## Abstract

Dean E. Davis. Morphometric Analysis of Myelinated Nerve Fibers in the Sympathetic Trunk of the Severely (Ketonuric) Diabetic Chinese Hamster. (Under the direction of Dr. Arthur R. Diani, Department of Anatomy) Department of Biology, East Carolina University, July 1980.

Myelinated nerve fibers from the abdominal sympathetic trunk of seven ketonuric diabetic Chinese hamsters and age-matched control nondiabetics were quantitatively analyzed to determine frequency distribution and density. Animals were perfused with 2.5% glutaraldehyde in 0.075 M cacodylate-HCl buffer and each sympathetic trunk was resected and embedded in Durcupan. Transverse 1$\mu$ sections were serially cut and randomly selected to eliminate observer bias. Fascicles of sympathetic trunks were photographed and combined into photomontages (875X). A plastic grid with sixteen uniform sectors was placed over the geometric center of each fascicle. External diameter of all myelinated axons within two randomly selected sectors was measured. Densities were determined from the total number of fibers and the area of the selected sectors.

Myelinated fiber distributions of diabetics were significantly different from those of matched controls. Alterations of myelinated fiber distributions appeared to be related to length of ketonuria. The mean myelinated fiber density of diabetic animals was significantly less than that of controls.

These data seemed to agree with previous qualitative studies which showed that demyelination and axonal degeneration were the principal pathologies in the sympathetic trunk of the diabetic Chinese hamster. The findings of this study also provide further structural evidence of diabetic autonomic neuropathology in this animal model.

# MORPHOMETRIC ANALYSIS OF MYELINATED NERVE FIBERS IN THE SYMPATHETIC TRUNK OF THE SEVERELY (KETONURIC) DIABETIC CHINESE HAMSTER

A Thesis

Presented to

the Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

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July 1980

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ACKNOWLEDGMENTS

I want to thank Dr. Arthur Diani not only for his help and guidance but also for his friendship during this thesis. I gratefully appreciate the review and recommendations made by my thesis committee (Dr. Charles Bland, Dr. Gerhard Kalmus and Dr. Everette Simpson). I want to express my gratitude to the faculty of the Department of Anatomy and especially Dr. Max Poole for their help in the computer analysis performed in this thesis. Finally, I want to thank my wife, Lynda, for her support and help.

TABLE OF CONTENTS

LIST OF TABLES

# LIST OF GRAPHS

LIST OF FIGURES

# INTRODUCTION

Previous studies of gastrointestinal innervation in the ketonuric diabetic Chinese hamster, Cricetulus griseus, have suggested that bowel dysfunction may be a manifestation of autonomic neuropathy (Diani et al., 1976; Diani et al., 1978; Diani et al., 1979). In a recent investigation, pronounced structural aberrations were observed in the Schwann cells and myelinated fibers of the sympathetic trunk of the severely diabetic Chinese hamster (Diani et al., 1978). Unfortunately, quantification of these pathologies was not carried out to further clarify the significance and complications of autonomic neuropathology in this animal model.

Therefore, this thesis is an initial attempt to morphometrically analyze the myelinated fibers in the sympathetic trunk of the ketonuric diabetic Chinese hamster. This study is designed to answer the following specific questions concerning autonomic neuropathology in the sympathetic trunk of the diabetic Chinese hamster:

1) What is the distribution (caliber spectra) of myelinated fibers?

2) What is the numerical density of myelinated axons?

3) Does a correlation exist between myelinated fiber distribution and age or duration of ketonuria?

# REVIEW OF LITERATURE

## Introduction

Diabetes mellitus has been recognized in man for at least 4000 years (Renold and Dulin, 1967), but due to the complexity of the disease, its exact cause and treatment remain uncertain. Recent statistics have indicated that diabetes mellitus has been diagnosed in 5% of the American population (Maugh, 1976). Diabetes and its accompanying pathologies are associated with 300,000 mortalities per year.

As described by Aretaeus of Cappaducia in the second century A.D., "Diabetes is a strange disease that consists of the flesh and bones running together into the urine" (Notkins, 1979). Today, diabetes may be broadly defined as a disease which affects carbohydrate, lipid, and protein metabolism. It has been suggested that diabetes is not a single disease but a heterogeneous group of diseases. Clinical findings have distinguished two major types of diabetes. The "juvenile onset" diabetes is characterized by a deficiency in insulin whereas "maturity onset" diabetes displays a reduction in insulin utilization (Notkins, 1979). Although there are a number of intermediate forms of diabetes between the two major types, there are some characteristics common to all forms. These traits are excess urine glucose (glycosuria), water and electrolyte loss, and elevated blood sugar (hyperglycemia). Insulin deficiency in juvenile diabetes enhances fat catabolism which results in

the production of ketone bodies. The ketone bodies lower the blood pH to produce diabetic ketoacidosis. Ketones also spill over into the urine (ketonuria). Diabetes mellitus is theorized to be primarily a genetic disorder (Maugh, 1975), but various environmental components such as diet and viruses may be involved in its etiology (Maugh, 1975; Notkins, 1979).

Diabetic man displays pathologies in virtually every organ system which has undergone serious study. These systems include the retina (Bloodworth, 1967), pancreas (Seltzer and Harris, 1964), testes (Schoffling et al., 1963), skin (Friedman et al., 1967), kidney (Kimmelstiel et al., 1962), peripheral nerve (Swallow, 1966), brain (Reske-Nielsen and Lundbaek, 1964), and aorta (Robertson and Strong, 1968). Investigation of these pathologies as well as the study of associated biochemical complications has been difficult and the data are fragmentary or controversial due to several factors:

1) Most studies lacked appropriate matched controls for diabetic patients.

2) The data have been generated from poorly fixed (block immersion) autopsy or biopsy specimens which result in fixation or preparation artifacts.

3) There have been few morphometric studies.

4) It has been difficult to control environmental factors such as diet.

In an attempt to circumvent these problems, various animal models have been developed. These animals are considered to

be appropriate models for the human disease due to the following factors:

1) Genetic control can be achieved through selective inbreeding.

2) The generation time is relatively short.

3) Terminal studies can be performed at any time during the life cycle.

4) Vascular perfusion fixation is possible.

5) Control of environmental factors is attainable.

Currently, two basic categories of animal models are utilized for the study of diabetes mellitus. "Induced-diabetics" display insulin deficiency due to selective destruction of beta cells by various agents (Lazarow, 1954). Two chemicals which induce the diabetic condition are alloxan and streptozotocin. Some studies have used hormones to cause "induced-diabetes" (Like et al., 1974). These induced models are appropriate for studies concerned with beta cell destruction and/or the effects of insulin deprivation. "Spontaneous diabetic" animals inherit the diabetic syndrome in a fashion similar to that of man. Although these animals may display some species dependent characteristics, most exhibit hyperinsulinemia followed by progressively severe hyperglycemia. The high serum insulin phase is unique to spontaneously diabetic animals since it cannot be reproduced in induced diabetics. The diabetic mutant mouse (C57 BL/Ks(db/db)) and the diabetic Chinese hamster are two such spontaneously diabetic animals.

The diabetic Chinese hamster has been shown to be an adequate model for human diabetes (Gerritsen and Dulin, 1967). Within the Chinese hamster colony, several phenotypes have been studied (Sirek and Sirek, 1967). A nondiabetic phenotype has not shown a positive test for urine glucose and never displayed diabetic symptoms for at least two or three generations. Hamsters of this phenotype have served as controls in most investigations. A nonketonuric diabetic phenotype has tested 4+ by Testape (urine glucose test) for a minimum of two to four tests within two months, but has never been positive for ketones. The clinical symptoms of a nonketonuric diabetic hamster are similar to those displayed by a maturity-onset human diabetic. A ketonuric diabetic phenotype has had consistent 4+ Testape and Ketostix (urine ketone test) results for at least one month. The clinical symptoms of a ketonuric diabetic hamster are similar to those in the human juvenile-onset diabetic.

Extensive investigation with the diabetic Chinese hamster has shown biochemical and physiological changes similar to those in man. Diabetic hamsters have less insulin that nondiabetics. Plasma insulin in severe diabetics fails to increase with glucose load. Fasting plasma insulin in mild nonketonuric diabetic hamsters is within normal limits (Gerritsen and Dulin, 1967). Diaphragm muscle and adipose tissue of diabetic hamsters have displayed normal utilization of glucose in vitro (Gerritsen and Dulin, 1967).

Organ systems of the diabetic hamster have shown numerous pathologies. Beta cell degranulation and/or destruction similar to that of diabetic man has been exhibited (Luse et al., 1967). Marked bladder distention in ketonuric hamsters is similar to the asymptomatic diabetic bladder syndrome of man (Gerritsen et al., 1974). The development of glomerulopathy in diabetic hamsters is suggestive of similar changes in diabetic man (Shirai et al., 1967). Gastric dilation and significantly delayed gastric emptying occurs in diabetic hamsters (Diani et al., 1979). In the testes, reduced thickness of the germinal epithelium and widening of the lumina of the seminiferous tubules have been displayed (Sirek and Sirek, 1967). In a study of the thoracic aorta, the internal elastic lamina was fragmented and calcium deposits were found in the media. Proliferation of smooth muscle was also observed (McCombs et al., 1974). Vascular lesions have been displayed in the retina and brain of diabetic Chinese hamsters (Soret et al., 1974; Luse et al., 1970; Federman and Gerritsen, 1970). These lesions included arteriolar and capillary aneurysms (Federman and Gerritsen, 1970) as well as thickening and reduplication of basement membranes (Luse et al., 1970). Study of the retina also revealed accumulation of glycogen in the outer nuclear layer (Soret et al., 1974). The mucopolysaccharide content of diabetic Chinese hamster skin was shown to be altered in comparison to control hamsters (Sirek and Sirek, 1967).

