JYVÄSKYLÄN YLIOPISTON
KANSANTERVEYDEN LAITOKSEN JULKAISUJA

UNIVERSITY OF JYVÄSKYLÄ
DEPARTMENT OF PUBLIC HEALTH PUBLICATIONS

No 29/1976

A. KIISKINEN AND E. HEIKKINEN

PHYSICAL TRAINING AND CONNECTIVE TISSUES IN YOUNG MICE

PART 1. BIOCHEMISTRY OF LONG BONES

PART 2. BIOCHEMISTRY OF ACHILLES TENDONS

UNIVERSITY OF JYVÄSKYLÄ

DEPARTMENT OF PUBLIC HEALTH PUBLICATIONS

PHYSICAL TRAINING AND CONNECTIVE TISSUES IN YOUNG MICE

PART 1. BIOCHEMISTRY OF LONG BONES

PART 2. BIOCHEMISTRY OF ACHILLES TENDONS

A. KIISKINEN AND E. HEIKKINEN

No 29/1976

ISBN 951-677-692-2

URN:ISBN:978-951-39-9669-7

ISBN 978-951-39-9669-7 (PDF)

ISSN 0357-4563

Jyväskylän yliopisto, 2023

PART 1. BIOCHEMISTRY OF LONG BONES

ABSTRACT. The effect of physical training on collagen, ground substance and nucleic acid concentrations in long bones was studied in male mice of NMRI-strain. The mice to be trained and their controls were about 2 weeks old at the beginning of training, which took place on a 5^{o} inclined treadmill 5 days a week for 3 to 22 weeks. The duration of daily exercise was increased progressively over 3 weeks. The final daily exercise bouts were 50 and 80 minutes for moderate programs and 180 minutes for the intensive program at a speed of 30 cm/s.

We found increased concentrations of nitrogen and hexosamines especially after prolonged training at both training intensities. The concentration of DNA, RNA-ribose and hydroxyproline tended to be reduced after some training programs. The hexosamine-hydroxyproline ratio was higher and the hydroxyproline-nitrogen ratio lower in the long bones of trained animals compared to the controls. We conclude that prolonged physical training contributes to maintaining a high glycosaminoglycan concentration in matured long bones.

LONG BONES, PHYSICAL TRAINING, COLLAGEN, GROUND SUBSTANCE, NUCLEIC ACIDS, GROWTH

INTRODUCTION

The scarcity of information dealing with the chemical composition of long bones after standardised physical training is striking especially as to the effects of training on the chemical composition of bones during growth. In adult animals running exercise accelerated the metabolism of minerals and organic substances (2, 14). Training has been reported either to increase or not to affect the concentrations of calcium and collagen in long bones after training (16, 30, 11, 28). We could not locate any report describing concentrations of nucleic acids and ground substance in the long bones of trained animals.

The aim of our study was to determine whether changes occurred in the concentrations of the nucleic acids and matrix components of the long bones of male mice trained either during growth only or until after reaching maturity. The concentrations of nitrogen, hydroxyproline, calcium, hexosamines, uronic acids, DNA and RNA-ribose were determined after moderate and intensive training programs. Changes in the physical parameters of the long bones as well as chemical changes of the Achilles tendons, skin, and heart have been published in other peports.

MATERIAL AND METHODS

Animals and physical training. A detailed description of experimental animals and training procedures is been published elsewhere (20). Two weeks old (14 ± 2 days) male mice of NMRI-strain were randomly assigned to test and control groups. The mice to be trained were gradually adapted to running on treadmill. The training took place on a 5^{o} inclined treadmill operating at a speed of 30 cm/s five days a week for 3 to 22 weeks. The duration of daily exercise as well as running speed were increased progressively during the first 3 weeks of training. The final daily exercise bouts were 50 and 80 minutes for moderate training programs I and II and 180 minutes for the/ intensive training program III. The daily exercise was divided into two sessions, one in the morning and the other in the afternoon.

