THE EFFECTS OF EXERCISE AND HIGH PROTEIN DIET ON THE RAT ACHILLES TENDON

by

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B.Sc. (Kinesiology), Simon Fraser University, 1969

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (KINESIOLOGY) in the Department of Kinesiology

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SIMON FRASER UNIVERSITY

April 1976

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ABSTRACT

Achilles tendonitis is a common occurrence in the endurance runner. The endurance runners who experience Achilles tendonitis have been shown to have an elevated serum uric acid and the question has been raised as to whether the inflammation of the tendon is precipitated by the presence of uric acid crystals in the tendon. This experiment was conducted to investigate whether uric acid crystals could be a causative factor in hyperuricemic tendonitis. A high protein diet was introduced into the experiment in an attempt to precipitate hyperuricemia to determine if a resultant inflammation of the tendonous region occurred.

A total of twenty male Sprague-Dawley rats, ranging in weight from 250 to 325 grams were randomly assigned to four groups of five rats each; a control group which was not exercised and was fed on regular lab chow, two exercise groups, one fed on high protein diet and one fed on regular lab chow and a second sedentary group fed on high protein diet. The exercise groups were run on a motorized treadmill five days per week for six weeks to near exhaustion. Samples of Achilles tendon were taken for determination of the presence of uric acid crystals and for electron microscopic study.

The electron microscopic studies showed little change in the fine structure of the tendon due to exercise or high
protein diet.

The tissue fixed for uric acid was observed under a Zeiss Photomicroscope II with polarized light attachments. It was found that various types of crystals were present in the tissue. The high protein exercise group exhibited more crystalline material than the other groups, followed by the regular diet exercise group, the sedentary high protein group and lastly the control group which exhibited only random crystals. Further specific staining for uric acid was carried out to eliminate the possibility of calcium and other crystals being mistaken for uric acid crystals. This staining method showed more uric crystals in the high protein exercise group than the other experimental groups. None of the experimental animals showed any symptoms of Achilles tendonitis (inflammation and swelling in the tendon region). It is proposed that trauma may be necessary as a precursor to the inflammatory process of hyperuricemic tendonitis. The increased uric acid deposits in the tendon of the rats fed on high protein diet and exercised indicated that accumulation of uric acid may be an important factor in hyperuricemic tendonitis in endurance runners.
To Jack
ACKNOWLEDGMENT

I wish to thank Dr. Doug Clement and Jack Taunton coauthors with myself on the study which led to this thesis. Their continued interest and support have been invaluable to me in the completion of this thesis. I also wish to thank Dr. N.M.G. Bhakthan, my senior supervisor, for his help in the final preparation of this thesis and for his technical advice on tissue preparation and also Dr. A.E. Chapman for his critical evaluation of the thesis. Final thanks to Margaret Savage for her encouragement and assistance and Brian Mist for his assistance.
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CHAPTER 1

INTRODUCTION

Achilles tendonitis is a major problem of long distance runners. The current view of its cause is of a mechanical nature, ascribing it to a structural weakening of the tendon due to the repeated forces of take-off and landing in running. However, Clement et al. (1975) reported a significant correlation between tendonitis and hyperuricemia in endurance athletes. They proposed that tendonitis is a gout-like syndrome resulting from the deposition of uric acid crystals into the tendon during intense and prolonged training and precipitated by trauma. The deposition of uric acid crystals in the tendons of patients with gout is well documented (Talbott, 1967).

I. Exercise

There is well documented information on the effects of exercise on the fine structure of skeletal muscle (Gollnick & King, 1969; Gollnick et al., 1969; Barnard et al., 1970 and Reitsma, 1970). However, little attention is paid to the structure of the tendon in these animals. Mechanically, tendon has been studied extensively and the forces applied to the Achilles tendon in running were found to be of the magnitude of one thousand pounds to the square inch (Della, 1950).
Endurance runners train from six to seven days per week at a very high intensity and have been shown to have an increased serum uric acid level as a result (Horvath, 1967). Clement et al. (1975) reported that exercise appeared to lead to an elevation of the serum uric acid (hyperuricemia) in two ways: by increasing the tissue nucleic acid breakdown and by decreasing renal excretion. Tissue nucleic breakdown was elevated by the increase in muscle tissue turnover, blood cell breakdown and mitochondrial breakdown. The decrease in renal excretion was effected by dehydration (causing decreased renal blood flow and decreased glomerular filtration rate), increased lactic acid and ketone bodies (causing a decreased urinary uric acid excretion) and an increase in catecholamines and local hypoxia (also causing a decrease in renal blood flow and decreased glomerular filtration rate) (Figure 1). The occurrence of hyperuricemia in endurance athletes was the basis for the proposal of Clement et al. (1975) that hyperuricemia and tendonitis were related (Figure 2).

II. High Protein Diet

High protein diets have been used by athletes in an effort to improve performance by increasing endurance and/or strength and also for rapid weight reduction. However, it does not appear to be universally agreed that extra dietary protein,
Exercise: Elevates Uric Acid (Hyperuricemia) by:

1. \( \uparrow \) tissue nucleic acid breakdown - \( \uparrow \) muscle tissue turnover, \( \uparrow \) RBC breakdown, \( \uparrow \) mitochondrial breakdown

2. \( \uparrow \) renal excretion
   a) dehydration - \( \uparrow \) renal blood flow, \( \uparrow \) GFR
   b) \( \uparrow \) lactic acid - \( \uparrow \) urinary uric acid excretion
   c) \( \uparrow \) ketone bodies - \( \uparrow \) renal blood flow and \( \uparrow \) GFR
   d) \( \uparrow \) catecholamines and local hypoxia - \( \uparrow \) renal blood flow and \( \uparrow \) GFR

Figure 1. The role of exercise in the alteration of uric acid levels (from Clement et al., 1975, p. 42).
Figure 2. Exercise, Hyperuricemia and Tendonitis.
beyond the daily necessary requirements improved performance. Yamaji (1951), Yoshimura (1961, 1965) and Cvorkov et al. (1974) found an increase in dietary protein during strenuous activity to be favourable for performance. However, Darling et al. (1944) and Rasch and Pierson (1962) found extra dietary protein to be of no value in improving performance.