The central and peripheral nervous systems of the diabetic hamster also have shown various abnormalities. In brain neuron processes, these aberrations consisted of megamitochondria in dendrites, dense fibrils in the axoplasm, and degenerate axons, dendrites and myelin (Luse et al., 1970). Segmented demyelination patterns were demonstrated in internode length/fiber size relationships in the distal peripheral tibial nerve of diabetic animals (Schlaepfer et al., 1974). Wallerian degeneration was displayed in axons of teased fiber preparations of the distal peripheral tibial nerve. In the study of the tibial nerve, a correlation was shown between severity of most structural alterations and length of diabetes.

## Diabetic Autonomic Neuropathy

The structural and/or functional impairment of the sympathetic and/or parasympathetic nervous systems has been observed frequently as an early consequence of diabetes (Martin, 1953; Rundles, 1945). In contrast to these studies, a more recent investigation has suggested that diabetic autonomic neuropathy may be related to long duration and severity of diabetes (Aagenaes, 1962). However, the paucity of investigations dealing with this disorder has left it the least understood and most controversial of the diabetic neuropathies.

In diabetic man, several organ systems have displayed autonomic neuropathology. Autonomic neuropathy has been implicated as a primary factor which leads to motor dysfunction in

the small intestine (Hensley and Soergel, 1968). A study of the prevertebral and paravertebral ganglia of diabetic patients showed the presence of giant sympathetic neurons in the paravertebral sympathetic chain ganglia and dendritic swelling of postganglionic neurons (Appenzeller and Richardson, 1966). The latter aberration was proposed as a major factor in the pathogenesis of diabetic diarrhea. A histological study of the intestinal tract detected some abnormalities in the autonomic ganglion cells but these were presumed to be an effect of post-mortem autolysis and/or fixation artifact (Berge et al., 1956). It has been suggested that gastric retention and atony are manifestations of neuropathic involvement of the gastrointestinal tract in diabetic diarrhea (Katz and Spiro, 1966).

Alterations in the autonomic innervation of the urinary bladder in diabetic man have been detected by histochemical and histological techniques (Faerman et al., 1973). The abnormalities included hyperargentophilia, beaded and vacuolated thickenings, and fragmentation of fibers. Some abnormally large chromatolytic and neurolytic neurons were present. Granules positive for Luxol fast blue and Periodic Acid Schiff were present in sympathetic prevertebral ganglion neurons.

Impairment of the vagus nerve has been suggested as the principle factor in the development of diabetic dysphagia (Forgacs et al., 1979). In human diabetics, vagus nerves displayed disrupted myelin sheaths and crowding of Schwann cell nuclei. The myelin sheaths were sequentially degenerated and

many axons were lost and/or broken up into granular fragments (Kristenson et al., 1971).

In a study of chronic alcoholics and diabetics (Appenzeller and Ogin, 1974), it was suggested that degeneration and regeneration, with axonal sprouting, occurred in myelinated fibers of the paravertebral sympathetic chain. Internodes in white rami communicantes of patients were about half the length of internodes on similar caliber fibers of controls. The uniformity of short internodes was attributed to complete degeneration followed by partial regeneration.

Sural nerve biopsies of diabetic patients with signs of sensory-autonomic-motor polyneuropathy were shown to be morphologically abnormal (Martin, 1953). Myelinated fiber populations and densities were reduced.

Respiratory sinus arrhythmia (the beat-to-beat variation) is generally reduced in diabetics (Hilsted and Jensen, 1979). This impairment of sinus arrhythmia is considered to be an indicator of cardiac vagal neuropathy.

Aside from the previously mentioned studies, autonomic neuropathology of diabetic man has been implicated as the major etiologic factor underlying many diabetic abnormalities. These abnormalities include amyotrophy (Garland and Taverner, 1953) and diabetic impotence (Ellenberg, 1971). The role of neuropathy in retrograde ejaculation has been established (Ellenberg and Weber, 1966).

Since most of these clinical studies lacked appropriate matched controls for diabetic patients, the results are inconclusive, controversial and fragmentary. The lack of morphometric analysis and appropriate statistical evaluation in human diabetics has not allowed systematic study of autonomic neuropathy. This type of approach has impeded the accumulation of knowledge concerning diabetic autonomic neuropathy and has left this subject the most controversial and least understood of all pathologies.

In a study with the spontaneously diabetic mutant mouse, sural and peroneal nerves displayed mitochondrial abnormalities and axonal swelling in unmyelinated fibers. Myelinated fibers exhibited various abnormalities. Clusters of membranous profiles were found in the axons as well as sequestered parts of the axoplasm by the inner lamina of the Schwann cell cytoplasm. It was suggested that these changes are identical with those of toxic dying back polyneuropathies (Sima and Robertson, 1979). Morphometric analysis of several nerves of the diabetic mutant mouse detected changes in both myelinated and unmyelinated fiber densities and distributions (Robertson and Sima, 1980). These alterations included an increase in myelinated fiber density in diabetics and a shift of larger myelinated axons in diabetics toward thinner diameters.

Pelvic visceral nerves of diabetic and normal Chinese hamsters were examined with histochemical and electron microscopic techniques to determine whether bladder dysfunction and

hydronephrosis were associated with autonomic neuropathy (Dail et al., 1977). Acetylcholinesterase activity was reduced in diabetic nerves. Increased microtubules in axons and circular profiles of Schwann cells were suggestive of axonal degeneration. Many myelinated fibers displayed aberrant myelination characterized by wide periaxonal spaces with unusual processes of Schwann cells.

Recent studies of the small bowel in the ketonuric diabetic Chinese hamster have revealed a number of changes in the innervation (Diani et al., 1979). Among the alterations were a diminution in the number of Auerbach's plexuses along with a reduction in cholinesterase activity. Qualitative ultrastructural analysis of the nerve fibers of Auerbach's plexuses revealed glycogen deposition, aggregation of neurofilaments, dense accumulation of lamellar inclusions and swelling of some axons and their terminals.

Quantitative barium x-ray patterns of ketonuric diabetic hamsters displayed marked dilation of the stomach, small and large intestine (Diani et al., 1979). Impaired peristalsis was characterized by considerable flocculation of barium in the small and large bowel, prolonged emptying of the stomach and delayed stool formation and passage. It was suggested that bacterial overgrowth in the small and large intestines was a result of impaired peristalsis. These radiologic and ultrastructural findings suggested that autonomic neuropathy was a significant factor underlying abnormal gastrointestinal motility.

A qualitative study of the abdominal sympathetic trunk of diabetic hamsters detected pronounced structural aberrations in the Schwann cells and associated myelinated fibers (Diani et al., 1978). Major structural abnormalities were Pi granules of Reich and lamellar inclusions in Schwann cells. Since the Pi granules have been classified as lysosomes due to acid phosphatase activity, it was suggested that these granules may represent an early degradative condition in the Schwann cell. Other major pathologies in nerve fibers included unraveling, disorganization, vesiculation and retraction of certain myelin sheaths. These previous studies have supported the contention that the diabetic Chinese hamster may be an acceptable model for diabetic autonomic neuropathy.

Until recently, quantitative analysis has not been attempted on the nervous system of the diabetic Chinese hamster. It is anticipated that such a study of perfusion-fixed, control-matched tissues will provide an extension of previous qualitative studies of the sympathetic trunk. It also should provide guidelines for future morphometric and physiological studies in the Chinese hamster. The baseline data of this investigation should provide critical guidelines for future nerve conduction velocity and insulin therapy studies. For example, these findings may suggest time coordinates for appropriate administration of insulin prior to manifestation of structural neuropathology. It will then be possible to determine if insulin

can prevent or retard diabetic autonomic neuropathy. This study should also provide a better understanding of diabetic autonomic neuropathy.

# MATERIALS AND METHODS

This study involved seven pairs of Chinese hamsters, which were obtained from the Upjohn Company (Kalamazoo, Michigan) Chinese hamster colony. Each pair consisted of a ketonuric diabetic (experimental) and a nondiabetic (control) matched for age, sex, and approximate body weight (Table 1). The controls had a weight range of 26.4 to 33.2 grams, whereas the diabetics ranged from 26.4 to 40.0 grams. Diabetics varied from five to eighteen months of age and had durations of ketonuria from three to ten months. Control hamsters were five to eighteen months old. All animals were maintained on Purina Mouse Breeder Chow and water ad libitum until sacrifice. The animals were weighed and tested for ketonuria (Ketostix) and glycosuria (Diastix) biweekly. Insulin therapy was not administered to any of the animals.

