabetics. Near the turn of the century, Panse and Wittmaack described degenerative changes in the cochlea and VII nerve of adult diabetics. The patients had, to varying degrees, clinical symptoms of which included decreased hearing acuity, sudden deafness, decreased irritability of vestibular apparatus, and menieriform attacks. Some patients improved when their blood sugar was normalized. Other independent researchers have found instances of degeneration of the cochlear nerve, spiral ganglia, and organ of Corti in diabetic subjects.

Jorgensen (1961) studied the temporal bones of 32 juvenile and adult-onset diabetics for abnormalities of the middle ear and the blood vessels and nerves of the inner ear. Jorgensen found exudate or otosclerosis in the middle ears of four subjects. PAS positive precipitate was observed in the stria vascularis of 29 diabetics as well as changes resembling those in severe atherosclerosis. Jorgensen found no relation between otopathology and age or blood pressure, but pathology was proportional to the duration of diabetes and the presence of other late complications such

as thickening of vessel walls in the modiolus and internal acoustic meatus. Jorgensen and Buch (1961) found 28 out of 60 diabetics had hearing losses. Ancona (Costa, 1967) found that 18 of 27 subjects of childhood diabetes aged 16 to 35 had bilateral hearing losses. Profazio and Barravelli (Costa, 1967) observed that 26% of diabetics aged 51 to 60 had hearing losses, and even more (37%) diabetics aged 60 to 70 had hearing losses. Costa (1967) noted that modiolar vessel walls were thicker than normal or blocked in six diabetic humans.

Makishima and Tanaka (1971) performed a histopathological study on the temporal bones of four long-term diabetic patients with no complaints of hearing loss. Their cochleae were normal except for accumulation of PAS positive substances around and atrophy of the spiral ganglion in the basal to middle turns. Also observed were demyelination and beading of the myelin sheaths of the VIII nerve. Naufal and Schuknecht (1974) also found recurring neuropathies of the III, VII, and VIII cranial nerves in a diabetic 86 year old female. She had a lower number of ganglion cells in both the inferior and superior divisions of the nerve compared to 15 older normal patients. Spratt and Hardin (Jorgensen, 1961) found that 14% of diabetics also had polyneuritis. Schuknecht (1974) postulated that neuropathologies are related to associated vascular disease. Kovar (1973) found evidence to support this, as did Kam-Hansen and Sorenson (1978). Kovar found two of the 14 diabetic temporal bones he studied had thickening of the walls of the vasa nervorum

of the VII and VIII cranial nerves, and all 14 had thickening of the strial walls, among other otopathologies. Kam-Hansen and Sorenson performed a post-mortem study on a diabetic 68 year old individual with sudden bilateral hearing loss, and discovered fresh infarctions localized in the cochlear and vestibular nuclei of the brain stem.

After performing various audiometric tests and investigating the auditory microcirculation with biomicroscopy on 130 diabetics, Rosen and Davis (1971) found high frequency hearing losses in the younger patients and hearing losses at all frequencies in the older patients. They found severity of microangiopathy to be correlated with duration rather than severity of diabetes, which corresponds with studies of diabetic microangiopathy of other locations throughout the body. Snashall (1977) also observed excessive tone decay at high frequency (8000 Hz) in two of eight diabetics. She found no hearing loss in her control group of 19 nondiabetics. Schuknecht (1974) proposed that investigators will give evidence of localized damage, specifically neuronal degeneration of the basal turn of the cochlea, in keeping with full high frequency hearing losses. In fact, Makishima and Tanaka (1971) have already produced supporting data.

Various animal studies have also been performed to study the relationship between diabetes and inner ear pathology. Costa (1967) studied the temporal bones of eight alloxan-diabetic rats which were sacrificed after periods of 45 days to 10 months of diabetes, and

compared them to the temporal bones of eight control animals. All the diabetics were found to have middle ear infections, and after six months of diabetes, capillary basement membrane thickening existed in the stria vascularis along with narrowing of lumina due to swollen light cells, and degeneration of endothelial-like cells of the vestibular margin of the spiral limbus.

Gladney (1978) sacrificed streptozotocin-induced diabetic chinchillas at one month of diabetes (two chinchillas), four months (one chinchilla), and six months (one chinchilla). He examined the cochleae microscopically and found no thickened basement membranes or vessel narrowing in the stria vascularis or spiral ligament. Marshak (1972) was unable to find any functional cochlear abnormalities in nine streptozotocin-induced diabetic chinchillas when studying their cochlear microphonics after twelve to eighteen months of diabetes. Marshak suggests angiopathy may be localized in the capillaries of the basilar membrane and not the stria vascularis. Oliveira, et al. (1977) found complete capillary occlusion of the stria, and decreased alkaline phosphatase activity (alkaline phosphatase is associated with transport mechanisms in the endothelium) in all five of their streptozotocin-treated chinchillas after six months of diabetes. Four of the five animals also had narrowing of the capillaries in the spiral ligament. No abnormalities were found in three other chinchillas after three months of diabetes nor in one animal which was diabetic for twelve months.

McIntire and Benitez (1981) found no significant changes in

the myelin or basement membranes in the inner ears of ten genetically diabetic Chinese hamsters which had been diabetic from nine to 24 months, although they did find that the total otopathology (severity and incidence) was three times greater in the diabetics than in the seven normal Chinese hamsters. In a follow-up study, Oostveen (1983) found total otopathology in 16 genetically diabetic Chinese hamster temporal bones to be 5.6 times the otopathology in normal Chinese hamsters. In addition, she found capillary basement membrane thickening in both the auditory and vestibular systems 5.5 times more often in the diabetics than in the normals.

The purpose of the present study was to compare the temporal bones of streptozotocin-diabetic Chinese hamsters with those of normal Chinese hamsters using light microscopy. The middle ears, cochleae, and vestibular systems were evaluated with special attention paid to the nervous tissue, blood vessels, and any signs of inflammation in order to determine the effects of long-term chemical diabetes on the middle and inner ear.