Since the time that the majority of these studies were carried out there has been an advancement in endurance race preparation which requires diet manipulation. This process, first established by Bergstrom et al. (1967), in Sweden, was called carbohydrate loading. It required an overloading of protein for three days followed by an overloading of carbohydrate for three days in the six days prior to the race. The purpose of this diet was not to increase performance through high protein intake but to increase the glycogen stores so as to allow the athlete to perform for a greater length of time using glycogen before he must utilize fatty acids.

A classical precipitator of gout (deposition of uric acid crystals in joints) was high protein intake (Hall, 1971). Clement et al. (1975) presented data indicating that Achilles tendonitis found in endurance runners was associated with hyperuricemia which is characteristic of gout. They postulated that high protein diet might precipitate tendonitis in the already vulnerable tendon of the endurance runner by further increasing
the urate concentration of the body fluids. Egan (1972) has reported that many rats, exercised while on a high protein diet, have developed a condition which he diagnosed as tendonitis. If this also occurred in endurance athletes the use of high protein diet by these athletes could be detrimental.

The use of high protein diets by endurance athletes may be further increased in the future as a result of two Japanese studies (Yamaji, 1951 and Shirahi et al., 1974). These authors reported that a large protein intake was necessary to correct the anemia found in endurance athletes as a result of intense training. The anemia occurred at the beginning of training and the degree of hemoglobin reduction and its rate of return to the pretraining level were influenced by protein intake. Yamaji (1951) believed that his results showed that muscle was formed at the expense of blood protein.

III. Summary

The purpose of this study was to determine the effects, both structural (electron microscopy) and histochemical (polarized light microscopy and selective staining), of exercise and high protein diet on rat Achilles tendon. It was hypothesized that exercise and high protein diet would lead to the deposition of uric acid crystals in the tendon.
I. Fine Structure of Tendon

There is a dearth of literature on the structure of normal tendon (see reviews by Hall, 1965 and Holmes, 1971). The gross appearance of the tendon is that of watered silk (Schafer, 1912) with alternating dark and light bands (Heringa and Lohr, 1924). These bands were found to be present in relaxation but disappeared under tension (Nauch, 1931 and Rigby et al., 1959).

"Tendon excites attention because it is virtually synonymous with collagen, a prime constituent protein of all connective tissue, including skin" (Crisp, 1972, p. 143). The composition of tendon is a package of collagen fibres and mucopolysaccharide (Schatzker and Branemark, 1969). The collagen fibres were laid approximately parallel to the long axis of a tendon (Elliott, 1965) and were found to be as long as the tendon itself, anastomosing with each other at acute angles (Schatzker and Branemark, 1969). An extracellular thread of protein, the fibril, was the structural unit of collagenous tissue (Elliott, 1965). The fibrils were grouped into bundles (primary bundles or fibres) which were aggregated into secondary bundles (Schneider, 1959) or fascicles (Edwards, 1946). The secondary bundles in turn formed tertiary bundles (Schneider, 1959) which together
comprised the tendon. The collagen fibres were thick with fibrillar structure and were parallel to one another (DiFiore, 1972).

The individual fibrils were characterized by a distinct pattern of fine cross-striations (Bear, 1942 and Schmitt, 1942). Light microscopy revealed these striations to be made up of alternating sets of bands and interbands (Grover, 1965). There were conflicting reports as to the inner structure of the collagen fibril. Kennedy (1955) (human), Grassman (1955) (rabbit) and Potz and Nemetschek (1956) (rabbit) showed the collagen fibrils from human periodontal membrane and rabbit skin to have a tubular structure. However, Borysko (1963) reported beef leg tendon fibrils to be solid. He concluded that the bands extended through the entire thickness of the fibril. Stromberg and Wiederheilm (1969) found the fibrils to be disoriented with respect to the long axis of the tendon when at resting length and they hypothesized that stress conditions caused some of the fibrils to be longitudinally aligned. This confirmed the earlier study of rat tail tendon by Elliott (1965) who found that the fibres followed a wavy course through the tendon bundle and only became straight and parallel to the axis when stretched. The other extracellular components of tendon included the amorphous ground substance which was a gel laying between the fibrillar elements and containing mucopolysaccharides (Elliott, 1965).
The cells of tendons were filamentous and lay parallel to the long axis of the tendon (Buck, 1953). There were two types of cells which predominated: tenoblasts (fibroblasts) and tenocytes (fibrocytes) (Holmes, 1971). The tenoblasts were described by Greenlee and Ross (1967) to have prominent nucleoli, rough endoplasmic reticulum and Golgi apparatus. Holmes (1971) reported that tenoblasts were more often found in old rats, which confirmed the studies of Viidik (1966) and Gould (1968) who distinguished tenocytes as the active cells which produce the collagen and tenoblasts as the major inert constituent of tendon. Many authors did not distinguish between tenoblasts and tenocytes, however, Holmes (1971) found that there were fine structure differences between tenoblasts and tenocytes. Tenoblasts appeared in longitudinal section as rhomboid cells arranged in rows with ovoid nuclei. There were one or more nucleoli present and a pale area in the cytoplasm opposite the nucleus. Tenocytes appeared to consist solely of elongated nuclei due to poorly staining cytoplasm.

The epitenon was a delicate layer of loose connective tissue which covered the tendon (Buck, 1953 and Crisp, 1972). It continued into the interior of the tendon as the endotenon or paratenon (Buck, 1953; Elliott, 1965 and Crisp, 1971). This covering surrounded the tendon bundles and held them together, allowing some movement of the bundles with relationship to each
other. It carried all the blood vessels, lymphatics and nerves (Schatzker and Branemark, 1969 and Elliott, 1965). The endotenon contained elastic fibres (Hirai, 1959 and Elliott, 1965). If lubrication of the tendon was essential due to friction or direction change, then there was a constraining sheath, the synovial sheath, which catered for one or several tendons (Crisp, 1972).