Preparation of samples. The mice were killed under ether anestesia by decapitation. The hind and fore limbs were dissected with scissors at room temperature and immediately chilled in ice in sealed plastic tubes and stored deep-frozen (at -20°C) until preparation. For the preparation the limbs were allowed to thaw at room temperature. Immediately after thawing the long bones were manually separated as carefully as possible using scissors, scalpel and forceps from muscle and soft connective tissue. The bone marrow was removed by means of compressing air through an injection needle fitted to a syringe into each half of the long bone severed in the middle shaft, and the pieces of femur and humerus were dried (at 95°C for 2 days) and weighed. The bones which were used for nucleic acid analyses were prepared at +4°C, freezed and dried for two days in a lyophilizer (Heto, Denmark), weighed, and homogenized manually in glass tubes.

Chemical methods. The dried long bones were hydrolyzed overnight at 103°C in 2 N HCl for the analyses of glycosaminoglycans. Aliquots were taken to determine the concentrations of hexosamines and uronic acids. Hexosamines were freed from interfering chromogens by means of Dowex-50 cation exchange resin according to Boas (7). The concentration of hexosamines was determined by Blix's (6) modification of the Elson-Morgan method, and the uronic acid concentration was analyzed by Dische's carbatsol-reaction as modified by Bitter and Muir (5). For the determination of nitrogen and hydroxyproline the hydrolyzing of the previous hydrolysate was continued in 6 N HCl at 130°C for 3 hours and thereafter evaporated completely. The residues were dissolved in distilled water and filtered. Aliquots were taken to determine the concentration of hydroxyproline by Stegemann's colour reaction as modified by Woessner (34) and calcium with an atomic absorbtion spectrophotometer (Unicam SP 90A, England) according the method used by Pybus et al. (26). After combustion of the samples in 8 N sulphuric acid the concentration of nitrogen was determined by the method introduced by Minari and Zilversmith (23). The DNA and RNA fractions were separated by Schmidt and Thannhäuser's method as modified by Munro and Fleck (24). The DNA concentration was determined by Burton's

colour reaction (8) and the RNA-ribose concentration by Ceriotti's colour reaction (10). The variation coefficients for the chemical methods in this experiment were: nitrogen 4.6%, hydroxyproline 2.5%, hexosamines 4.1%, uronic acids 3.9%, DNA 2.0%, RNA-ribose 2.4%, and calcium 1.5%. The results are given in relation to the dry weight of samples.

In order to ensure the reliable comparison of the results between the trained and control animals special care was taken to analyse the respective samples of the trained and control animals within the same test-series. The comparability of the results between different training programs and age groups is somewhat impaired by the relatively large variation observed in the initial and final body weights of mice taken from different litters (Tables 1 and 2) and by some variation in the chemical methods over the three year experimental period.

Statistical methods. The results were statistically evaluated by Student's t-test for non-correlating means (two sided test).

RESULTS

The dry weight of long bones analysed chemically was significantly heavier only after 7 weeks' moderate training for the trained mice vis a vis the controls (Table 1). After intensive training, however, the dry weight of long bone tended to be on average lower for the trained mice compared to the controls (Table 2). The chemical results are presented in Tables 3 and 4. The concentrations of nucleic acids and hydroxyproline tended to decrease after some training programs. The mean concentration of nitrogen was higher in the long bones of trained mice after 22 weeks of moderate training and after 3, 5, 7 and 12 weeks of intensive training compared to the controls. The hexosamine concentration was also higher on the average in the bones of trained animals after prolonged training programs (12 and 22 weeks) at both training intensities. The uronic acid concentration increased by moderate training after 3, 12 and 22 weeks' training, but did not change after 3 and 5 weeks of intensive training or even decreased after 12 weeks' intensive training. No significant differences between the groups

were found in the calcium concentration after any of the two training programs. The hydroxyproline-nitrogen ratio tended to decrease and the hexosamine-hydroxyproline ratio to increase after prolonged training programs (Figure 1).