CHAPTER IV

DESIGN AND METHODOLOGY

Animals

The study sample consisted of 20 normal (nondiabetic) Chinese hamsters (*Cricetulus griseus*, M-line) donated by The Upjohn Company. There were nine females and 11 males ranging in age from 10 to 11 months old. The animals were individually housed in plastic cages in a room at 68° F with a 12 hour light/dark cycle. They were given Wayne Lab-Blox Rat Chow and water ad libitum. The sample was randomly divided into a control group of six hamsters (four females, two males) and an experimental group of 14 hamsters (five females, nine males). The experimental group was larger than the control to allow for attrition due to the toxic effects of streptozotocin.

Inducement of Experimental Diabetes

There are many methods of experimentally inducing diabetes. The pancreatic methods include pancreatectomy and B-cell destruction with the use of chemicals such as streptozotocin, alloxan, dithizone, and their homologues. Extra-pancreatic means of diabetic initiation include hormonal injections (e.g., growth hormone, adrenocorticotrophic hormone, glucagon, thyroxin, and steroids (such as cortisone to enhance gluconeogenesis), insulin antisera, and neural damage (Okamoto, 1970). Spontaneous diabetes has been

noted in most domesticated and several wild animal species, and has been selected for in a few species, most notably the Chinese hamster (Meier and Yerganian, 1959; Chang, et al., 1977).

Because many of the extrapancreatic methods of experimental diabetes involve chronically high level of hormones or antibodies of which the specific effects on the inner ear have not been adequately examined, and because an analogous study was concurrently performed using spontaneously diabetic Chinese hamsters (Oostveen and McIntire, 1983), a pancreatic method of diabetes induction was used. Chemical means are more specific and convenient than total pancreatectomy, and streptozotocin was chosen over alloxan because of wider species effectiveness (Brosky, 1969) and wider margin between diabetogenic and lethal doses (Junod, et al., 1967). In addition, Arison, et al. (1967) maintained, as a result of light and electron microscopic studies, that the streptozotocin-induced diabetic condition is associated with extrapancreatic lesions found in the human diabetes, and causes no immediate vascular changes.

Streptozotocin is an antibiotic derived from *Streptomyces acromogenes* and has the N-nitrosomethylamide function (Vavra, et al., 1959). The empirical formula is $C_{14}H_{27}N_5O_{12}$ (Rakieten, et al., 1963). A single injection causes rapid degranulation of B-cells without necrosis (Brosky and Logothetopoulos, 1969; Anson, et al., 1967). Other effects may include hepatotoxicity, thymic atrophy (Evans, et al., 1959), development of cataracts, accumulation of glycogen in the proximal convoluted tubules of the

kidney, lesions in exocrine cells of the pancreas at high doses, and persistence of granules in the golgi zone of B-cells (Arison, et al., 1967). Ketonuria is rarely exhibited (Junod, et al., 1967).

Before streptozotocin injection, the hamsters were fasted 24 hours, and the urine of each animal was tested for glucose with Testape (Eli Lilly). No signs of glucosuria were evident. The animals in the experimental group were injected with streptozotocin (125 mg/kg i.p.) in citrate buffer, pH 4.55. The streptozotocin was used within 30 minutes after being buffered to avoid degradation which is indicated by formation of a straw to deep brown color. The animals in the control group were injected i.p. with the citrate buffer only. All 20 hamsters were maintained until their death or for 26 weeks to simulate the long-term, insulin dependent diabetic state. No insulin was administered. Each animal was tested for glucosuria with Testape at two, six, 10, and 26 weeks. A Testape reading of 1+ or more was accepted as indicative of the diabetic state (Lilly Research Laboratories, 1973). The control animals showed constant negative results for glucosuria, while all the experimental animals gave Testape readings of 1/4% or more.

Histologic Procedure

Six months after injection, the hamsters were sacrificed by ether inhalation. Temporal bones were removed in block (right and

left remaining together as one tissue block) and placed in Heidenhain-Susa fixative within 20 minutes after death. After 24 hours of fixation, the bones were rinsed in cold running tap water for one and one-half hours and placed in 5% trichloroacetic acid (TCA) at room temperature to decalcify. By the end of 12 hours, calcium precipitation end point tests gave negative results and the bones were rinsed 30 minutes under cold tap water. The minimum time necessary for neutralization is 3 hours, although leaving the bones in sodium sulfate for up to 48 hours does no harm. The bones were neutralized for seven hours in 5% sodium sulfate, rinsed with distilled water for five minutes, and placed in 50% ethanol with 2 ml saturated iodine dissolved in 70% ethanol, added to each 100 ml 50% ethanol for 12 hours. At this point an attempt to trim the bones revealed they were not fully decalcified, contrary to the calcium end point test results. The early negative calcium end point test was most likely due to the ammonium oxalate being in solution for too long a time before the end point test was run. The bones were rinsed in running cold tap water for one and one-half hours, and placed in 5% TCA to complete the decalcification. The solutions were changed every 48 hours for six days. End point tests were run on the second, fourth, fifth, and sixth days. At the end of the fifth and sixth days, the calcium end point test was negative, the test on the sixth day being a check. Once again, the bones were rinsed 30 minutes in cold running tap water, neutralized four hours in 5% sodium sulfate, rinsed in

distilled water for ten minutes, and placed in 50% ethanol with iodine for eight hours. The bones were further dehydrated in increasing (70, 80, 95, 100%) concentrations of ethanol over a period of five days. Saturated iodine was added to the 70% and 80% ethanol solutions to remove any mercury there as a result of the fixative. The bones were put in two changes of ethanol and anhydrous ethyl ether (1:1). Finally, the bones were infiltrated with increasing concentrations of celloidin (1.5%, 3%, 6%, 12%) over a period of three weeks. The celloidin was dissolved in a one to one mixture of absolute ethanol and anhydrous ethyl ether. The bones were placed in the 12% celloidin with the superior petrous side toward the bottom of the jar, and allowed to harden slowly by loosening the lids to permit evaporation of the ether-ethanol solvent. When the celloidin around the embedded specimens was no longer sticky when touched the specimens were mounted on wood blocks and placed in chloroform for 24 hours to complete the hardening process. The bones were serially sectioned in the horizontal plane at 20 micrometers. Sections were collected commencing at or near the beginning of the ampullae of the superior semi circular canals, and finishing when the round window niche was disappearing. The first section and every fifth section thereafter were saved for staining with hematoxylin and eosin, for overall histopathologic evaluation (see Appendix A for procedure). The second section and every fifth section following were saved and stained using allochrome technique (PAS) to detect basement

membrane changes in the blood vessels due to increased glycoprotein content (see Appendix B for procedure). The third section and every fifth section thereafter were saved in 80% ethanol for possible future studies. The remaining sections were discarded.