The Achilles tendon was the common tendon of the soleus and gastrocnemius muscles and inserted into the calcaneus. This tendon did not change direction and so had no need for a synovial sheath (Kiely, 1963 and Corrigan, 1967). It was, however, surrounded by areolar fatty connective tissue in which elastic fibres were interposed (the epitenon) (Arner and Lindholm, 1959).

II. Effects of Exercise on Tendon

"Tendon is a dynamic link between bone and muscle, vital to the efficient translation of the body's energy into effective action" (Welsh et al., 1971). Jewell and Wilkie (1958) concluded that the tendon played a role in damping the force produced by a muscle and thereby modified the effect of muscular contraction. When external forces were applied, greater than the accustomed load of a muscle, the tendon dampened the force. In this way it played the role of a safety mechanism. The
tendon was two times stronger than its muscle (Elliott, 1967) yet Walker et al (1964) found that in vivo the stress on a tendon was never more than one quarter of its ultimate tensile strength.

Comparatively few investigations were reported on the effects of exercise on the structure of tendon. Elliott (1964, 1965) studied the tendon of three week old rabbits following exercise. He found that the tendon hypertrophied and that this growth was related to the degree of tension to which the tendon was subjected. The increase in tendon thickness was proportional to the increase in the muscle cross sectional area. Inglemark (1948) reported an increase in the concentration of nuclei in hypertrophied tendon of exercised growing mice and rabbits. Adult mice and rats exhibited only a small nuclear increase. A later study by Viidik (1967) on tendons of exercised rabbits found no change in weight, water content and collagen content from the control to the trained. His conclusion was that training induced qualitative but not quantitative changes in tendon.

III. Effects of High Protein Diet on Tendon

The main structural constituents of tissues were proteins. They combined with lipid to form the internal and external cellular membranes and interfaces. Free amino acids were
liberated by digestion of dietary protein and were taken up by the tissues, especially the liver, intestine and kidney. Amino acids were metabolized by incorporation into protein and reserves of protein were found in the liver, kidney, plasma and muscle. The amino acids which were not incorporated into protein were deaminated. The nitrogen was excreted in the urine as urea and carbon was metabolized through a variety of pathways to tricarboxylic acid cycle intermediates (Harper, 1969).

Figure 2 (p. 4b) shows the point at which high protein diets may influence the purine pool. This type of diet has been common for some athletes who were attempting quick weight loss and for some who were looking to increase performance. Egan (1972) has reported that high protein diet was seen to induce what he diagnosed as tendonitis in exercising rats. This resulted in inflammation about the Achilles tendon and a cessation of running.

High protein diets have been thought to increase the performance of athletes due to increased endurance and strength (Cvorkov et al., 1974). Some students of nutrition do not agree with this and feel that variations in protein intake affect neither physical fitness nor muscle mass (Rasch, 1960). This was in agreement with an earlier study by Darling et al. (1944). Rasch and Pierson (1962) confirmed these results in a study on male college students. They divided the students
into two groups, one given a daily dietary protein supplement and the other given a placebo. The subjects were given three weight training sessions per week for six weeks. The body weight, arm volumes, arm girths and total weight lifted were measured before and after training. At the end of six weeks training there was no significant differences \( (P < .01) \) between the pre and post training body weights, arm volumes or upper arm girths, for either group. The total weight lifted was significantly increased for both groups but there was no difference between the groups. Although these authors concluded that the protein supplement had no effect on muscular strength or hypertrophy they also reported a need for further studies in this area to cover a longer period of time. Yamaji (1951), Kraut et al. (1953) and Yoshimura (1961 and 1965) found protein supplementation, during strenuous exercise, favourable for performance. Cvorkov (1972) agreed with these findings. He studied the endurance capabilities of rats on high protein diet as compared to rats on a normal lab chow diet and found a significantly higher endurance capacity in the high protein diet group.

Dietary protein level has been reported to influence the serum urate concentration, but to a lesser degree than the protein content (Bien et al., 1953). The products of protein metabolism contributed precursors for urate biosynthesis.
Seegmiller et al. (1961) subjected twenty-two normal and sixty gouty individuals to a purine free diet and noted average falls in serum urate concentration of 0.63 and 1.01 mg%, respectively. A later study (Seegmiller et al., 1963) showed that the ingestion of a diet rich in purines enhanced serum urate production. Seven nongouty subjects were given a high purine diet (four grams of ribonucleic acid per day) with a resultant increase in serum urate from an average of 4.6 mg% to 8.3 mg%.

Unfortunately, there is no information reported in the literature about the effects of high protein diet on tendons.

IV. Uric Acid and Tendonitis

A. Introduction

Hyperuricemia is the presence of sodium urate (uric acid) in the plasma at a greater than normal concentration (>7.0 mg%), resulting in a supersaturated solution. Under appropriate conditions, the excess urate will precipitate and result in sodium urate crystal deposition in a variety of tissues including synovium and tendon (Goldfinger, 1971). Hyperuricemia has been shown to be present in gout and in gouty arthritis (Hall, 1971). It has been hypothesized that hyperuricemia, with a resultant deposition of crystals, was the cause of the all too common Achilles tendonitis found in endurance runners (Clement et al., 1975) (Figure 3). As there
ENDURANCE EXERCISE
between 30 miles and 120 miles per week

HYPERURICEMIA
URIC ACID is a metabolic excretory product of purine degradation. Hyperuricemia is present when serum uric acid exceeds 7.0 mg%.

MONOSODIUM URATE CRYSTAL DEPOSITION
gouty arthritic state

TENDONITIS
mechanical and biochemical irritation

FIGURE 3. Theoretical Explanation of Hyperuricemic Tendonitis (from Clement et al., 1975)
was no synovium associated with the Achilles tendon, the crystals would have been deposited in the tendon itself, setting up an inflammatory reaction which in turn resulted in pain and swelling.