Prior to sacrifice, tests for ketonuria and glycosuria were performed (Table 2). At this time, 50 microliters of blood were removed from the orbital sinus with a microcapillary tube and blood glucose was analyzed with a Technicon Auto-Analyzer. All animals were anesthetized with .02 cc Nembutal (60 mg/ml). The thoracic and abdominal cavities were exposed with a mid-ventral incision. The hamster tissues were exsanguinated via the left ventricle with Earle's basic salt solution (Appendix A) and then perfused with 175 ml of cold 2.5% glutaraldehyde in 0.075 M cacodylate-HCl buffer. After perfusion, both

sympathetic trunks from the diaphragm to the pelvic brim were resected. The sympathetic trunks were then placed on a cold petri dish and quickly cut into 1.5-2.0 mm segments with a single edge razor blade. All segments were immersed in cold 2.5% glutaraldehyde in 0.075 M cacodylate-HCl buffer for two hours. The tissues were then left for twelve hours in cold 0.075 M cacodylate-HCl buffer. All tissues were then postfixed in 1% osmium in 0.05 M cacodylate buffer (cold). The tissues were placed in two 0.05 M cold maleic acid-NaOH (pH 5.2) buffer rinses for ten minutes each. En block staining was accomplished by placing the tissues in cold 0.5% uranyl acetate in 0.05 M maleic acid-NaOH (pH 5.2) buffer for one hour. Trunk segments were then rinsed twice in maleic acid buffer as previously described. The tissues were then dehydrated in ethanol in the following manner:

1) 70% ethanol--ten minutes (cold)
2) 95% ethanol--two fifteen minute changes at room temperature
3) 100% ethanol--three twenty minute changes at room temperature

After dehydration, all tissues were placed in two changes of room temperature propylene oxide for ten minutes each. The tissues were embedded in Durcupan (Appendix B) with the following protocol:

1) 1:1 ratio of propylene oxide and Durcupan--two ten minute changes at room temperature

2) 1:1 ratio of propylene oxide and Durcupan--twelve hours at room temperature

3) Durcupan in a Beem capsule

Each Beem capsule was then incubated for twenty-four hours at 60° C in a vacuum oven.

After incubation and cooling, the tissue blocks were removed from the Beem capsules. Each block was then assigned a code to eliminate observer bias during experimental procedure. The total number of blocks per animal varied from eight to ten. All blocks from one animal were collected and one block was randomly selected for microtomy. The selected block was then trimmed with a single edge razor to remove excess Durcupan. All sections were cut with glass knives on a Dupont-Sorvall MT2-B ultramicrotome. One micron transverse sections were cut from each block and every fortieth section was placed on a glass slide. The slides were appropriately coded and divided into ten sectors. Each tissue section was situated within a single sector on the slide. The entire length of each block was sectioned. All sections were stained with Toluidine blue (.5% Toluidine blue in 1% sodium borate) for forty-five seconds at 50° C to clearly delineate myelin sheaths.

The total number of sections per block usually ranged from forty to fifty. In order to assure unbiased sampling and proper representation of the entire tissue block, the forty to fifty sections were divided into four equal size groups and one

section from each was randomly selected to be photographed. Photographs were taken with a light microscope at a magnification of 125X on S0115 35mm film (Eastman Kodak). Prints were processed on Kodabromide (8 inch by 10 inch) F-5 paper at a final magnification of 875X. The prints of an entire fascicle from each tissue section were combined into a photomontage for measurement of myelinated axon diameters and numbers.

Fascicles were sampled with a 1/8 inch clear plexiglass grid divided into sixteen equal sectors. Each sector was subtended by an angle of $22.5^{o}$ (Fig. 1). The center point of the grid was placed over the geometric center of each fascicle (Fig. 2). One of sixteen sectors was randomly selected and all fibers within that sector and its opposite sector were counted. The measurement of all axons within two sectors assured that the sample size was at least ten percent of the total in each fascicle. It was difficult to discern whether some fibers on the borders of a sector were actually within that sector. To circumvent this problem, it was decided that all fibers in contact with the lead line (Fig. 1) of a sector would be counted and all fibers in contact with the trail line (Fig. 1) of a sector would be excluded.

Individual fiber diameters were measured with a 7X Lupe. Measurements were made from the outer edge of the myelin sheath to the opposite outer edge. With respect to irregularly shaped fibers, long and short axis measurements were made and the

average of these measurements was recorded. A Burroughs 6800 computer was used to collocate the measurements of each sector into individual data files. To determine the fiber distribution of a sympathetic trunk, all four data files for that trunk were run through a sort program. This program calculated the relative percentage of fibers at one micron intervals.

To obtain numerical fiber density (number of myelinated fibers per unit area), the total number of fibers per sector and the area of the sector were recorded. The outer border of each sector was formed by the perineurium of each fascicle. Since the two sides of the sector and the perineurium formed a triangle, the area was obtained by application of the basic geometric equation for area of a triangle (area = ½ base X height). Density was determined by dividing the number of fibers in a sector by the area of the sector. The densities of eight sectors per animal were averaged to establish its numerical density.

The Kolmogorov Smirnov test (Siegel, 1958) was employed to determine differences in myelinated fiber distributions for each pair of animals. The Wilcoxon test for paired means (Siegel, 1958) was used to analyze the grand mean density of controls versus diabetics. The Spearman Rank Correlation test (Siegel, 1958) was utilized to determine correlations among the following:

1) Ketonuria and fiber distribution

2) Ketonuria and fiber density

3) Age and fiber distribution (control)

4) Age and fiber distribution (diabetic)

5) Age and fiber density (control)

6) Age and fiber density (diabetic)

TABLE 1

ANIMAL CHARACTERISTICS AT SACRIFICE

| DIABETIC | | | | | CONTROL | | | |
|---|---|---|---|---|---|---|---|---|
| Subline | Sex | Age (Months) | Weight (Grams) | Duration of Ketonuria (Months) | Subline | Sex | Age (Months) | Weight (Grams) |
| AC18-61 | F | 12 | 26.4 | 10 | M00-836 | F | 12 | 26.4 |
| AC18-28 | F | 18 | 26.6 | 9 | M00-796 | F | 18 | 26.6 |
| X21-22 | M | 14 | 40.0 | 4 | M20-45 | M | 15 | 28.2 |
| AH18-26 | M | 9 | 38.0 | 6 | M00-851 | M | 9 | 32.7 |
| BD03-76 | M | 5 | 35.2 | 3 | AV14-33 | M | 5 | 31.5 |
| ZM09-74 | M | 12 | 36.6 | 7 | AV13-23 | M | 11 | 31.5 |
| ZM09-37 | M | 16 | 36.2 | 4 | AV11-37 | M | 18 | 33.2 |
| | | RN 5-18 | RN 26.4-40 | | | | RN 5-18 | RN 26.4-33.2 |

Figure 1. Photograph of sampling grid showing sectors to be sampled (C), lead line of each sector (A), and trail line of each sector (B).