DISCUSSION

Physical training did not induce any changes in the calcium concentration of long bones. The result confirms those of comparable studies on rats (30, 11, 33), but conflicts with many human and animal studies in which training was started after prolonged inactivity (immobilization, bed rest) or after manifest osteoporosis (1, 16, 32). Also in our earlier experiments (19) an increase in the concentration of calcium was observed. This was not, however, confirmed by later more extensive experiments. The difference is probably related either to impaired growth in one group of mice or to exceptionally low initial body weight in another group of mice. According to our later experiments a larger increase in bone density was observed after 7 weeks intensive training in the bones of mice who started training about 2-3 days younger compared to the bones of older similary trained mice. Dalén and Olsson (12) have demonstrated that three months physical training was not enough to increase bone mineral content in healthy men, but competition runners active for more than 25 years had higher bone mineral content than their sedentary controls. Nilsson and Westlin (25) showed, in addition, that bone mineral content varies from one type of sport to another: the long bones of weight lifters were the densest, then those of runners, and least dense were the bones of swimmers. King and Pengelly (22) have stated that sprint running is more effective in increasing the cortical density of rat tibias than endurance running. Issekutz et al. (17) showed that pressure equal to body weight is essential and even more important than physical activity in maintaining a normal calcium and nitrogen balance during prolonged bed rest. Similar conclusions can be drawn from the results of the bipedal rat experiments (29). Thus it seems obvious that at least pressure equal to body weight is needed to maintain a normal calcium balance in bone tissue, but for increased

calcium concentrations evoked by physical activity in healthy animals and human beings relatively long and intensive training is needed with the possible exception of very young strenuously trained animals, although acceleration of mineral metabolism has been found to occur already after short training programs (2).

We found either no change or a slight reduction in bone hydroxyproline concentration after training. Chvapil et al. (11) also failed to observe significant changes in bone collagen concentration in young trained rats after 7 weeks' training, but after the prolonged training of young rats, and after 7 weeks' training of old rats they found an increase in the hydroxyproline concentration. Our results might differ from those of Chvapil et al. (11) because of differences in the age and/or species among the experimental animals: our mice were growing and the rats observed by Chvapil et al. (11) were already mature at the beginning of training.

The hydroxyproline-nitrogen ratio decreased after prolonged training in the bones of trained animals (Figure 1), which indicates that these bones contained relatively more noncollagen proteins than the controls. This difference appears to be due partly to a higher concentration of glycosaminoglycans, which resulted in increases also in the hexosamine-hydroxyproline ratio. The high ratios of hexosamines and uronic acids to hydroxyproline have been considered as an indication of biologically young connective tissues and even as an indication of the biological age of an organism (31, 9, 18). Since physical training seemed to slower the loss of glycosaminoglycans in bone tissue with age it can be concluded that prolonged physical activity may retard the rate of ageing. The unchanged DNA concentration in the bones suggests that physical training maintains the synthetic activity of cells on a higher level without changes in mitotic activity.

There is no clear explanation for mechanisms through which bone tissue adapts to physical training. Rosenfeld et al. (28) have demonstrated that calciferol plays a role in regulating the gain of body weight and affects also the accumulation of calcium in bone tissue. The observation

that the growth hormone concentration in blood is increased by physical exercise (27) is also of great interest given the findings of Asboe-Hansen (3, 4) that administering a growth hormone restores a 'young' glycosaminoglycan pattern in the ground substance of bones and intervertebral discs. The observations that physical activity accelerates the metabolic turnover rate of minerals and organic substance (2, 14) and activates some enzymes of energy metabolism (13) in bone also deserve attention as well as that an enchanced vascularity in bones may occur with physical training (21). Apparently, the above
preconditions for faster fracture healing after training (15).

As a conclusion we state that prolonged physical activity affects the organic matrix of long bones by maintaining above average concentrations of glycosaminoglycans in matured bones.

TABLE 1 . Effects of 3 to 22 weeks' moderate training (I+II) on the gain of body weight and dry weight of long bones. Mean ± SD and the number of observations are given together with Student's t-test.

Variable	Training time (weeks)	Animals						p
		Trained			Control			
Initial body weight (g)	(3)	11.1	±	1.6 (33)	11.0	±	1.6 (43)	N.S.
	(5)	11.0	±	1.6 (21)	10.8	±	1.9 (20)	N.S.
	(7)	12.7	±	2.2 (28)	9.9	±	1.8 (24)	<.001
	(12)	10.6	±	3.2 (51)	10.4	±	1.9 (22)	N.S.
	(22)	11.9	±	5.5 (85)	12.4	±	5.4 (63)	N.S.
Final body weight (g)	3	28.4	±	2.8 (30)	28.6	±	3.1 (28)	N.S.
	5	33.9	±	2.6 (28)	38.9	±	3.8 (22)	<.001
	7	34.5	±	2.8 (21)	33.9	±	2.9 (11)	N.S.
	12	37.1	±	3.8 (19)	37.9	±	2.3 (18)	N.S.
	22	41.8	±	4.6 (20)	42.1	±	5.2 (22)	N.S.
Dry weight of long bones (mg) (femur + humerus)	3	45.17	±	5.05 (28)	45.39	±	5.23 (28)	N.S.
	5	61.75	±	8.23 (22)	63.11	±	6.81 (22)	N.S.
	7	63.75	±	7.18 (21)	53.54	±	6.32 (10)	<.001
	12	73.22	±	10.20 (19)	72.43	±	6.10 (18)	N.S.
	22	68.16	±	8.16 (19)	75.58	±	12.09 (22)	<.05