B. Uric acid and acute exercise

Elevated serum uric acid has been reported to be associated with exercise since 1921 when Rakestraw reported elevations in serum uric acid in women after acute exercise. His results showed increases in serum uric acid of 0.9 mg% in brief and 1.5 mg% in prolonged periods of exercise. In a study by Levine et al. (1924) of runners in the Boston marathon, pre race blood samples were taken on five runners with a resultant mean serum uric acid of 3.94 mg% while post race samples on eleven subjects yielded a mean of 5.70 mg%. One runner was re-examined forty-eight hours later. His serum uric acid had returned to the pre-race level.

These results have been supported by Nichols et al. (1951) who reported a close correlation between serum uric acid and inhibition of uric acid excretion. These authors observed an increase in serum uric acid levels after strenuous physical activity (mean increase of 0.5 mg% following thirty minutes of running) whereas moderate exertion like walking did not influence uricemia. Even light exercise (light gymnastics with
running, walking and apparatus work for thirty minutes) has been reported to result in a small rise in serum uric acid (0.16 ± 0.7 mg%) in twenty young men (Zachau-Christiansen, 1959). Severe strain such as marathon running resulted in a much greater increase in serum uric acid (1.4 mg%) (Horvath, 1967).

A number of reasons have been put forward to explain the increase in serum uric acid with muscular exertion, the earliest being that there was a reduction in urinary excretion of uric acid due to an increased excretion of fluids as sweat. However, Horvath (1967) reported that other factors must be involved because the decrease in uric acid excretion was also present in subjects whose diuresis remained at a high level during stress. Rakestraw (1921) and Levine et al. (1924) found that excessive strain was accompanied by a decrease in the clearance of uric acid which was associated with a reduction of glomerular filtration and tubular dysfunction. The tubular dysfunction was seen to be caused by hyposecretion of uric acid (Nichols et al., 1951). A further factor was introduced by Horvath (1967) who studied a group of International sportsmen (light athletes, heavy athletes, hockey players, footballers, cyclists and gymnasts) during rest and during intensive training. Twenty-three of the athletes were studied during a period of no training, and had a mean serum uric acid level of 3.05
mg% while the remaining twenty-seven were studied during intense training for their particular sporting event and showed a mean serum uric acid of 4.90 mg%. Horvath (1967) concluded that the increased serum uric acid in the intensive training group was due to an increased destruction and new formation of protein. The author presented the question as to whether the increased uricemia of muscular exertion would eventually lead to hyperuricemic syndrome or gout. Clement et al. (1975) postulated that this increase in uricemia lead to tendonitis when the conditions were right. Figure 2 (p. 4b) outlines the exercise factors which could lead to an increased serum uric acid level. Banister et al. (1971) reported mitochondrial breakdown in animals following strenuous exercise which also suggested an increased turnover of tissue protein.

There was contradictory evidence that showed that physical exertion in high school sports lead to a decrease in serum uric acid (Montoye et al., 1967). However, the high school athletes studied had resting serum uric acid levels significantly higher than non-athletes. The serum uric acid levels of twenty male college athletes were measured twice, seven months apart. These athletes had a substantially elevated serum uric acid when compared to other non-athletic groups (Greenleaf et al., 1969). A study by Bosco et al. (1970) on the effect of eight weeks of chronic physical exercise on thirty
males (ten - athletic group - athletes in training - extremely active; ten - training group - soccer class - moderately active; ten - control group - bowling class - sedentary) showed that chronic exercise lowered uric acid in eighty per cent of the athletic and training groups' subjects. The exercise training period lasted for eight weeks and consisted of two hour daily physical conditioning sessions of sprint swimming, resistive exercise, water polo drills and weekly school competition for the athletic group. The moderate activity group ran one mile per day, played one hour of soccer per week and did one-half hour of circuit training twice a week for muscular endurance. The control group had two one-half hour bowling sessions per week, participated in little or no organized physical activity and led relatively sedentary lives. The athletes' activity was estimated, in terms of caloric expenditure, to be six times more than the soccer class and twenty-five times more than the bowling class. However, the intensity of the exercise in this study did not closely approximate that of endurance athletes, many of whom train twice a day resulting in an increase in metabolic demands and cellular turnover. This increased the quantity of purines and proteins added to the total purine pool (Figure 2, p. 4b) which increased uric acid concentration.

See: miller and Howell (1963) proposed a self-propogating inflammatory reaction theory involving urate crystals.
The initiating event of the cycle could be stress, trauma, adrenocorticotropic hormone withdrawal or exercise. All these factors, especially exercise, involve the development of a pH gradient between tissue and blood, favouring the precipitation of urate (Clement et al., 1975). Seegmiller, Laster and Howell (1963) stated that "strenuous exercise increased the serum urate level through the urate-retaining action of the elevated lactic acid levels".
MATERIAL AND METHODS

Twenty male albino rats of the Sprague-Dawley strain, averaging 280 grams in weight, were used in the study. The rats were randomly divided into four groups of five rats each.

I. Treatment of the Animals

Group I (n = 5) - Exercise High Protein Diet

This group of rats was exercised by running to near exhaustion five days per week for a six week period. They were maintained on a high protein diet (powder) given ad libitum.

Group II (n = 5) - Sedentary High Protein Diet

Group II were maintained in their cages for a period of six weeks. They were fed on a daily diet of high protein powder given ad libitum.

Group III (n = 5) - Exercise Regular Diet Group

These rats were exercised by running to near exhaustion five days per week for six weeks. They were fed on a rat lab chow (mixed) diet ad libitum.