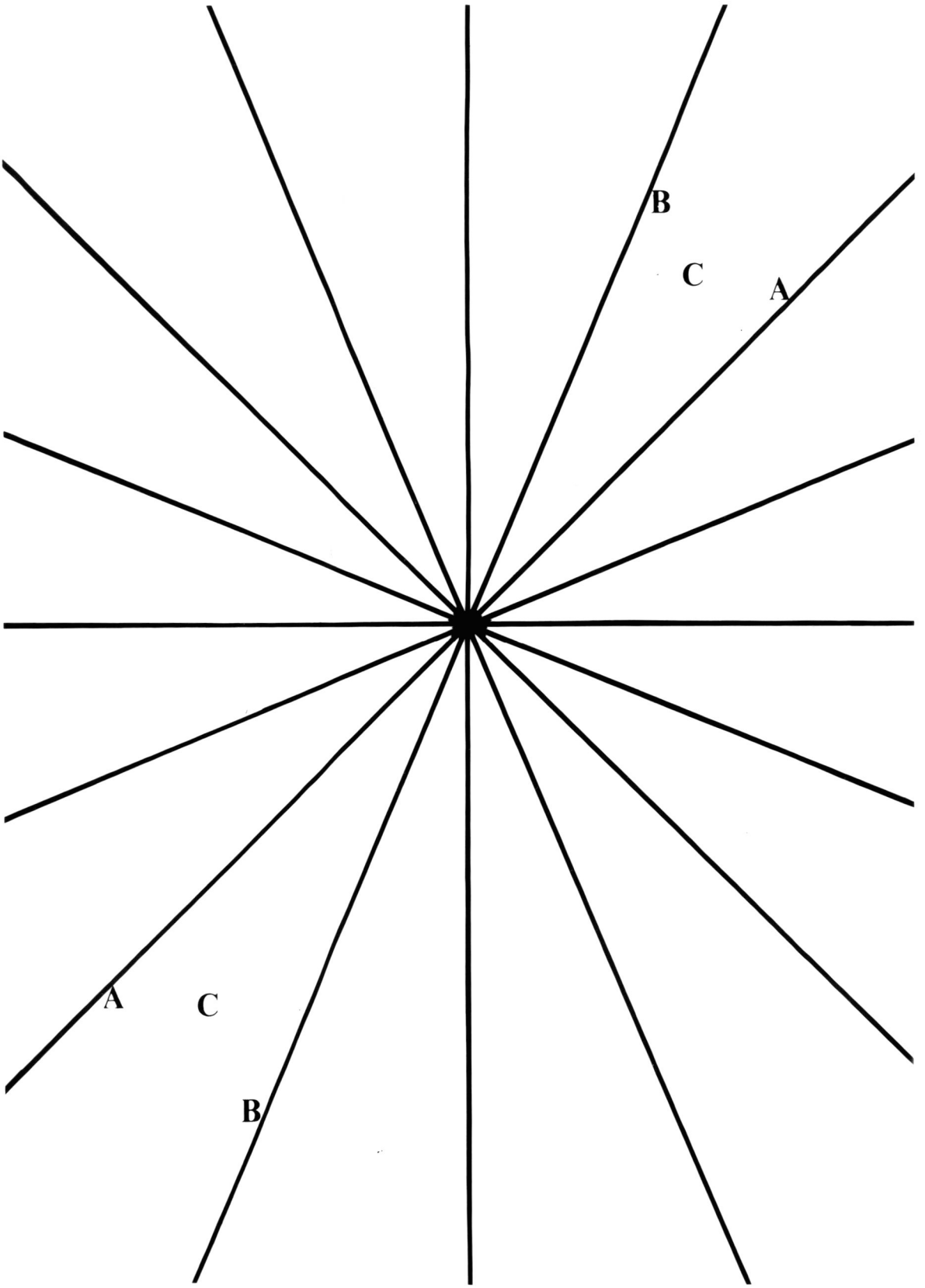
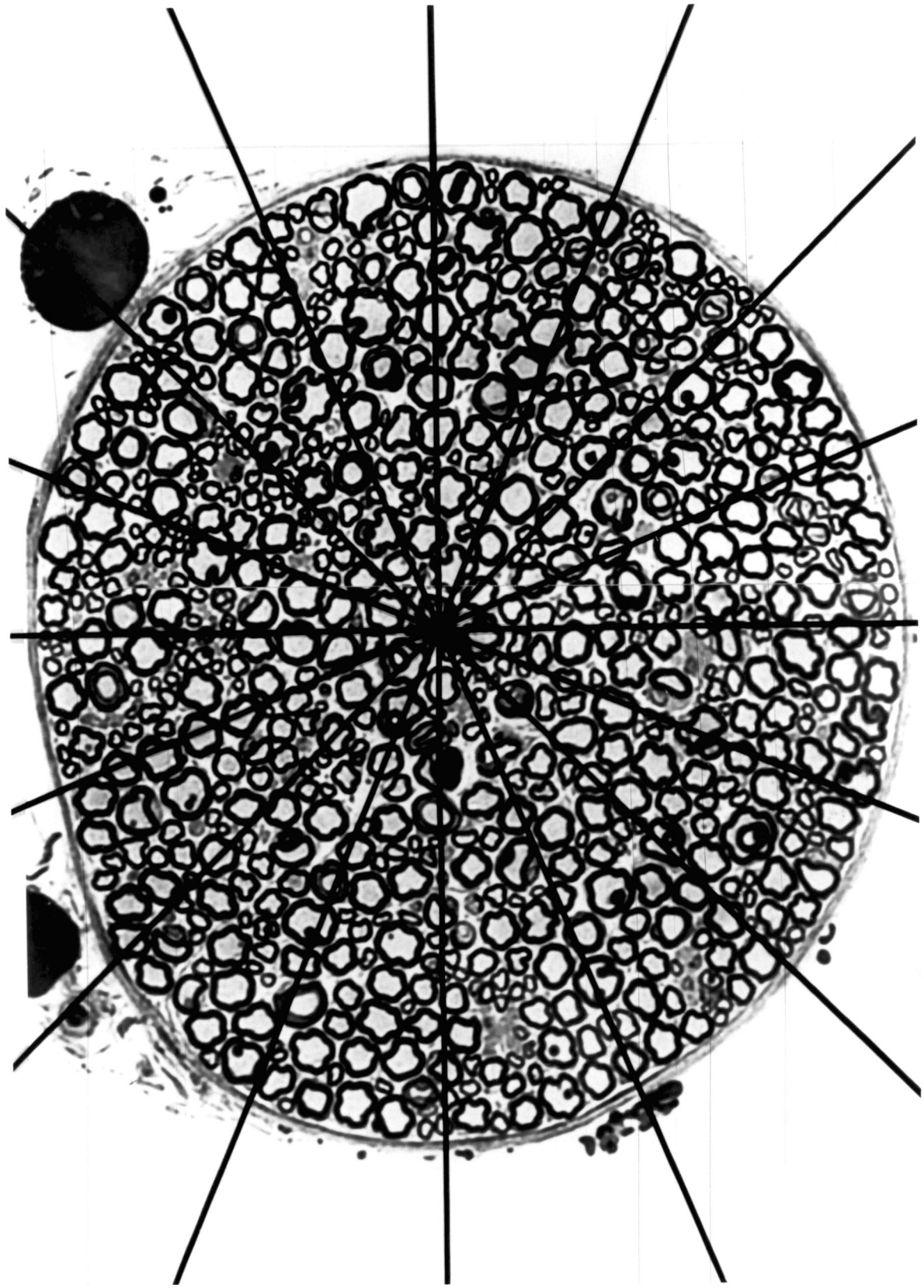

B
C
A
A
C
B

Figure 2. Typical photomontage of a nerve fascicle with sampling grid centered over the fascicle.



# RESULTS

Terminal measurements are found in Table 2. Control animals exhibited negative Diastix and Ketostix results. All diabetic animals displayed 4+ Diastix values and large Ketostix values. Blood glucose measurements ranged from 57 to 136 mg./dl. in controls and 328 to 597 mg./dl. in diabetics.

On the basis of axon diameter, there seemed to be two populations of myelinated fibers in the sympathetic trunk of control hamsters. The large population was found in the 9 to 11 micron interval whereas a small population was located between 3 and 5 microns (Graphs 1-7). Myelinated fiber distributions in diabetic hamsters also displayed two populations, but the large population tended to shift to the left and fall in the 7 to 9 micron interval (Graphs 1-7). In all but one pair of animals, the diabetics exhibited a significant difference in fiber distribution when compared to matched controls (Table 3). It should be pointed out that the one pair, which did not show an alteration in fiber distribution, consisted of relatively young animals (5 months) and a short-term ketonuric (3 months).

In general, the mean numerical myelinated fiber density of each control was greater than that of the matched diabetic (Table 4). Statistical analysis of the grand means showed that the numerical fiber density of diabetics was significantly less than that of controls (Table 4).

When duration of ketonuria was analyzed against the shift in myelinated fiber distribution of diabetics, there appeared to be a strong correlation between these two parameters (Table 5). Significance was not found, however, between length of ketonuria and numerical fiber density (Table 5). Significant correlations did not exist between age and fiber distribution or density in diabetic or control animals.

The morphology of fascicles in short duration ketonuric hamsters appeared unremarkable and was similar to that of matched controls. Diabetics with long term ketonuria showed marked alteration in structure of fascicles. The diabetic fascicles were characterized by fibers with unraveled or distended myelin sheaths.

TABLE 2

MEASUREMENTS AT SACRIFICE

| DIABETIC | | | | CONTROL | | | |
|---|---|---|---|---|---|---|---|
| Subline | Ketostix | Diastix | Blood Sugar (mg/dl) | Subline | Ketostix | Diastix | Blood Sugar (mg/dl) |
| AC18-61 | Large | 4 | NM | M00-836 | - | - | NM |
| AC18-28 | Large | 4 | 328 | M00-796 | - | - | 57 |
| X21-22 | Large | 4 | 597 | M20-45 | - | - | 69 |
| AH18-26 | Large | 4 | 550 | M00-851 | - | - | 46 |
| BD03-76 | Large | 4 | 406 | AV14-33 | - | - | 136 |
| ZM09-74 | Large | 4 | 384 | AV13-23 | - | - | 104 |
| AM09-37 | Large | 4 | 412 | AV11-37 | - | - | 116 |
| | | | RN 328-597 | | | | RN 57-136 |

TABLE 3

MYELINATED AXON DISTRIBUTIONS

| Subline | N(Pair) | Maximum Difference (Percent) | Duration of Ketonuria (Months) | Significance Level |
|---|---|---|---|---|
| BD03-76<br>AV14-33 | 2031 | 5 | 3 | $p > .05$ |
| X21-22<br>M20-45 | 1381 | 8 | 4 | $p < .025$ |
| ZM09-37<br>AV11-37 | 1710 | 15 | 4 | $p < .001$ |
| AH18-26<br>M00-851 | 1182 | 15 | 6 | $p < .001$ |
| ZM09-74<br>AV13-23 | 3700 | 11 | 7 | $p < .001$ |
| AC18-28<br>M00-796 | 545 | 20 | 9 | $p < .001$ |
| AC18-61<br>M00-836 | 1644 | 9 | 10 | $p < .025$ |

TABLE 4

NUMERICAL DENSITY OF MYELINATED AXONS

| Subline | CONTROLS ($N/mm^2$) | Subline | DIABETICS ($N/mm^2$) |
|---|---|---|---|
| M00-851 | 22929 | AH18-26 | 19126 |
| M00-836 | 18612 | AC18-61 | 16412 |
| M00-796 | 31271 | AC18-28 | 20274 |
| AV11-37 | 17211 | ZM09-37 | 16216 |
| M20-45 | 20426 | X21-22 | 20793 |
| AV13-23 | 21372 | ZM09-74 | 19220 |
| AV14-33 | 26274 | BD03-76 | 24550 |
| | MEAN $\pm$ SEM<br>22586 $\pm$ 1826 | | MEAN $\pm$ SEM<br>19513 $\pm$ 1072<br>$p < 0.025$ |

TABLE 5

CORRELATIONS

| PARAMETERS | CORRELATION COEFFICIENT | SIGNIFICANCE LEVEL |
|---|---|---|
| Ketonuria vs. Fiber Distribution | .86 | $p < .05$ |
| Ketonuria vs. Density | .50 | $p > .05$ |
| Age vs. Fiber Distribution (Controls) | .32 | $p > .05$ |
| Age vs. Fiber Distribution (Diabetics) | .18 | $p > .05$ |
| Age vs. Density (Controls) | .10 | $p > .05$ |
| Age vs. Density (Diabetics) | .10 | $p > .05$ |

# DISCUSSION

Morphometric analysis of myelinated axons in the sympathetic trunk displayed a significant difference between fiber distributions of control and diabetic Chinese hamsters. In diabetics, the large fiber population had a reduced size (8-9$\mu$) compared to its counterpart (10-11$\mu$) in matched controls. This shift in the large fiber population appeared to be a manifestation of previously observed segmental demyelination and/or axonal degeneration in the sympathetic trunk of the diabetic Chinese hamster (Diani et al., 1978). Demyelination was characterized by unraveling, vesiculation and retraction of myelin sheaths whereas the axoplasm of myelinated fibers also exhibited fibrillar degeneration. In pelvic visceral nerves of diabetic hamsters, increased microtubules in axons and circular profiles of Schwann cells were suggestive of axonal degeneration (Dail et al., 1977). A possible effect of demyelination has also been displayed in the db/db mouse. Quantitative analysis of dorsal root myelinated fibers in the diabetic mutant mouse showed a significant shift of these axons toward thinner diameters (Robertson and Sima, 1980). Demyelination of autonomic nerve fibers has been found in studies of diabetic man. The vagus nerve of human diabetics has shown disrupted and degenerate myelin sheaths (Kristensson et al., 1971). Diabetic patients have also exhibited nonuniform internode lengths in sympathetic chain fibers (Appenzeller and Ogin, 1974).