After staining, the sections were placed on slides, cover-slipped, and put under weights for seven to 14 days to prevent air bubble formation and to keep the sections pressed flat between the glass. Then the slides were placed in serial order from the superior to the inferior aspects of the temporal bone. The slides were evaluated in a blind assessment in two stages. The first stage consisted of a general otopathological evaluation of the H & E stained slides. Each serial section of every animal was evaluated in the middle ear, cochlea, and vestibular system. A sample evaluation can be found in Appendix C. The results of H & E evaluations on all temporal bones were compiled and summarized in Table 1. The second stage consisted of assessment of the PAS slides. The blood vessels of selected cochlear and vestibular structures, and the membranes lining the middle ear were observed for basement membrane thickening. Appendix D contains a sample assessment. The PAS evaluations for each animal are compiled and summarized in Table 2.

Statistical Analysis

The results were analyzed using the Wilcoxon rank sum test for independent samples to compare the probability distributions of the diabetic and control sampled populations. This nonparametric

TABLE 1
TEMPORAL DATA FOR CHINESE HAMSTERS

Animal Number	Sex	Birth date	Diabetic Date	Diabetic Time (Months)	Death or Sacrifice Date	Age at Time of Sacrifice	Testape Reading at Time of Death
Diabetics							
2. MAA01-44	M	1-27-81	11-20-81	N.A.	12-1-81	N.A.	N.A.
3. MAA01-57	M	1-30-81	11-20-81	6	5-15-82	15m/19d	2+
4. MAA01-45	M	1-27-81	11-20-81	N.A.	12-1-81	N.A.	N.A.
5. MAA01-47	M	1-27-81	11-20-81	N.A.	3-8-82	N.A.	N.A.
6. MAA01-54	F	1-30-81	11-20-81	6	5-18-82	15m/19d	3+
8. MAA01-41	M	1-5-81	11-20-81	N.A.	12-1-81	N.A.	N.A.
9. MAA01-56	M	1-30-81	11-20-81	N.A.	12-1-81	N.A.	N.A.
12. MAA01-51	M	1-29-81	11-20-81	N.A.	1-8-82	N.A.	N.A.
13. MAA01-52	M	1-29-81	11-20-81	6	5-18-82	15m/20d	4+
16. MAA01-55	F	1-30-81	11-20-81	6	5-18-82	15m/19d	3+
17. MAA01-81	F	2-25-81	11-20-81	6	5-18-82	14m/21d	3+
18. MAA01-50	M	1-29-81	11-20-81	N.A.	5-1-82	N.A.	N.A.
19. MAA01-80	F	2-25-81	11-20-81	6	5-18-82	14m/21d	3+
20. MAA01-82	F	2-25-81	11-20-81	N.A.	1-6-82	N.A.	N.A.
Controls							
1. MAA01-46	M	1-27-81	N.A.	0	5-18-82	15m/22d	Negative
7. MAA01-49	F	1-29-81	N.A.	0	5-18-82	15m/20d	Negative
10. MAA01-53	F	1-30-81	N.A.	0	5-18-82	15m/19d	Negative
11. MAA01-59	F	1-30-81	N.A.	0	5-18-82	15m/19d	Negative
14. MAA01-65	M	1-30-81	N.A.	0	5-18-82	15m/19d	Negative
15. MAA01-60	F	1-30-81	N.A.	0	5-18-82	15m/19d	Negative

Note: m = months
d = days

TABLE 2

NUMBER OF ANIMALS WHICH EXHIBIT EACH OTOPATHOLOGICAL CONDITION

Condition: Middle ear - H & E	DIABETIC			CONTROL		
	sl	mo	se	sl	mo	se
Round cell infiltration	0	0	0	0	0	0
Fibroblasts & cholesterol crystals	0	0	0	0	0	0
Tympanic membrane thickening	2	0	0	2	0	0
Fibrous attachments to tympanic nerve	0	0	0	0	0	0
Fibrosis around ossicles	0	0	0	1	0	0
Polyp formation	0	0	0	0	0	0
Cholesterol granuloma	0	0	0	0	0	0
New bone formation	0	0	0	0	0	0
New gland formation	0	0	0	0	0	0
Hemorrhage	3	2	0	4	1	0
Inflammatory cells in stapedial cavity	0	0	0	0	0	0
Fibrous attachments in stapedial cavity	0	0	0	0	0	0
Fibroblasts in stapedial cavity	0	0	0	0	0	0
Fibrous attachments to the round window niche	0	0	0	0	0	0
Inflammatory cells in the round window niche	0	0	0	0	0	0
Fibroblasts in the round window niche	0	0	0	0	0	0
Polyp formation in the round window niche	0	0	0	0	0	0
Condition: Cochlear and Vestibular - H & E						
Strial atrophy	0	0	0	0	0	0
Precipitate	5	0	0	5	1	0
Hemorrhage	0	0	0	0	0	0
Reissner's membrane thickened	0	0	0	0	0	0
Sensory epithelium changes	0	1	4	1	4	1
Round window niche thickened	0	0	0	0	0	0
Endolymphatic Hydrops	0	0	0	1	0	0
Nerve changes	3	0	0	0	0	1
Condition: Vascular - PAS						
Thickened stapedial artery	0	0	0	0	0	0

TABLE 2 (continued)

	DIABETIC			CONTROL		
	sl	mo	se	sl	mo	se
C.B.M.T.	1	0	0	0	0	0
Strial blood vessel diameter enlarged	0	0	0	0	0	0

Note: sl = slight
 mo = moderate
 se = severe

Six animals total in each group

statistical analysis was chosen because the sample sizes were small, random, and independent, and the observations could easily be ranked in order of magnitude. Statistical significance at $\alpha = 0.10$ was determined for pathological difference between treatment and no treatment groups for middle ear (H & E), cochlear and vestibular system (H & E), and basement membrane thickening (PAS).