Group IV (n = 5) - Control Group

This group of rats were maintained in their cages for a six week period. Their diet was rat lab chow (mixed) and was given ad libitum.
II. High Protein Diet

This powdered diet was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. The high protein diet contained:

- Vitamin Free Casein: 64%
- Sucrose: 22%
- Vegetable Oil: 8%
- Brewers Yeast U.S.P.: 2%
- Salt Mixture U.S.P.: 4%

The lab chow was obtained from Purina Company, St. Louis, Mo. The content, according to the manufacturer's specifications was:

- Crude protein: 22%
- Crude fat: 4%
- Crude fiber: 5%
- Ash: 9%

III. Exercise Procedure

Groups I and III were exercised five days per week, for six weeks, to near exhaustion, on motor driven small animal treadmill (Quinton Instrument Co., Seattle, Washington). The point of cessation of exercise was determined by the rats' behaviour. They were removed from the treadmill at near exhaustion, when they lay on the treadmill and would allow
themselves to be carried into the shock electrodes (Holloszy, 1970). The grade of the treadmill was maintained at zero percent while the speed was increased progressively to maintain the time of exercise at approximately sixty minutes per exercise period (Cvorkov et al., 1974). Initially, the speed of the treadmill varied between 0.5 and 1.0 km/hr and at the end of the training period the rats were running at varied speeds between 1.9 and 3.5 km/hr.

IV. Excision of the Tissue

The five rats from each group were sacrificed under a light anaesthesia induced with ether and tissue was taken for light and/or electron microscopic study. When the animals were lightly sedated (determined by a lack of eyelid reflex when the eye was gently touched) they were placed ventral surface down on the operating board and their limbs were secured in an outstretched manner. An incision was made at the midpoint of one lower leg and this was extended in both directions to include the entire lower leg. The skin and fascia were removed to reveal the Achilles tendon.

Four per cent glutaraldehyde in phosphate buffer was pipetted into the area immediately following exposure and the tissue was periodically bathed with this solution to preserve the tendon in a resting state. Glutaraldehyde is used as
a good protein fixative (Pease, 1964).

Small pieces of the Achilles tendon were excised and placed in vials containing buffered glutaraldehyde. Three separate pieces of tendon were taken.

The second leg of each rat was prepared for tissue excision as above. The muscle and tendon of the lower leg were exposed and the tendon was immediately excised and placed in one hundred per cent alcohol. This tissue was prepared for uric acid demonstration. Again, three separate pieces of each tendon were used for the study. One hundred per cent alcohol has been shown to be the fixative of choice as uric acid is soluble in water (Merck, 1972).

V. Treatment of the Tissue

A. Electron microscopy

The excised tendon was post fixed in two per cent buffered osmium tetroxide in phosphate buffer for two hours (Millonig, 1962). Following post fixation the tissue was processed through grades of ethyl alcohol (dehydration), propylene oxide (clearing) and was flat embedded in Epon 812 (Weakley, 1972). The tissue was oriented on blank blocks for longitudinal sections and thin sections were cut on a Porter-Blum MT-1 ultramicrotome. The sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and were
examined under an RCA EMU-3H/Zeiss 9A electron microscope.

The three specimens from each of three animals of all four groups were scanned for fine structure changes.

B. Light microscopy

The excised tissue was cut into pieces and was fixed in cold absolute alcohol in order to preserve uric acid crystals if they were present. Fixation was for forty-eight hours. This tissue was processed through toluene as the clearing agent. Washing and dehydration were not carried out as washing would have dissolved any uric acid crystals present and the tissue was fixed in absolute alcohol so dehydration was not necessary. Embedding was in paraffin. Sections, five μm thick, were cut and passed through xylene and alcohol and were mounted on glass slides with Canada balsam.

Uric acid crystals have been easily identified in synovial fluid by the use of compensated polarized microscopy (Phelps, 1968). Polarized light was attained by use of a polarizing microscope which had two polarizing filters, one positioned below the condenser (polarizer) and one inserted at some point above the objective (analyzer). A removable first-order red compensator was placed between the objective and the analyzer. In practice, a crystal was centred in the field of view and the stage was rotated so that the long axis
of the crystal was parallel to the direction of vibration of the slower component of light in the compensator.

The polarizing filter passed light in only one plane and the polarizing filter was set so that no light passed through it and there was a black background. If a crystal (optically anisotropic) was placed between the polarizer and the analyzer the light passed by the polarizer was rotated by the crystal allowing light to pass through the analyzer. Only the light which passed through the crystal was visible and the crystal appeared white.

In order to identify one crystal from another (i.e. sodium urate from calcium pyrophosphate) it was necessary to add a first order red compensator (Phelps, 1971). This device separated light according to components of fast and slow vibration. It was inserted between the objective and the analyzer and retarded red light so that the background became red instead of black (Phelps, 1968). The urate crystals were short and rod-shaped with rounded ends and parallel sides in the joint fluid but were long and needle-shaped in tophaceous material (Phelps, 1971). Urate crystals were strongly negatively birefringent such that under compensated polarized light they were yellow when the long axis of the crystal was parallel to the axis of slow vibration of the compensator; at the perpendicular they were blue. Calcium pyrophosphate, which was weakly
positively birefringent, exhibited the opposite colours.

Slides prepared from the three pieces of tendon from each of the rats were examined under compensated polarized light. As the tissue had been sectioned the crystals were not oriented such that the long axis could be determined. More sections were prepared. The slides were then placed in twenty per cent silver nitrate solution and were exposed to strong sunlight for one to four hours (urates become bright rose colour). A solution of ten ml. of three per cent gelatin in hot water, three ml. of twenty per cent silver nitrate and two ml. of two per cent hydroquinone was poured over the slides until the urates turned black and the connective tissue was yellow. The slides were washed in hot water (50°C), dehydrated and mounted with Canada Balsam. They were observed under the light and polarized light microscope.
CHAPTER 4

RESULTS

I. Light Microscopy

The results of the light microscopic study are presented as colour photomicrographs. Initially, all of the slides prepared for light microscopy were scanned under the polarizing microscope for the presence of crystals. Figures 4 to 7 are photomicrographs taken under the compensated polarized microscope. Figure 4 shows the presence of crystals in the tendon of a high protein exercise rat. The crystals, which are scattered through the tissue, are seen as blue and yellow while the tissue is red. These colours are due to the presence of the first-order red compensator plate as well as the polarizing filter and analyzer in the path of the beam. It is not possible from this micrograph alone to differentiate the various crystals. Figures 5, 6 and 7 are photomicrographs of tendon from regular diet exercise, high protein sedentary and control rats, respectively. These micrographs also demonstrate the presence of crystals which cannot be differentiated.