Unfortunately, data from human diabetics are inconclusive since most of the reports lacked appropriate matched controls for diabetic patients. Furthermore, the data have been generated from poorly fixed autopsy and biopsy specimens. It seems possible that some of the alterations in the autonomic nervous system of diabetic man were fixation or preparation artifacts.

It has been suggested that myelin sheath abnormalities of diabetics develop through alterations in Schwann cell metabolism and concomitant changes in metabolism within the axon or neuron cell body (Bischoff, 1968; Thomas and Lascelles, 1965). Abnormal function of the sorbitol pathway has been implicated as a possible cause of myelin sheath aberrations (Field, 1966; Clements, 1979). This pathway converts glucose to sorbitol which is then converted to fructose (Field, 1966). Due to a lack of fructokinase (Bischoff, 1968), excessive amounts of fructose accumulate and may exert a harmful osmotic effect on the nerve cell or fiber (Field, 1966). Accelerated utilization of the sorbitol pathway has been reported in nerve and lens tissue of the diabetic Chinese hamster (Holcomb et al., 1974). Similar findings have been shown in alloxan and streptozotocin-induced diabetic rats (Varma and Kinoshita, 1972; Stewart et al., 1967). It has been reported that Schwann cell lamellar bodies and lipid aggregates in the sympathetic trunk of the Chinese hamsters may be manifestations of these metabolic alterations (Diani et al., 1978). These lamellar bodies have

also been described in sural, facial, and femoral biopsy specimens of diabetic patients (Bischoff, 1968).

The alteration in myelinated fiber distributions within the sympathetic trunk of diabetic Chinese hamsters was significantly related to length of ketonuria. The relationship of length and/or severity of diabetes to the manifestation of neuropathology was further supported by the nonsignificant shift of fiber distribution in the short duration (3 month) ketonuric hamster and its matched control. In a study of distal tibial nerves in the diabetic Chinese hamster, the severity of lesions was directly correlated with length of diabetes (Schlaepfer et al., 1974). Morphologic abnormalities in the retina, pancreas, and kidney of the diabetic Chinese hamster have shown a similar relationship to duration of diabetes (Soret et al., 1974). It has been suggested that the incidence of pathologies in the small intestine of the diabetic hamster may also be related to severity of the disease (Diani et al., 1979). In man, it has been established that the appearance of diabetic symptoms is more acute in juvenile diabetics than in maturity-onset diabetics.

Analysis of myelinated fiber densities in the sympathetic trunk of Chinese hamsters revealed that the grand mean of diabetics was significantly reduced compared to that of controls. In a study of fiber densities in the greater splanchnic nerves of diabetic patients, a significant reduction in fiber density was also established (Low et al., 1975). In contrast to the above findings, db/db mice were reported to

display an increase in myelinated fiber density of peripheral nerves when compared to that of controls (Robertson and Sima, 1980). Since total fascicular area of diabetic nerves was less than that of controls, it is conceivable that this reduction in area could produce an elevation in myelinated fiber density of db/db mice. Total fascicular areas were not calculated for the sympathetic trunk of Chinese hamsters. It would be of interest to measure fascicular areas and correlate these data with fiber densities of autonomic nerves in the diabetic Chinese hamster.

With a decrease in myelinated fiber density and associated demyelination, impairment of nerve conduction velocity would be a likely consequence in the sympathetic trunk of the diabetic Chinese hamster. In diabetic man, the diabetic mutant mouse, and induced diabetic rats, reduction of nerve conduction velocity (NCV) has been shown to be a result of early metabolic changes in nerve fibers (Ellenberg, 1973; Robertson and Sima, 1980; Eliasson, 1964; Greene et al., 1975). Administration of insulin therapy can reverse this functional disorder in nerve fibers by retarding prolonged periods of hyperglycemia (Clements, 1979). These data also imply that long-standing diabetes is directly related to manifestation of structural neuropathologies. When nerves undergo definitive structural derangement, insulin apparently does not have an ameliorative effect on disrupted NCV. Unfortunately, conduction velocity studies have not yet been attempted in the Chinese hamster to determine if altered

NCV precedes structural complications or vice-versa.

Although a significant correlation was not found between reduced fiber density and length of ketonuria in the sympathetic trunk of the diabetic Chinese hamster, a distinct trend was observed. A similar tendency seemed to exist between altered fiber distribution and age. It is conceivable that these trends may have become statistically significant if larger numbers of animals had been employed.

In this study, diabetic Chinese hamsters displayed a reduction in size of large diameter fibers. On the basis of standard fiber classification, these 8-10$\mu$ diameter fibers would most likely be categorized as visceral afferent fibers (Barr, 1979). Visceral afferents generally function as pressure receptors in the gastrointestinal tract (Kuntz, 1947). These fibers also convey information into the central nervous system concerning the degree of emptiness (fullness) of the stomach and intestines (Kuntz, 1947; Noback and Demarest, 1975). Structural impairment of visceral afferents would presumably have a deleterious effect on autonomic reflexes. Since the function of the motor limb depends upon sensory input, impairment of the sensory limb would presumably cause malfunction of the efferent response. Impaired function of efferent fibers would thereby alter the activity of smooth muscles and sphincters of the gastrointestinal tract. Barium x-ray analysis of the diabetic Chinese hamster has shown delayed peristalsis of the small intestine and prolonged emptying of the stomach (Diani et al.,

1979). Bacterial overgrowth in the small intestine of the diabetic Chinese hamsters has been attributed to delayed peristalsis (Diani et al., 1979). The digestive tract in diabetic man has also shown abnormal x-ray patterns in the esophagus (Forgacs et al., 1979), stomach and intestine (Katz and Spiro, 1966). Although these findings have suggested that there may be a neurogenic etiology for gastrointestinal dysfunction in man, such a relationship has not been documented.

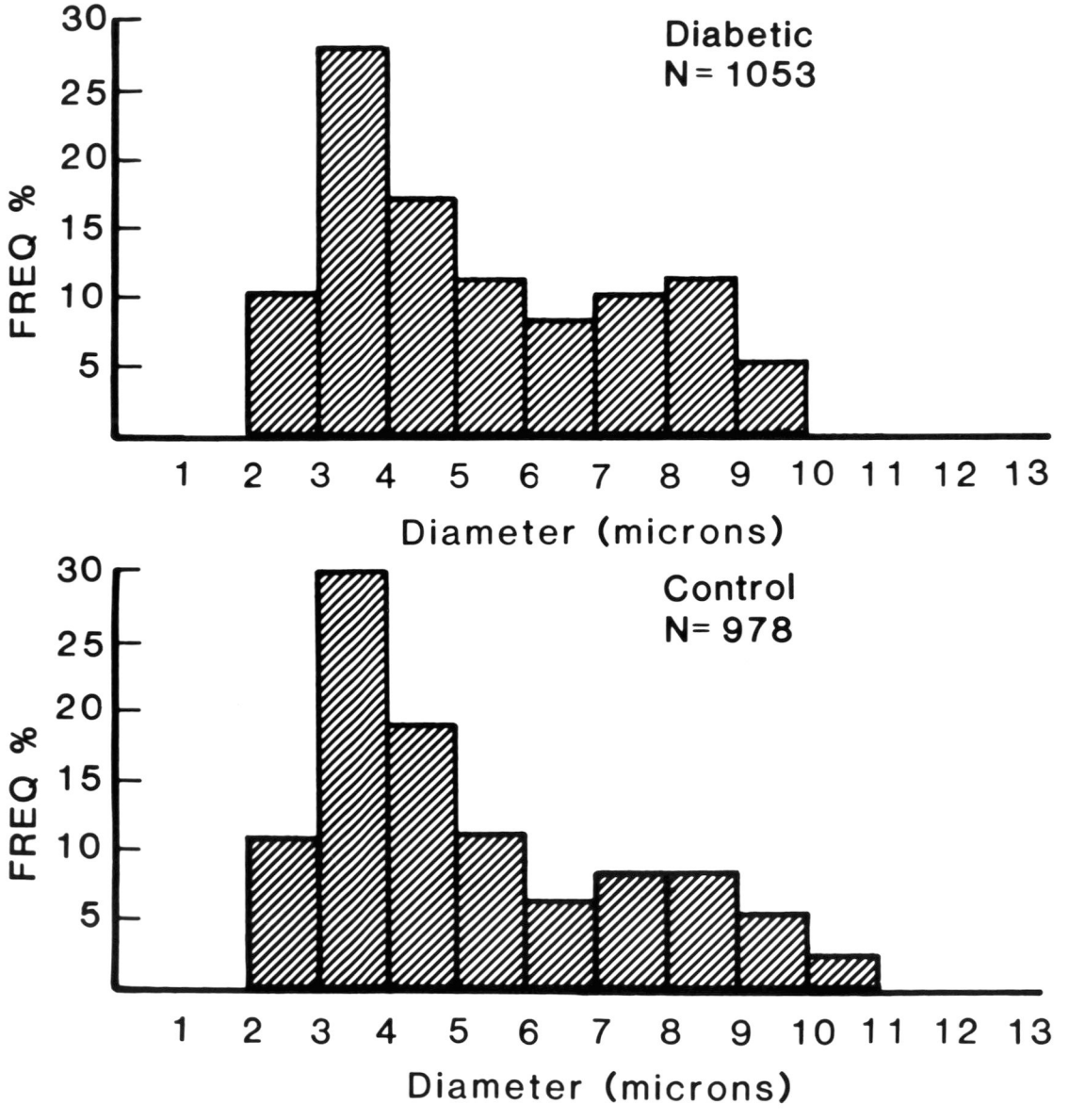
The quantitative data of this study provide further evidence of autonomic neuropathy in the sympathetic trunk of diabetic hamsters. Structural damage to visceral afferent fibers has been observed and quantified. It would be of interest to determine if visceral efferent fibers in the sympathetic trunk are also in a pathologic condition. The morphologic state of the vagus nerve should also be evaluated to determine the relative contribution of parasympathetic and sympathetic systems to autonomic neuropathy in the Chinese hamster. Nerve conduction velocity studies on autonomic nerves should be performed in conjunction with morphologic and morphometric studies to determine if reduced nerve function is primarily a metabolic or morphologic disorder. Insulin therapy should also be attempted to determine whether such treatment retards, reverses, or ameliorates autonomic neuropathy in the diabetic Chinese hamster.