CHAPTER V

RESULTS

Of the original hamsters injected with streptozotocin, six survived the inducement of diabetes for six months and their temporal bones were evaluated for middle and inner ear pathologies. Each of the six survivors showed glucosuria within 24 hours after the injection, which remained until time of sacrifice. At sacrifice they had Testape readings of 2+ through 4+. All of the controls survived the six-month period, and their temporal bones were likewise assessed. At time of sacrifice, all animals were between 14.5 and 15.7 months in age.

General Otopathology

Each set of temporal bones was evaluated for signs of past or present inflammation in the middle ear, for abnormalities in the cochlear and vestibular systems which could possibly affect sensory abilities and for basement membrane thickening and other vascular changes (see Table 2 for complete list). All assessments were blind, meaning the observers did not know the group to which the animal belonged. Each type of pathology for a given ear was judged to be slight, moderate, or severe according to the extent and frequency with which it was encountered. In order to tabulate the total otopathological involvement for the middle and inner ear, numerical values (1 = slight, 2 = moderate, 3 = severe) were assigned to each otopathological incident, and the values were

then summed for each animal (see Table 3).

The hematoxylin and eosin set of serial sections showed no significant differences in total otopathology between the diabetic and control groups.

There were three types of inner ear pathologies observed. As in the middle ear, there were no types of otopathology in the diabetic group that were not also found in the control group, although the degree of involvement varied between groups. All animals had some type of inner ear pathology.

The most common inner ear abnormality was a form of acellular, protein-like precipitate found in all the control animals (5 slight, 1 moderate) and five of the six diabetic animals (all slight). This precipitate was found in both the scalae vestibulae and tympanae of the cochlea, and in the perilymph of the utricle and saccule. The precipitate resembles the precipitate formed by proteins during fixation (Schuknecht, 1974), which is considered a normal histological background. As a result, the acellular, protein-like precipitate was not considered a pathology when computing statistical differences between groups.

Also fairly common were changes in the sensory epithelium. Such changes were seen in the form of edema of the hair cells of the various cristae and maculae. There were no observed changes in number of hair cells nor was edema found in the support cells. The diabetic group had one moderate and four severe cases of sensory epithelium changes. The control group had one slight, four moderate, and one severe case of edema in the sensory epithelia.

TABLE 3

TOTAL OTOPATHOLOGICAL INVOLVEMENT

<u>Animal number</u>	<u>Sum of H & E for middle ear</u>	<u>Sum of H & E for coch.vest.</u>	<u>Total H&E Pathology</u>	<u>Total PAS Pathology</u>	<u>Total Pathology</u>
<u>Diabetics</u>					
3. MAA01-57	0	4	4	0	4
6. MAA01-54	3	5	8	0	8
13. MAA01-52	1	4	5	2	7
16. MAA01-55	1	5	6	0	6
17. MAA01-81	2	4	6	0	6
19. MAA01-80	2	3	5	0	5
<u>Controls</u>					
1. MAA01-46	1	3	4	1	5
7. MAA01-49	1	2	3	0	3
10. MAA01-53	3	3	6	0	6
11. MAA01-59	2	7	9	0	9
14. MAA01-65	1	5	6	0	6
15. MAA01-60	1	3	4	0	4

Such edema is likely to be the result of fixation and temporal bone processing.

Pathology of the auditory or vestibular nerves was observed in one control animal and three diabetic animals to a slight degree. The vestibular nerve in the control animal was absent bilaterally. This may be attributed to tearing during histologic processing rather than to pathology. In two diabetic animals the nerve was slightly pulled away from the nerve capsule. In the third diabetic animal, there was evidence of hemorrhage within the nerve capsule, although because of the composition of the hemorrhagic material, the bleeding looked fresh and was probably the result of blood vessels torn after sacrifice.

Finally, one control animal had a slight amount of endolymphatic hydrops in the middle turn in the right cochlea, as evidenced by a bowing outward of Reissner's membrane.

In summary, there were no significant differences of types, severity, or frequency of otopathology between the diabetic animals and the control animals. Furthermore, there was little within-group variability. With a possible high middle ear pathology score of 51, the diabetic animals had scores ranging from zero to three. The control animals had scores of one to three. With a possible high inner ear pathology score of 24, the diabetic animals ranged from three to five, and the controls ranged from two to seven. The total otopathology was consistent within and between groups for the middle ears.

Microvascular Pathology

Each set of PAS-stained temporal bones was evaluated for vascular basement membrane thickening. Special attention was paid to the capillaries within the stria vascularis, spiral limbus and within the sensory epithelia of the vestibular system. On at least one slide all capillaries were observed at each location. Also, several vessels outside the auditory and vestibular systems were observed to obtain a range of vascular membrane thickness in each animal. After the first round of observations were made on each set of slides, when it became clear that the vascular membranes were consistently in the normal range, several sets of slides exhibiting vascular membrane thickening were observed to reassure the researcher that no changes were overlooked. All assessments were blind, meaning that observers did not know the number or the group to which the slides belonged. After two assessments, a third evaluation was made with the help of an individual other than the scorer of all questionable slides. Membrane thickness was judged to be slight, moderate, or severe according to the degree of thickening and frequency within a particular location (see Table 2).

The PAS set of serial sections showed no significant differences ($\alpha = .10$) in basement membrane thickening or angiopathy between the treatment and no treatment groups.