As uric acid crystals are strongly negatively birefringent and calcium crystals are weakly positively birefringent it was necessary to rotate the slides and observe whether the crystals changed colour from yellow to blue or from blue to yellow.
FIGURE 4. Achilles tendon of a high protein exercise rat under compensated polarized light. Crystals are seen as yellow and blue (X 1250).
Figure 5. Achilles tendon of a regular diet exercise rat under compensated light. Crystals are seen as yellow and blue (X250).

Figure 6. Achilles tendon of a high protein sedentary rat under compensated polarized light. Crystals are seen as yellow and blue (X500).

Figure 7. Achilles tendon of a control rat under compensated polarized light. Crystals are seen as yellow and blue (X500).
Figures 8, 9, and 10 are of one slide, that of a high protein exercise rat. The first micrograph (Fig. 8) has only the polarizing analyzer and filter in place, causing the black out of light. The crystals are illuminated and appear as a bright cluster in the upper right hand corner of the micrograph. The next two figures (9 and 10) were taken with the first-order red compensator in place and were taken at right angles to one another (i.e. the stage was rotated ninety degrees after the first micrograph was taken). A close observation of the crystals present in these two micrographs shows that the yellow crystals have turned blue and the blue crystals yellow. The possibility was considered that the yellow crystals present in the first micrograph were uric acid and the blue calcium. However, this was not valid as it was not possible to identify the long axis of the crystals in the sectioned tissue which is a requirement for positive identification (uric acid crystals are yellow when their long axis is parallel to the axis of slow vibration of the compensator plate). A further step in the identification process followed.

Tissue was sectioned for selective staining for uric acid, a procedure which turns the crystals coral and then black in colour. The remaining colour photomicrographs are of the selectively stained tendon (Figures 11 to 27). A quantitative analysis of the relative amounts of uric acid in the
Figure 8. Achilles tendon of a high protein exercise rat under compensated polarized light. Crystals are white (X500).

Figure 9. Achilles tendon of a high protein exercise rat under compensated polarized light. Crystals are yellow and blue. This micrograph is at right angles to Figure 7 (X500).

Figure 10. Achilles tendon of a high protein exercise rat under compensated polarized light. Crystals are yellow and blue. The micrograph is at right angles to Figure 6 (X500).
tissue of the four groups was not possible, photomicrographs from three animals of each group are presented.

The tendon of the high protein exercise rats exhibited the most uric acid crystals. Figures 11 and 12 show crystals with the analyzer and filter in place and with the analyzer, filter and compensator in place, respectively. The third micrograph (Fig. 13) was taken without the analyzer, filter or compensator. This micrograph shows a number of black deposits to be present as well as a number of coral coloured deposits which are more evident in Figure 11. It would appear that the black and coral coloured deposits are both uric acid, the coral ones having not been washed with the silver nitrate, gelatin and hydroquinone mix for a long enough period. The presence of other crystalline material is evident from Figures 11 and 12. Figures 14 and 15 (p. 35b) are of high protein exercise rat tendon from two other rats. They were taken without the polarizing equipment in place and show the uric acid as black deposits.

The regular diet exercise tissue (Figs. 16 and 17, p. 36b) also showed the presence of uric acid, but to a lesser degree than the high protein exercise group. There are no coral coloured deposits visible; all the uric acid is black. Figure 16 with the analyzer and plate in place shows the presence of other crystalline material. Photomicrographs of tendon from
Figure 11. Achilles tendon of a high protein exercise rat under uncompensated polarized light. Stained for uric acid. Crystals are white, uric acid is black and coral (X500).

Figure 12. Achilles tendon of a high protein exercise rat under compensated polarized light. Stained for uric acid. Crystals are yellow and blue. Uric acid is black (X500).

Figure 13. Achilles tendon of high protein exercise rat under the light microscope. Stained for uric acid. Uric acid is black and coral (X500).
Figure 14. Achilles tendon of high protein exercise rat under the light microscope. Stained for uric acid. Uric acid is black (X500).

Figure 15. Achilles tendon of high protein exercise rat under the light microscope. Stained for uric acid. Uric acid is black (X500).
FIGURE 16. Achilles tendon of regular diet exercise rat under uncompensated polarized light. Stained for uric acid. Crystals are white. Uric acid is black (X31).

FIGURE 17. Achilles tendon of regular diet exercise rat under the light microscope. Stained for uric acid. Uric acid is black (X31).
two other regular diet exercise rats are presented in Figures 18 and 19. Uric acid is stained black.

Fewer deposits of uric acid were present in the high protein sedentary rats as observed in Figure 21 (p. 39b), taken without the polarizing equipment. Again, there is some other crystalline material present (p. 39b, Fig. 20) and the uric acid has been changed from coral to black. Figures 22 and 23 (p. 40b) are photomicrographs from two different high protein sedentary rats. Uric acid crystals are demonstrated.

The final four colour micrographs (Figs. 24, 25, 26 and 27; pps. 41b and 42b) are of tendon from three control group rats. This group exhibited the fewest deposits of uric acid and the smallest amount of other crystalline material.

Positive identification of the presence of uric acid deposits in the tendons of all the rats was made. Other, non-identifiable crystals were also present. The tissue was not useful for the study of fine structure changes.

II. Electron Microscopy

Exercise has been reported to have little effect on tendon fine structure. However, as the literature in this field of investigation is limited, it was decided to examine the Achilles tendon under the electron microscope.
Figure 18. Achilles tendon of a regular diet exercise rat under the light microscope. Stained for uric acid. Uric acid is black (X500).