# CONCLUSIONS

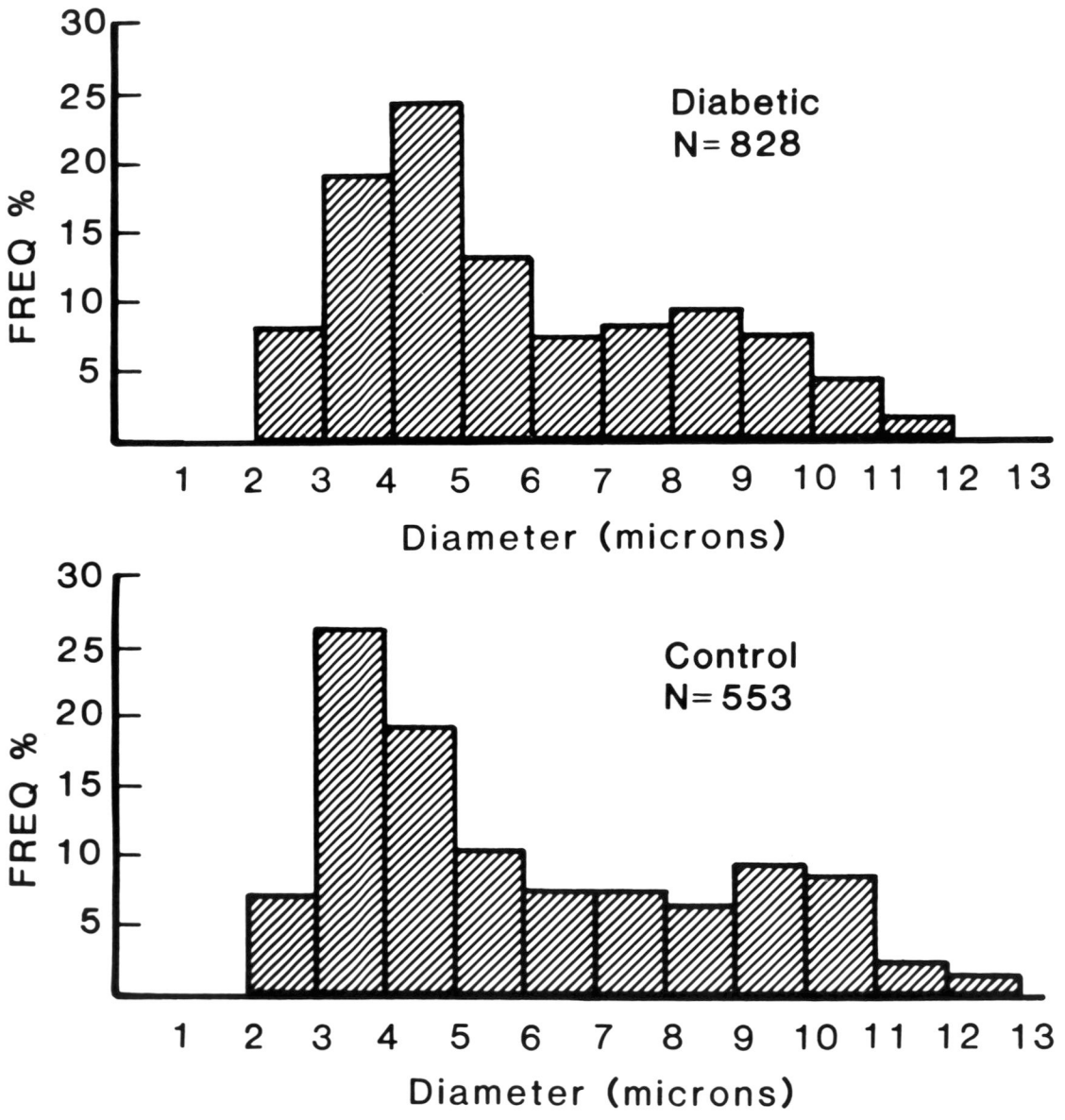
Morphometric analysis of the abdominal sympathetic trunk of the ketonuric diabetic Chinese hamster divulged the following results:

1) Reduction in frequency of large myelinated axons
2) Reduction in numerical density of myelinated axons
3) Correlation between length of ketonuria and reduced frequency of large myelinated axons

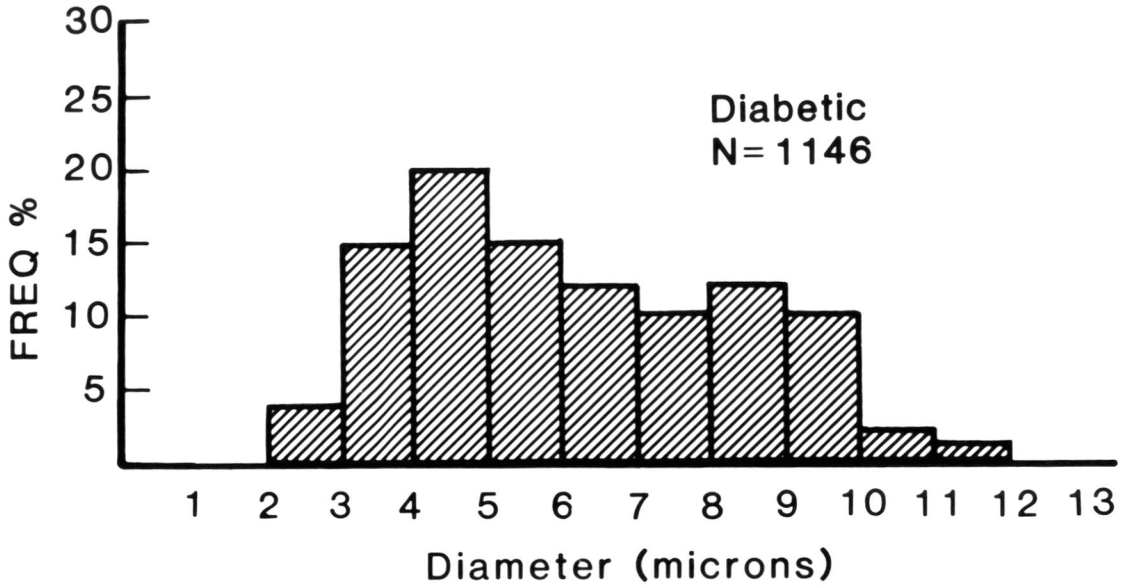
Graph 1. Myelinated fiber distributions of three month ketonuric animal, BD03-76, (top) and matched control, AV14-33, (bottom). ($P > .05$)

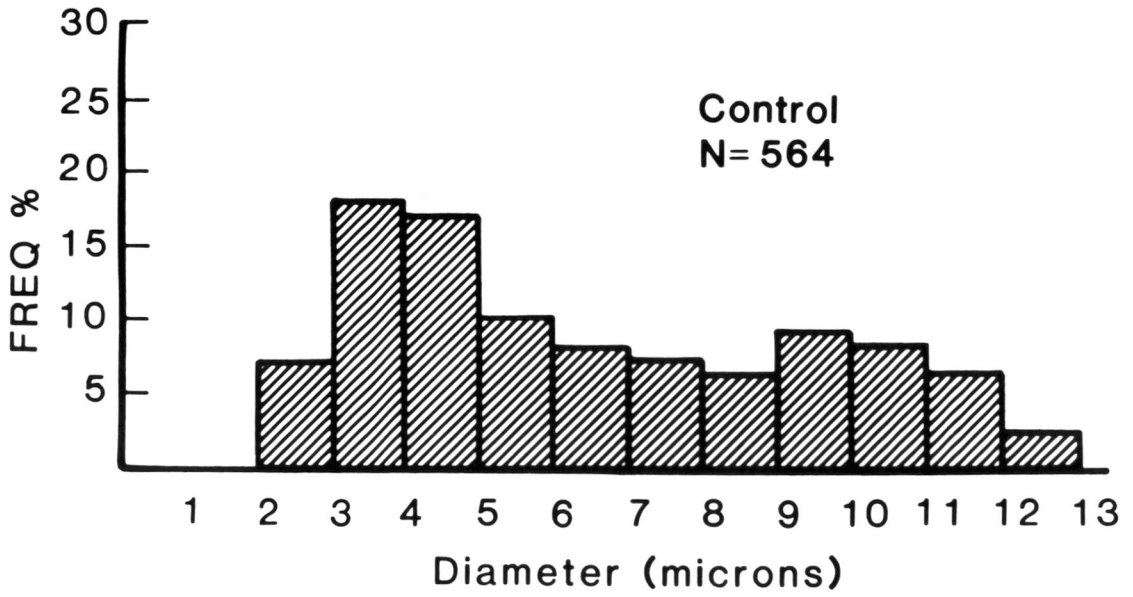

Diabetic
N= 1053
FREQ %
30
25
20
15
10
5
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)
Control
N= 978
FREQ %
30
25
20
15
10
5
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)