There was no evidence of macroangiopathy visible in either group of animals. There was one example of capillary basement

membrane thickening in a diabetic hamster, located unilaterally within the stria vascularis of the basal turn of the cochlea. Sophisticated and quantitative methods of evaluating vascular membrane thickness were not possible, and there is no universally accepted method in the literature. Two experienced observers, however, noted the strial capillary mentioned earlier in this paragraph appeared outside the normal range of basement membrane thickness, although it was not as thickened as were the thickest basement membranes observed. Because of the slight degree of thickening and solitary appearance, the PAS temporal bone was given a rating of 1.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

There were no significant differences found in the capillary basement membrane thickness or in the overall otopathology with respect to severity, frequency, or type of pathology observed between the middle and inner ears of the streptozotocin-diabetic and the normal Chinese hamsters.

One possible explanation of these results is that six months of experimental diabetes is not long enough to cause the manifestations which result from long-term adult-onset diabetes. However, the normal life span of the Chinese hamster is from two to four years, three being average (Gunderson, 1967). Six months is a large proportion of the adult life of a Chinese hamster, which is generally believed to be a time period sufficient to represent the state of long-term diabetes. In addition two studies using streptozotocin-diabetic chin-chillas (Oliveira, 1977) and alloxan-diabetic rats (Costa, 1967) showed changes in blood vessel walls after six months of the diabetic state.

No comprehensive study has been performed to determine the anatomic and biochemical aspects which are peculiar to the Chinese hamster middle and inner ears, although histopathological assessment in two independent investigations (McIntire and Benitez, 1978; Oostveen and McIntire, 1983) revealed no notable differences between Chinese hamster and other rodent temporal bones. Minor biochemical

differences within the species may exist which protect the auditory and vestibular systems from the effects of chemical diabetes. However, such differences, if present, do not safeguard the temporal bones against the effects of genetic diabetes. McIntire and Benitez (1981) and Oostveen and McIntire (1983) found a greater degree of otopathology in two lines of genetically diabetic Chinese hamsters, and Oostveen and McIntire also found significantly more capillary basement membrane thickening in the diabetic lines. Thus, a more plausible explanation may be that chemical diabetes is not as true a model as is spontaneous diabetes for the study of long-term otopathological complications of diabetes. Arison, et al. (1967) found streptozotocin-induced diabetes mimicked the human diabetic state with respect to extra pancreatic lesions. In the present study there was sustained hyperglycemia and greatly increased urine output, (the increased urine output manifested as saturated bedding which smelled of urine and turned the shavings green while drinking water effected no color change) conditions found in genetically diabetic Chinese hamsters (Gunderson, 1967). Nonetheless, the vast majority of papers published announcing the results of studies observing the effects of spontaneous diabetes on the inner ear showed there was a positive correlation between diabetes and changes in the inner ear. In contrast, of four studies on the effects of chemical diabetes on the inner ear, only two (Costa, 1967; Oliveira, 1977) were able to link diabetes with any form of otopathology. These effects were localized to the blood vessels and surrounding tissues of the stria vascularis, spiral limbus, and spiral ligament.

It is reasonable to conclude from the results of the present study that it would be more productive to use a model other than streptozotocin-induced diabetes when studying the effects of diabetes on the inner and middle ears of Chinese hamsters.

Finally, comparisons of the present study of the otopathology of chemical diabetes with the Oostveen and McIntire study of genetic diabetes lends credibility to the idea that capillary basement membrane thickening is the result of genetic determinants possibly associated with, but independent of, diabetes. Both studies used similar techniques within the same species.

A study of the effects of streptozotocin-diabetes on the retina, kidney, and selected enzyme concentrations (e.g., glucose kinase, phosphoenolpyruvate) for comparison with known parameters in the genetically diabetic and normal lines of the Chinese hamster would be beneficial. Further study might be performed using electron microscopy to observe microanatomy which is only grossly visible with light microscope techniques, particularly the capillary basement membranes.

APPENDIX A

Hematoxylin and Eosin Procedure

1. Tap water	rinse
2. Tap water	rinse
3. Lugol iodine	10 minutes
4. 2.5% sodium thiosulfate	5 minutes
5. Tap water	rinse
6. Tap water	rinse
7. Harris's hematoxylin	12 minutes
8. Tap water	rinse
9. Tap water	rinse
10. 70% acid alcohol 1.0%	10 seconds
11. Running tap water	30 seconds
12. 0.5% ammonium hydroxide	rinse
13. 0.5% ammonium hydroxide	rinse
14. 0.5% ammonium hydroxide	rinse
15. Tap water	rinse
16. Tap water	rinse
17. Eosin Y	30 seconds
18. 80% ethanol	1 minute
19. 80% ethanol	1 minute
20. 80% ethanol	1 minute
21. 95% ethanol	30 seconds

APPENDIX A (continued)

100% ethanol and chloroform, 1:1	1 minute
Xylene and terpineol 1:1	may leave
Coverslip with permount	
Weight slides	1-2 weeks

Solutions

1. Lugol iodine:	distilled water	100 ml
	potassium iodide	2 g
	iodine crystals	

Dissolve potassium iodide in water, then add iodine.

2. Harris's hematoxylin

hematoxylin crystals	1 g
95% ethanol	5 ml
ammonium or potassium alum	20 g
distilled water	200 ml
mercuric oxide	0.5 g

Dissolve the hematoxylin in the ethanol and the ammonium in the water by heating. Mix the two solutions. Bring the mixture to a boil as rapidly as possible. Remove from heat. Add the mercuric oxide. Reheat until the solution turns dark purple, and then remove immediately from the heat and plunge it into a basin of cold water. The solution is ready to use when cool. Add 2-4 ml glacial acetic acid if desired, to keep away metallic luster

APPENDIX A (continued)

and brighten nuclear structure.