Figure 19. Achilles tendon of a regular diet exercise rat under the light microscope. Stained for uric acid. Uric acid is black (X500).
FIGURE 20. Achilles tendon of high protein sedentary rat under uncompensated polarized light. Stained for uric acid. Crystals are white. Uric acid is black (X125).

FIGURE 21. Achilles tendon of high protein sedentary rat under the light microscope. Stained for uric acid. Uric acid is black (X125).
Figure 22. Achilles tendon of a high protein sedentary rat under the light microscope. Stained for uric acid. Uric acid is black (X500).

Figure 23. Achilles tendon of a high protein sedentary rat under the light microscope. Stained for uric acid. Uric acid is black (X500).

FIGURE 25. Achilles tendon of control rat under the light microscope. Stained for uric acid. Uric acid is black (X125).
Figure 26. Achilles tendon of a control rat under the light microscope. Stained for uric acid. Uric acid is black (X500).

Figure 27. Achilles tendon of a control rat under the light microscope. Stained for uric acid. Uric acid is black (X500).
The ultrathin sections of the Achilles tendon were scanned under the electron microscope. It was noted that there were no differences in the tendon of one group as compared to any other group. The exercise and high protein diet had little effect on the fine structure of the tendon. The collagen fibres were still regularly arranged and fibroblasts were seen between the fibre bundles. It is quite possible that the number of collagen bundles may have changed as an effect of exercise, however no quantitative analysis was made on the size and number of these fibres because it was beyond the scope of this study.

Collagen microfibrils were seen, with the characteristic pattern of cross striations of axial periodicity (Ham, 1969). They demonstrated little units of structure along their lengths which repeated themselves every 640 Å. These units of structure were made up of tropocollagen molecules. Hodge and Petruska (1964) proposed a hypothesis to explain the periodicity. According to these authors each microfibril had light and dark segments which repeated themselves, with each light segment accounting for slightly more than half a period and each dark segment slightly less than half a period. The tropocollagen macromolecules were arranged side by side in a staggered manner in such a way that gaps between their ends fell in the dark region.

Electron micrographs of the Achilles tendon tissue are
presented as Figures 28 to 32. Figures 28 and 29 are longitudinal sections showing the collagen fibres. The wavy distribution of light and dark bands may be seen in Figure 28. This tissue is from a high protein sedentary rat. The higher magnification of Figure 29 (p. 46b) enables one to see the axial periodicity of the collagen fibres of a high protein exercise rat. The tissue of one high protein rat showed crystals to be present (Figure 30, p. 47b), however it is not possible to identify the crystals.

A section of tendon from a regular diet exercise rat is presented in Figure 31 (p. 48b). This micrograph shows normal transverse and longitudinal sections of collagen fibres. Sections from a control rat showed portions of fibroblasts between the collagen fibres.

The experimental conditions did not appear to induce any conspicuous change in the fine structure of the rat Achilles tendon. Therefore, only a few representative micrographs which show the tendon fine structure are presented.
FIGURE 28. Electron micrograph of the Achilles tendon of a high protein sedentary rat (X20,900)
Figure 30. The Achilles tendon of a high protein exercise rat under the electron microscope. Crystals can be seen as dark irregular shaped bodies (X20,900).
FIGURE 32. Electron micrograph of Achilles tendon of a control rat (X7300).
I. Light Microscopy

A. Polarized light microscopy

The identification of uric acid crystals in the tendon itself was initially attempted using polarized microscopy. The presence of crystals within the tissue was confirmed, however, their identification was not possible as it was impossible to identify their long axis.

A suitable stain which was specific for uric acid was chosen (De Galantha, 1935). The slides obtained showed the presence of both uric acid and other crystals when studied under uncompensated polarized light.

B. Exercise and uric acid

Although the presence of uric acid was confirmed in all of the rat tissue it was most prevalent in the high protein exercise group, and less so in the regular diet exercise group. This indicates a relationship between exercise and uric acid deposition in the Achilles tendon of rats. Uric acid crystals have been seen in the tendon of a patient who had a long history of tendonitis leading to tendon rupture (personal communication with Clement, 1975).
The presence of elevated serum uric acid levels in athletes has been reported by a number of authors (Rakestraw, 1921; Levine et al., 1924; Nichols et al., 1951 and Horvath, 1967). Evidence, contradictory to this finding, has also been presented (Montoye et al., 1967 and Bosco et al., 1970), however, the extent of exertion in the last two studies did not closely approximate the sustained physical stress of endurance athletes. Many of these athletes trained twice a day with the result that there was less chance for the serum uric acid, elevated during the training period to return to a lower level. The training sessions increased the metabolic demand and cellular turnover which in turn increased the amount of purines and protein added to the total pool with a resultant increase in serum uric acid concentration (Clement et al., 1975).

Clement et al. (1975) proposed that the increased uric acid concentrations could lead to Achilles tendonitis in the endurance athlete. They viewed tendonitis as a gout-like syndrome, with hyperuricemia and a resultant crystal deposition in the tendon itself. Horvath (1967) and Greenleaf et al. (1969) had proposed investigation on the relationship of the elevated serum uric acid levels seen in sportsmen to the occurrence of gout in later life.

Serum uric acid levels were not measured in the rats of this study and it was therefore not possible to correlate the
presence of the uric acid crystals in the rat Achilles tendon with serum uric acid levels.

There were no gross signs of tendonitis in the rats. It would appear that tendonitis may be precipitated by trauma. Clement et al. (1975) suggested trauma induced the uric acid deposition. However, it appears that in this study the crystals may already be present in the tendon, with no resultant inflammation. It seems logical therefore to suggest that the inflammatory process may be initiated by trauma in the tendon. A second possibility is that the trauma may cause a further precipitation of uric acid crystals and other crystalline material into the tendon of the hyperuricemic athlete and then the inflammatory process is initiated.