Graph 2. Myelinated fiber distributions of four month ketonuric animal, X21-22, (top) and matched control, M20-45, (bottom). ($P < .025$)

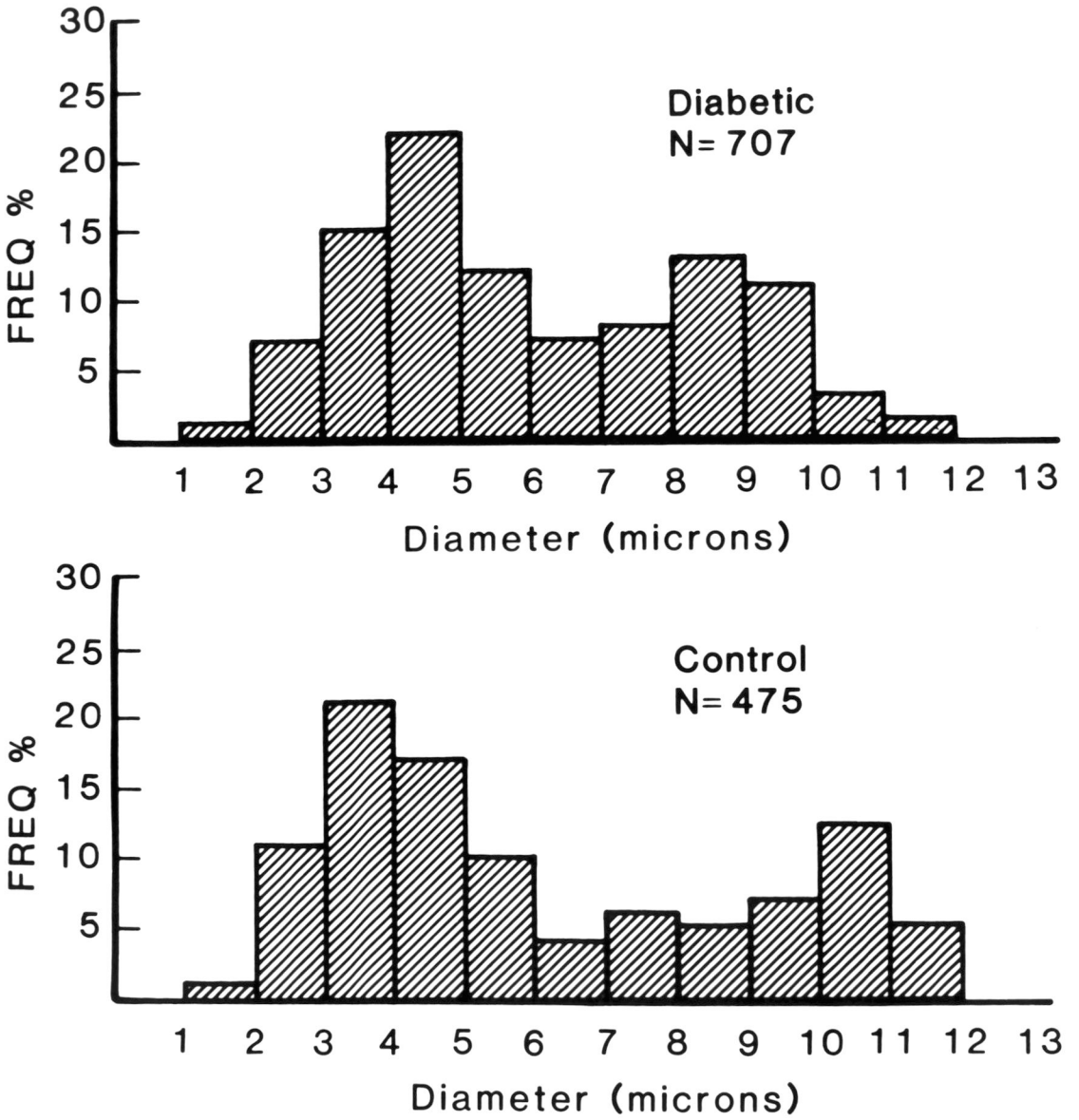

30
25
20
15
10
5
FREQ %
Diabetic
N= 828
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)
30
25
20
15
10
5
FREQ %
Control
N= 553
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)

Graph 3. Myelinated fiber distributions of four month ketonuric animal, ZM09-37, (top) and matched control, AV11-37, (bottom). ($P < .001$)

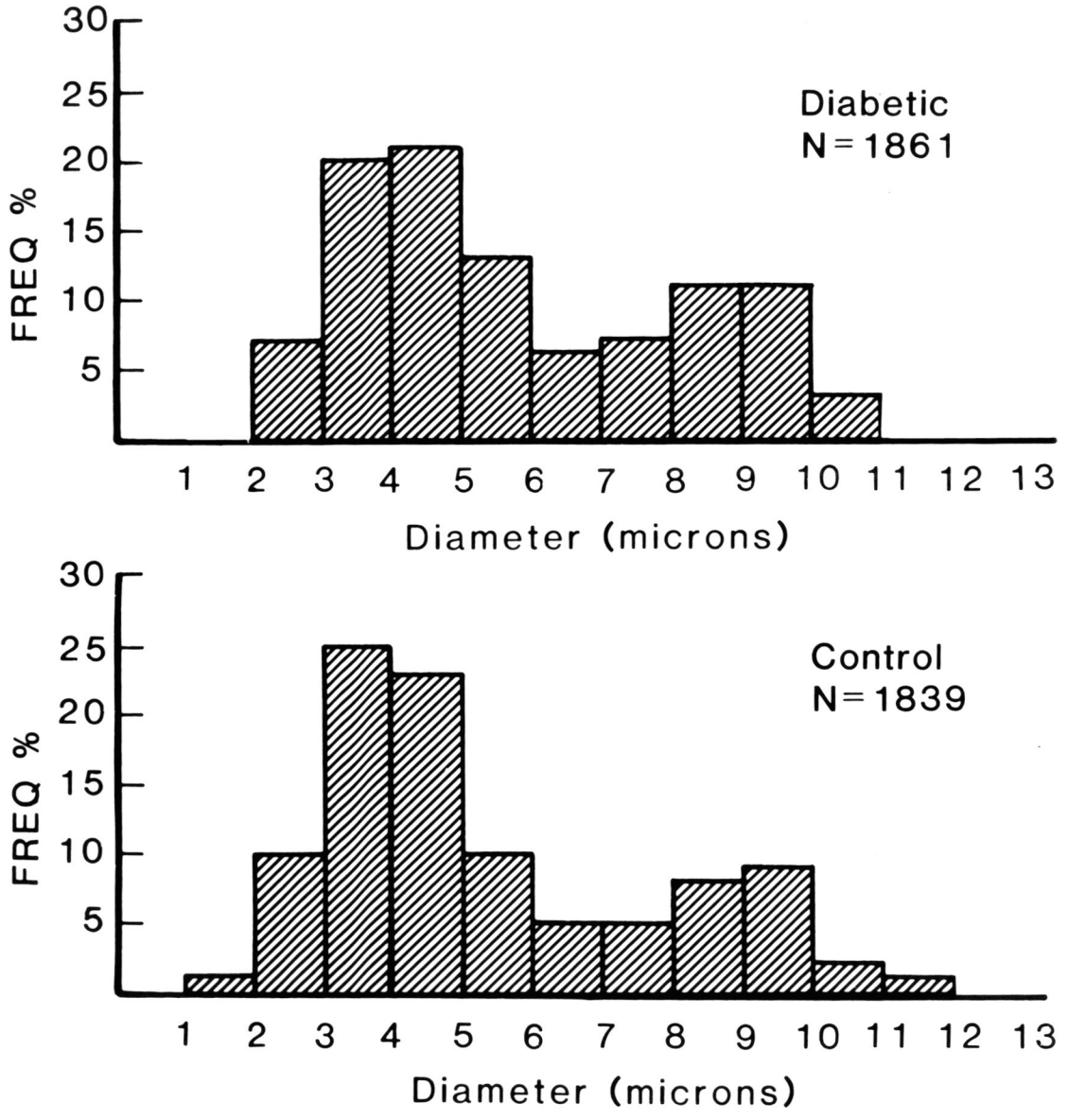

30
25
20
15
10
5
FREQ %
Diabetic
N= 1146
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)


30
25
20
15
10
5
FREQ %
Control
N= 564
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)