70% acid alcohol

concentrated HCl	1 ml
70% ethanol	100 ml

Eosin Y

eosin Y	0.5 g
70% ethanol	500 ml
glacial acetic acid	2.5 ml

APPENDIX B

Periodic Acid - Schiff - Allochrome Procedure

1. Tap water	rinse
2. Tap water	rinse
3. Lugol's iodine	10 minutes
4. 2.5% sodium thiosulfate	5 minutes
5. Tap water	rinse
6. Tap water	rinse
7. Periodic acid 0.5%	11 minutes
8. Running tap water	5 minutes
9. Schiff reagent	7 minutes
10. 0.5% sodium metabisulfite	30 seconds
11. 0.5% sodium metabisulfite	2 minutes
12. 0.5% sodium metabisulfite	2 minutes
13. 0.5% sodium metabisulfite	2 minutes
14. Running tap water	5 minutes
15. Weigert's iron hematoxylin	6 minutes
16. Running tap water	4 minutes
17. Picric acid/methyl blue 0.02%	2 minutes
18. 95% ethanol	1 dip
19. 95% ethanol	1 dip
20. 100% ethanol	1 dip
21. 100% ethanol	1 dip

APPENDIX B (continued)

- | | |
|--------------------------------------|-----------|
| 22. 100% ethanol and chloroform, 1:1 | 1 dip |
| 23. Xylene and terpineol 1:1 | may leave |
| 24. Coverslip with permount | |
| 25. Weight slides | 1-2 weeks |

Solutions

1. Periodic acid solution 0.5%

periodic acid	0.5 g
distilled water	100 ml

2. Schiff reagent

basic fuchsin	1.0 g
sodium metabisulfite	1.9 g
distilled water	85.0 ml
N HCl	15.0 ml

Mix above reagents together. Shake at intervals until clear or straw-colored. If necessary, store overnight in a dark place. Add 0.5 g activated charcoal. Shake one minute. Filter into a fresh bottle. The filtrate should be clear. Store in refrigerator. Good until solution shows reddish-purple tinge.

3. Weigert's iron hematoxylin

Solution A: hematoxylin	1 g
95% ethanol	100 ml
Solution B: ferric chloride	
29% aqueous solution	4 ml

APPENDIX B (continued)

distilled water	95 ml
-----------------	-------

concentrated HCl	1 ml
------------------	------

Just before use, add solution B slowly to Solution A in equal parts. Good for approximately 30 minutes.

4. Picric acid - methylene blue

0.04% methyl blue in saturated aqueous picric acid (1.2%). Dilute 1:30.

APPENDIX C

Sample, Qualitative Assessment of Serial Sections (H & E) Animal #6

Mid-modiolar section, Slide #55. The overall preservation and staining looks good. There is little artifact, except concerning the stapes on both sides which will be commented on later. On the right hand side, the cochlea is well seen. There is a small amount of acellular, protein-like precipitate on the scale vestibule side of Reissner's membrane probably filling less than an eighth of each scale vestibuli. The two basal turns and one middle turn can be seen. In all three, the tectorial membrane is seen except in the one of the basal turns and in the other two where the tectorial membrane is seen it is not connected to the external hair cells. In all three turns, the external and internal hair cells and the spiral limbus appear normal as does the stria vascularis. Reissner's membrane also looks normal. Spiral ganglia and cochlea nerve appear normal. In the middle ear, the tympanic membrane is well seen although it is broken in several places. This looks to be due to histological artifact. All membranes appear to be normal except for a small part near the anterior-medial side where the membrane looks thicker and removed from the bony wall. The middle ear is clear. The vestibular nerve appears normal although there is some doubt to me as to whether it fills the entire channel that it should fill. With regards to the vestibular system, no sensory epithelia can be

seen. Only a small bit of the saccule can be seen. It appears clear. Membranes appear normal. There is a small bit of the acellular, protein-like, precipitate in the perilymph surrounding the saccule. All other membranes appear normal. The stapes can be seen on the right side. The foot plate and the annular ligament both appear normal. The anterior crura cannot be seen probably due to histological artifact. The stapedia artery cannot be seen on the right side. One last note, in the middle turn of the cochlea the basilar membrane is partially removed or disconnected.

On the left side of Slide #55, the middle ear is clear of any precipitate. All membranes appear normal and in place. The tympanic membrane is broken and mostly gone except for a small part of the pars tensa, which appears of normal thickness. The stapes cannot be seen except for the foot plate and it is hard to tell the condition of this because it is folded. It is a histological artifact. The stapedia artery cannot be seen and neither can either the posterior or anterior crura of the staples.

On the left side, two basal turns and one middle turn of the cochlea can be seen. In all three turns, Reissner's membrane looks within range of normal although they are all three slightly curved inward toward the scala media. In all the external and internal hair cells are of normal number and appearance. The tectorial membrane is normal in one basal turn. In the other basal turn the tectorial membrane is missing. In the middle turn, the tectorial membrane is perhaps stretched a little too tight. In that same turn, the middle turn, the stria is normal except it is a little bit squashed. In the

other two turns the striae are normal. All the turns of the scala media are clear but in all three turns, the scalae vestibulae have acellular, protein-like precipitate filling between $1/8$ and $1/2$ of each. The spiral ganglia appear normal. Scarpa's ganglia appear normal except that they fill only half of their allotted space within the bone with regards to the vestibular system on the left side, no sensory epithelia can be seen. All membranes appear normal and in the perilymph of the saccule there is also some of the acellular, protein-like precipitate filling less than $1/8$ of the perilymph. Brain appears normal.

There is some protein-like, acellular precipitate seen in the saccule. The utricle is clear. All membranes look good and no sensory epithelia in either the saccule or utricle is visible. Foot plate of the stapes is visible although this researcher can't see the annular ligament on either side. Part of that may be caused by a fold in the stapes right where the annular ligament might be. The stapedia artery is not visible. The tympanic membrane can be seen although it is broken, it looks normal. Middle ear is clear. All membranes look as usual. There are some dark, precipitate that looks to me like histological artifact on the anterior side and on the bone near where the tympanic membrane connects. End of the mid-midiolar section #55.