The positive identification of the presence of uric acid crystals in the tendon puts the problem of treatment of choice in tendonitis closer to resolution. At present, tendonitis is treated with oral anti-inflammatory drugs (i.e. phenylbutazone) and corticosteroid injections which may be responsible for weakening the tendon. Clement et al. (1975) treated seven of their subjects who had hyperuricemic tendonitis with the uricosuric agent probenecid in combination with the gout specific anti-inflammatory drug colchicine. Abatement of symptoms was seen in four to twenty weeks and all subjects returned to full training and competition without relapse
while remaining on the drug therapy.

II. Fine Structure of Tendon

The lack of fine structure changes in the Achilles tendon due to exercise and high protein diet agreed with earlier studies (Inglemark, 1948 and Viidik, 1967). The tendon was composed almost entirely of dense collagenous connective tissue. The collagen fibres are gathered into coarse bundles in an orderly parallel arrangement. The tendon is subjected to stress and its bundles are bound into strong fascicles with little intervening ground substance and relatively few cells or fibroblasts. A small number of elastic fibres are present in tendon.

The tendon seen in the micrographs (Figs. 28 and 29; pps. 45b and 46b) is in an unstrained state. Abrahams (1967) studied the effects of straining tendon on its fine structure. He cut a horse extensor tendon into three parts, two tensile test specimens and one control specimen. The two tensile test specimens were strained to a predetermined level. One was fixed in formalin while still under the stress while the other was allowed to relax before fixation. The control was also fixed in formalin. The control tissue, under the light microscope, showed collagen fibres which have a clearly defined wavy pattern. The strained fibres, fixed while under strain, at the
3.5 per cent strain level, exhibited parallel rows of collagen fibres with the fibres oriented in the direction of the applied load. The second strained tissue was also strained at the 3.5 per cent level but was then allowed to relax before fixation. This specimen showed some wavy collagen fibres and some parallel collagen fibres which had not returned to their original unstrained orientation. The author concluded that some permanent structural change may have occurred either in the collagen network or in the bonding of the mucopolysaccharides in the collagen network due to passing the elastic limit of the tendon. The exercise situation in this present study did not stress the tendon to the point of causing a permanent deformation. Also, fixation of the tissue occurred, primarily, with the tendon excised.

A number of factors have reported to lower the strength of the tendon: syphilis, gonorrhea, tuberculosis, uratic arthritis and pyogenic infections (Ljungqvist, 1968). Also implicated are tumours and cysts (Christiansen, 1954) and tendonitis (Silfvershiold, 1941). The present study was designed with the administration of high protein diet to half of the rats in an attempt to verify Egan's (1972) findings of tendonitis in exercising rats fed on high protein diet. However, there were no gross signs of tendonitis or edematous swelling observed in any of the rats.
Although exercise per se has not been shown to cause fine structural changes in Achilles tendons, it may indirectly cause a pathological process which might lead to rupture of the Achilles tendon. Trauma, in the form of direct and indirect violence, can cause tendon rupture. The more common cause is indirect violence. Arner and Lindholm (1959) proposed three main types of indirect violence which occurs in the Achilles tendon:

(1) pushing off with the weight bearing foot flexed in a plantar direction while extending the leg at the knee,

(2) violent dorsiflexion of the ankle when the foot is in intermediate position, and

(3) violent dorsiflexion of the foot flexed in a plantar direction.

It has been noted that tendon degeneration is common in patients with ruptured tendon (Arner et al., 1959). The histological changes observed were attributed to impaired blood supply and this was supported by angiographic studies which showed Achilles tendon rupture to occur frequently in the most avascular portion of the tendon (Lagergren and Lindholm, 1959). All of Arner and Lindholm's (1959) Achilles tendon rupture studies were done on former athletes. They concluded that the increased stress implied by strenuous exercise may result in degenerative changes in the tendon tissue with a decrease in its resistance.
Ricklin (1962) maintained that the exhaustion of physical training played an important role in causing structural changes in the Achilles tendon leading to a greater risk of rupture. This might be associated with the deposition of uric acid crystals in the tendon of endurance athletes. One case of ruptured quadriceps tendons as a result of gouty affectation of the tendon has been reported (Levy et al., 1971).

The ruptured tendons studied by Ljungqvist (1968) showed histological degeneration in the form of partially devitalized tendon tissues with obliterated fibrin structure and some fraying of the collagen bundles. There was loose granulation tissue, rich in collagen and of a wavy structure unlike normal tendon collagen attached to the devitalized tissue.

Although an attempt was made to induce Achilles tendonitis in rats through the administration of high protein diet, no inflamed or swollen tendons were observed. The stress on the tendon was induced by exercising on the motorized treadmill, however, no elevation of the treadmill was used. The stress of running may not be sufficient to cause strain changes or degenerative changes in the rat Achilles tendon.
CHAPTER 6

CONCLUSIONS

Uric acid crystals were found to be present in the tendon of all the rats studied in this experiment. These crystals can be positively identified by selective staining. The exercise condition led to a greater uric acid deposition in the tendon when compared to the non exercise groups. High protein diet also increased the deposition of uric acid crystals over regular diet. These findings have important implications in the study of the hyperuricemic tendonitis reported by Clement et al. (1975) in endurance runners. They postulated that trauma precipitated the deposition of uric acid crystals in the tendon with the resultant inflammatory reaction. From the findings of the present study it would appear that the uric acid crystals may be present in all tendon and to a greater degree in the exercised tendon, leading the author to postulate that trauma causes an inflammatory reaction and possibly the precipitation of further uric acid crystals. It is recommended that studies be carried out on normal tendon from sedentary humans and on ruptured tendon from endurance athletes to confirm the above findings for humans.

Fine structure studies showed exercise to have little effect on the tendon. Some crystals were observed under the
electron microscope but it was not possible to identify them. These crystals were present in the Achilles tendon of one of the high protein exercise rats.
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