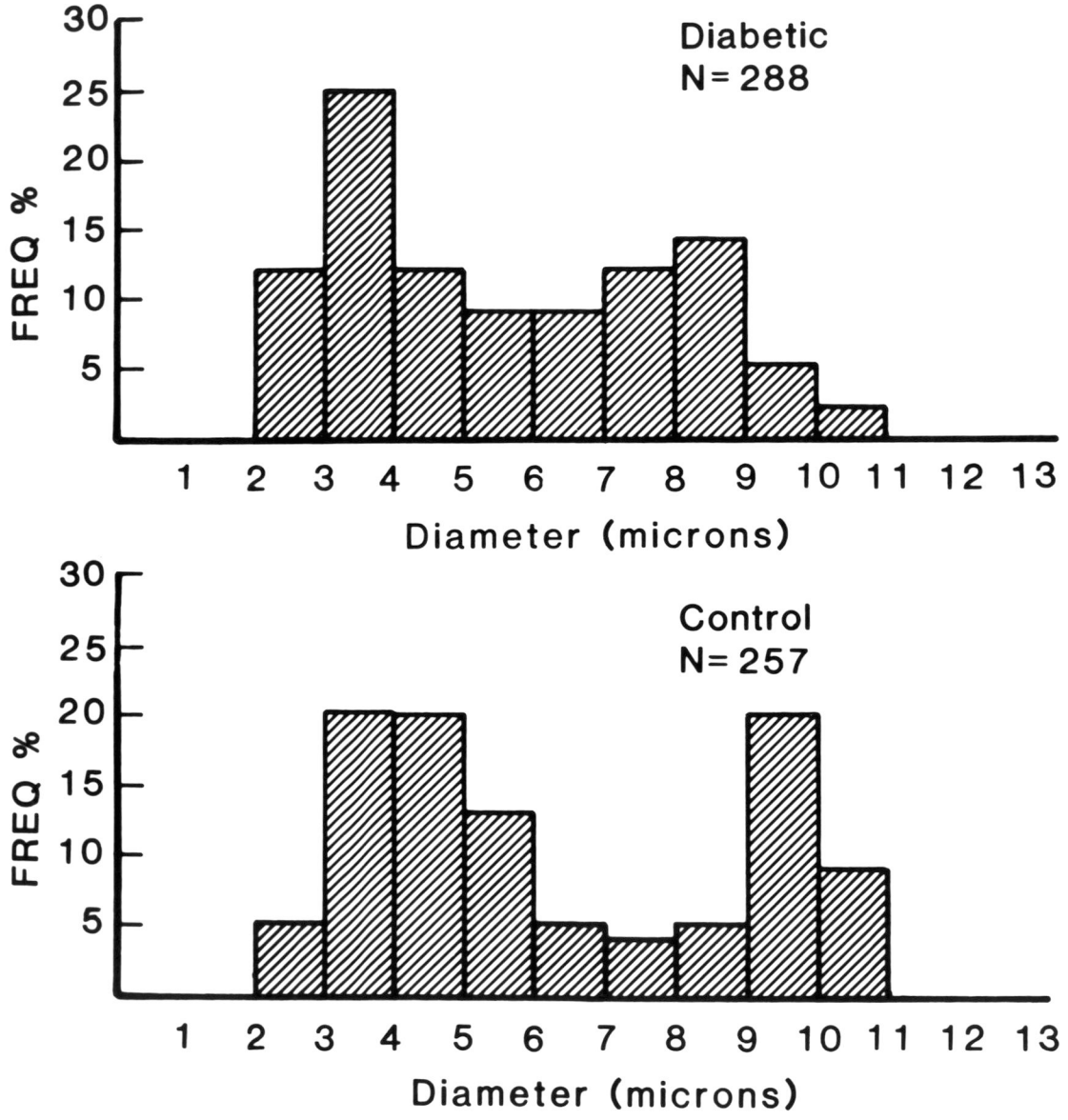
Graph 4. Myelinated fiber distributions of six month ketonuric animal, AH18-26, (top) and matched control, M00-851, (bottom). ($P < .001$)


30
25
20
15
10
5
FREQ %
Diabetic
N= 707
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)
30
25
20
15
10
5
FREQ %
Control
N= 475
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)

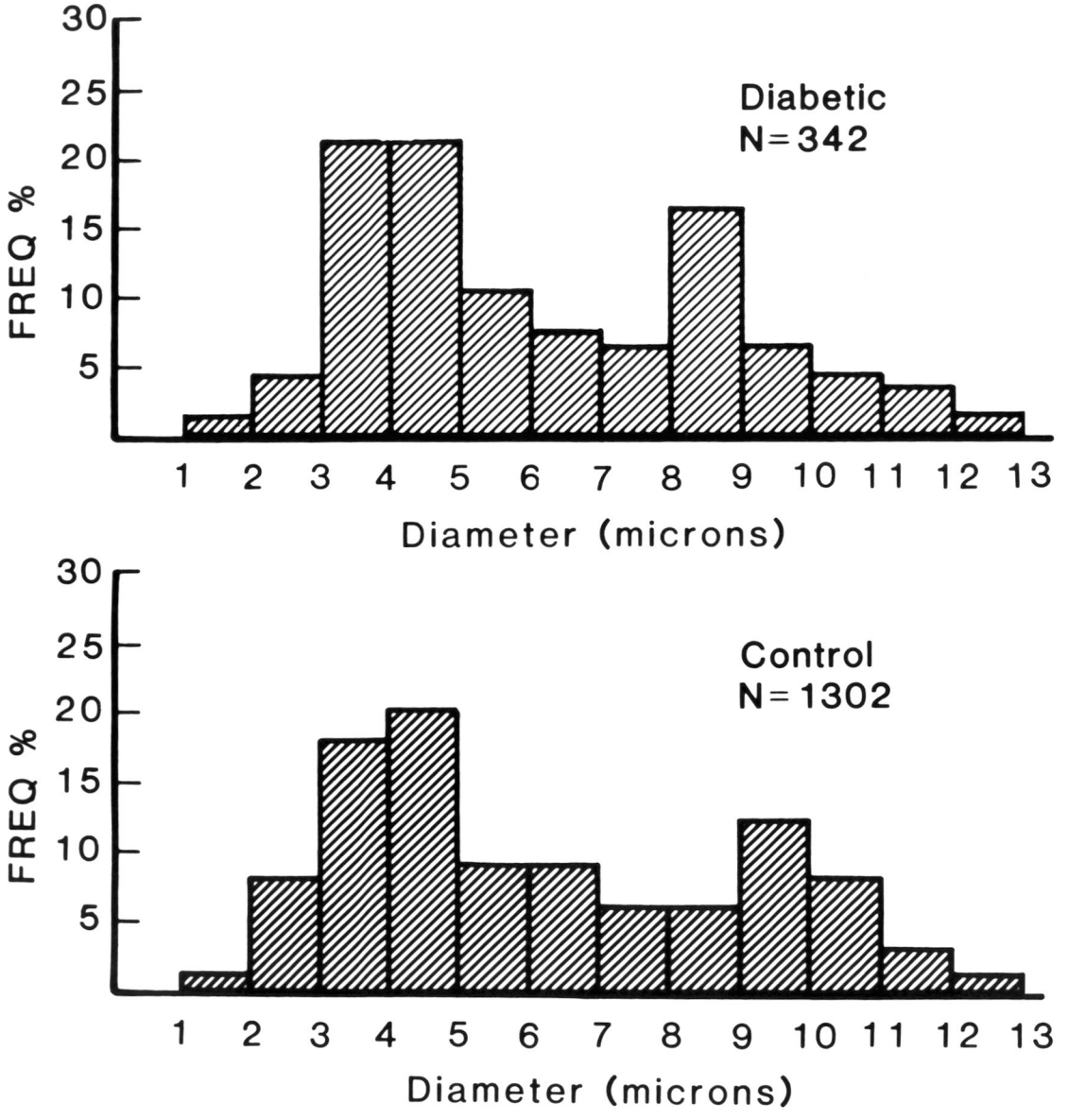
Graph 5. Myelinated fiber distributions of seven month ketonuric animal, ZM09-74, (top) and matched control, AV13-23, (bottom). ($P < .001$)


30
25
20
15
10
5
FREQ %
Diabetic
N = 1861
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)
30
25
20
15
10
5
FREQ %
Control
N = 1839
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)

Graph 6. Myelinated fiber distributions of nine month ketonuric animal, AC18-28, (top) and matched control, M00-796, (bottom). ($P < .001$)


Diabetic
N= 288
FREQ %
30
25
20
15
10
5
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)
Control
N= 257
FREQ %
30
25
20
15
10
5
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)

Graph 7. Myelinated fiber distributions of ten month ketonuric animal, AC18-61, (top) and matched control, M00-836, (bottom). ($P < .025$)


30
25
20
15
10
5
FREQ %
Diabetic
N=342
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)
30
25
20
15
10
5
FREQ %
Control
N=1302
1 2 3 4 5 6 7 8 9 10 11 12 13
Diameter (microns)

# Appendix A

Earle's Basic Salt Solution:

| | | |
|---|---|---|
| A. | NaCl | 6.80 g. |
| B. | $CaCl_2 \cdot 2 H_2O$ | .27 g. |
| C. | KCl | .40 g. |
| D. | $NaH_2PO_4 \cdot H_2O$ | .14 g. |
| E. | $NaHCO_3$ | 2.20 g. |
| F. | $MgSO_4 \cdot 7 H_2O$ | .20 g. |
| G. | Glucose | 1.00 g. |
| H. | $H_2O$ | 1000 ml. |

# Appendix B

Durcupan:

| | | |
|---|---|---|
| A. resin | Araldite 6005 | 5.62 g. |
| B. hardener | DDSA (dodecenyl succinic anhydride) | 5.02 g. |
| C. accelerator | DMP-30 (2,4,6-tris(dimethylaminomethyl)phenol) | .20 ml. |
| D. plasticizer | DBP (dibutyl phthalate) | .05 ml. |

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