Hamster #6, slide #10, section #10, the incudo-malleal joint is visible although it is not easy to be read because the bone is bent and is out of place, likely the result of histological artifact. The middle ear is clear. The cochlea of the superior semi-circular

canal is visible on the right side. The hair cells look to be normal in number although most of them are slightly swollen within the cochlea, the hair cells look slightly scrambled or slightly in a less orderly appearance than they usually are. The support cells look normal in density and they are not swollen at all. In the gelatinous membrane it looks good although it might be slightly more spread out than usual. There is some dark, greenish precipitate on the lateral side as you come down the slope of the cochlea, probably histological artifact. All other membranes look normal. On the left side the epitympanic cavity is clear. The membranes look normal and of normal thickness. The incudo-malleal joint is visible and looks normal. The cartilage lining appears normal as does the bone. So on the left side, the cochlea of the superior, semi-circular canal is visible. The hair cells look normal although a few of them are slightly swollen. Fewer hair cells on the left side than on the right side are swollen. The support cells and gelatinous membrane looks slightly spread out especially on the lateral side. Again there is a slight, greenish yellow dark precipitate below the level of support cells on either side of the cupula.

On Slide #15 on the right hand side, the incudo-malleal joint is visible and looks normal. The cartilage and the bone appear normal. It looks slightly displaced and it looks to be the result of histological technique as the bone and the ligament and the tympanic membrane are all broken and displaced, slightly superior and medial to what they should be. The cupula of the superior

semi-circular canal is also visible. The hair cells are so swollen. Everything else is normal, although the dark greenish precipitate is again visible on both sides of the cupula. The geniculate ganglia are visible on the right side and appear normal. On the left side, the epitympanic cavity is again clear. The incudo-malleal joint looks normal. The tympanic membrane is again visible but broken in one section. The tympanic membrane appears normal and of normal thickness. The cupula of the superior, semi-circular canal on the left side is no longer visible.

On Slide #20 on the left hand side, the tympanic membrane is visible. There is a section of the pars tensa that is missing. Probably 1/3 of it is missing, although the pars tensa is much thinner than in the previous two slides of the more normal thickness. The cochlea is just appearing on the left side.

For Slide #25, on the right hand side, the hair cells of the macula of the utricle are normal in density. It looks as though at least 3/4 of them are swollen more than usual. The support cells are normal. Gelatinous membrane is good. The otoconia are normal in both density and appearance. On the far lateral side of the macula is a little bit more of the dark green precipitate. The cochlea is just beginning on the right hand side. The incudo-malleal joint is visible and appears normal. On the left hand side of the macula of the utricle is also visible. The hair cells are normal in density. They look to be a little swollen but not as swollen as they were on the right side. The support cells and gelatinous membrane appear normal as do the otoconia. The macula of the saccule

is also visible. This time the hair cells appear normal, are of normal density, and are not swollen at all. The support cells look normal and so does the gelatinous membrane and the otoconia. Both sections of the saccule that is, the perilymph and the endolymph, are 1/2 filled with protein-like acellular precipitate. There is also some dark, greenish, precipitate in the endolymph of the utricle. This looks like histological artifact. The cupula of the lateral semi-circular canal is visible. Ninety percent of hair cells appear swollen although they appear in good order in that they are lined up well. There isn't the lack of organization that there was on the right side in the superior semi-circular canal. The support cells and the gelatinous membrane all appear normal. There is also some more of the dark green precipitate on either side of the cupula. The left hand side lateral, semi-circular canal is free of all precipitate.

On Slide #30, the cupula of the right hand side lateral, semi-circular canal is visible. The hair cells are well seen. They are normal with regards to density and organization, although most of them are swollen, especially toward the tip of the cupula. The support cells all look normal and so does the gelatinous membrane. There is some dark, greenish precipitate on either side of the cupula. The macula of the saccule on the right side is also visible. Nearly all of the hair cells here are swollen. Support cells and gelatinous membranes are normal. So are the otoconia. Near the macula of the saccule is a slight amount of precipitate. It is hard to tell what it is, whether it is artifact or just fibrous precipitate. On the

left side, the macula of the saccule is also well seen. The otoconia of the gelatinous membrane, and support cells all look normal. Fifty percent of the hair cells are slightly swollen, not as swollen as they are on the right side. It looks as though less than 10% of the otoconia are displaced and are freed from where they normally are. There is also a slight amount, less than 10%, of fibrous precipitate on the anterior and on the macula of the saccule just above the otoconia, between the otoconia and the membrane. There is also some acellular, protein-like precipitate on the perilymph side of the saccule filling less than 1/4 of that part of the saccule. On the left side between the malleus and the tympanic membrane is also some precipitate. It looks as though it is mostly red blood cells clumped together, or packed very tightly. There are probably between 10 and 20 white blood cells that look like monocytes. It also looks as though the red blood cells are somehow held together by fibers. This is most likely the result of hemorrhage at the time of sacrifice. At this point, the tympanic membrane is also separated and except for one strand, almost completely severed. Except for histological artifact, the rest of the epitympanic cavity is clear on the left side.

The basal turn on the left hand side of the cochlea is visible now in Slide #35. The stria vascularis, tectorial membranes, hair cells, spiral limbus all look normal. On the scala vestibuli side on the Reissner's membrane on both sides, 25% of the cavity is filled with acellular protein-like precipitate. All Reissner's

membranes appears somewhat loose and are curved towards the scala media on both sides of the basal turn of the cochlea.

On Slide #40, on both sides, the VII nerve appears normal. On the left side, the middle turn of the cochlea has just become visible. The stria vascularis appears normal as do all the hair cells and the tectorial membrane. The basal turn of the cochlea is visible on the right side, and there is also the acellular protein-like precipitate on the scala vestibuli side of Reissner's membrane. Reissner's membrane looks to be within normal limits although it slightly goes inward toward the scala media. On the lateral side of the basal turn there is one white blood cell, although the nucleus is not dark so it is indistinguishable as to type.

On Slide #45, the saccule on the left side is about 50% filled with acellular, protein-like precipitate. There are no cells visible. The foot plate and the annular ligament are both seen and appear normal as does the stapedial artery. The stapedial artery is a little bit misshapen and the crura of the stapes are not visible, although part of the head of the stapes is visible. The condition of the stapedial artery and the stapes due to histological artifact rather than any kind of malformation of the animal. The tympanic membrane is visible although it is broken in several pieces. All membranes lining the cavity look normal. On the right hand side, more of the tympanic membrane is visible. It looks normal. The foot plate of the stapes is just beginning to be visible. It looks normal as do all membranes lining the epitympanic cavity.

On Slide #50, on the right hand side, the foot plate of the stapes is visible and so is the annular ligament. They both look good. Again the head of the stapes is visible but neither crura are and stapedia artery flattened against itself. This is due to histological artifact. The walls don't look any thicker than as usual. The lining of the epitympanic cavity in the tympanic membrane look normal on the right side. On the left side both crura of the stapes are visible as is the incudostapedial joint that looks normal. The stapedia artery here is flattened against itself also, which is the result of histological artifact. Again the saccula on the left side is 1/3 to 1/2 filled with the protein-like precipitate.

On Slide #60 on the right hand side, the entire stapes is visible. Both crura, the foot plate, the head, and the incudostapedial articulation all look normal. The stapedia artery in normal position although it is still flattened against itself, and one end is torn, appearing to be the result of histological artifact. The tympanic membrane is present although broken, and of normal thickness. All membranes look normal in the epitympanic cavity on the right hand side.

On the left hand side the medial turn of the cochlea is visible. The tectorial membrane, inner and outer hair cells, stria, and Reissner's membrane all look normal and clear of any kind of precipitate. Reissner's membrane is more flaccid than usual and is slightly bowed toward the scala media side.

On Slide #65 on the right hand side, the basal turns and one medial turn of the cochlea are visible. The stria vascularis, the

tectorial membranes and all internal and external hair cells look normal as do the Reissner's membranes. In the medial basal and the middle turns there is a slight amount of protein-like precipitate acellular on the scala vestibuli side of the Reissner's membrane. On that same basal turn, the Reissner's membrane is slightly wrinkled on one end. Other than that all three are perfectly straight and look normal.

In the epitympanic cavity on the right side, the tympanic membrane is well seen and appears normal and of normal thickness. Between the tympanic membrane and the bone there is a slight amount of dark blue acellular precipitate that looks to be the result of histological artifact.

On Slide #70 both sides, both right and left ears are filled with bluish gray precipitate between the bone and tympanic membrane.

On Slide #80, on the left hand side, the cristae of the posterior semi-circular canal are visible. The hair cells are slightly swollen although they are normally well organized. The support cells and the gelatinous membrane all look fine. There is some dark green precipitate on the lateral side of the down slope of the cristae and beneath the support cell.

On Slide #85, the cristae of the posterior semi-circular canal is visible. The hair cells again are swollen although they are well organized. The support cells look normal and so does the gelatinous membrane. There is very little of that dark green precipitate on the lateral side of the down slope of the cristae.

APPENDIX D

Sample, Qualitative Assessment of Serial Sections (PAS) Animal #6

Slide #5 is well stained including PAS, hematoxylin, and allochrome. No air bubbles are visible. The blood vessels in the cristae of the left superior semi-circular canal appear of normal thickness and are unoccluded.

Slide #10. The blood vessels are normal in the right hand side cristae of the semi-circular canal.

In Slide #15 on the right side, one blood vessel is visible in the VII nerve and it appears normal. Walls are of normal thickness and the lumen is unoccluded. One blood vessel in the VII nerve is also visible on the left side, and appears normal in wall thickness and lumen size. The left hand side macula of the utricle can be seen, and the capillaries look normal. There is some PAS positive precipitate which appears to be histological artifact.

On Slide #20 on the right hand side, the macula of the utricle has appeared. The blood vessels look normal. On the left hand side, both the macula and the saccule and the cristae of the lateral semi-circular canal have appeared. The blood vessels in both look normal in thickness and number. There is a great deal of the PAS positive precipitate on the cristae of the lateral canal. This precipitate is most likely histological artifact. Also in the utricle and part of the saccule there is more of the acellular

protein-like precipitate that was seen in the H & E slides, enough to fill 1/4 of the perilymph.

On Slide #25, the cristae of the lateral semi-circular canal and the macula of the saccule are both well seen and the blood vessels appear normal in both the blood thickness of the walls and quantity. On the left side in the basal turn of the cochlea, the blood vessels of the stria and the spiral limbus all look normal in thickness and number. There are a few pieces, maybe four pieces of PAS positive precipitate along the stria vascularis on the lateral side of the posterior end of the basal turn.

On Slide #35 on the right hand side, in the basal turn of the cochlea, the blood vessel of the stria and spiral limbus appear normal. There is no PAS positive precipitate.

On Slide #45 on the left hand side, the stapedial artery is normal in thickness. The vessels in the middle turn of the cochlea in the stria vascularis and spiral limbus are all normal as are the vessels feeding the cochlear nerve.

On Slide #50, on the left hand side of the cristae of the posterior semi-circular canal is well seen. The blood vessels all appear normal in thickness and number. There is quite a bit of the PAS positive precipitate along the membrane walls in the posterior semi-circular canal.

On Slide #55, on the right hand side in the middle turn of the cochlea, all vessels in the stria and spiral limbus look normal. All vessels feeding the cochlear nerve appear normal in thickness.

In the perilymph of the saccule on the right side, and also in the basal turn of the cochlea there is some protein-like non-cellular precipitate filling 1/2 of the space. The stapedial artery is visible although squashed, probably due to histological technique. The stapedial artery vessel walls are of normal thickness. On the left hand side, there is more of the acellular protein-like precipitate, especially in the perilymph and the saccule. There is some in the middle and basal turn of the cochlea on this slide also.

In Slide #65 on the left hand side, in the apical turn of the cochlea all blood vessels in the stria and spiral limbus appear normal.

On Slide #80, on the cristae of the posterior semi-circular canal, all blood vessels appear normal.

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