Note Some of the above comparison data are taken from Kiiskinen (20)

TABLE 2 . **Effects of 3 to 12 weeks' intensive training (III) on the body weight and dry weight of long bones.** Mean ± SD and number of observations are given together with Student's t-test.

Variable	Training time (weeks)	Animals		p
		Trained	Control	
Initial body weight (g)	(3)	9.3 ± 2.3 (45)	9.6 ± 2.4 (31)	N.S.
	(5)	11.1 ± 1.6 (33)	11.1 ± 1.5 (32)	N.S.
	(7)	8.5 ± 1.0 (24)	8.5 ± 1.0 (43)	N.S.
	(12)	11.5 ± 1.1 (30)	11.8 ± 0.9 (20)	N.S.
Final body weight (g)	3	25,3 ± 4.0 (22)	28.7 ± 3.4 (15)	<.02
	5	33.1 ± 2.9 (22)	37.7 ± 2.9 (22)	<.001
	7	30.7 ± 2.9 (14)	35.3 ± 3.8 (17)	<.005
	12	40.7 ± 1.7 (11)	42.0 ± 2.7 (14)	N.S.
Dry weight of long bones (mg) (femur + humerus)	3	36.7 ± 6.1 (22)	33.6 ± 8.4 (15)	N.S.
	5	64.34 ± 4.69 (21)	66.24 ± 8.82 (21)	N.S.
	7	52.96 ± 7.32 (14)	56.72 ± 8.03 (17)	N.S.
	12	72.38 ± 7.44 (11)	75.61 ± 6.31 (13)	N.S.

Note Some of the above comparison data are taken from Kiiskinen (20)

TABLE 3. Effects of 3 to 22 weeks' moderate training (I+II) on the consentrations of DNA, RNA-ribose, nitrogen, hydroxyproline, hexosamines, uronic acids, and calcium in the long bones

Variable (µg/mgDW) and tissue	Training time (weeks)	Animals: Trained	Animals: Control	p
DNA (femur)	3	7.32 ± 0.44 (12)	7.02 ± 0.99 (9)	N.S.
	7	5.06 ± 0.81 (21)	5.57 ± 0.76 (10)	N.S.
	12	6.06 ± 1.32 (19)	6.39 ± 1.07 (12)	N.S.
RNA-ribose (femur)	3	7.39 ± 0.73 (12)	8.02 ± 0.56 (9)	<.10
	7	6.03 ± 0.76 (21)	6.23 ± 0.76 (10)	N.S.
	12	3.94 ± 0.49 (19)	4.48 ± 0.52 (14)	<.01
Nitrogen (femur + humerus)	3	41.52 ± 6.14 (28)	41.51 ± 11.32 (28)	N.S.
	5	51.07 ± 2.46 (22)	51.69 ± 3.11 (21)	N.S.
		48.71 ± 4.20 (11)	51.68 ± 6.77 (9)	N.S.
	12	53.17 ± 4.47 (19)	49.87 ± 4.17 (15)	N.S.
	22	47.20 ± 6.34 (19)	42.09 ± 5.01 (21)	<.01
Hydroxy-proline (femur + humerus)	3	25.04 ± 1.69 (28)	25.29 ± 1.68 (28)	N.S.
	5	27.84 ± 0.96 (22)	28.67 ± 0.77 (21)	<.05
	7	28.55 ± 2.51 (11)	30.08 ± 4.35 (9)	N.S.
	12	30.14 ± 2.38 (19)	29.43 ± 2.08 (15)	N.S.
	22	28.08 ± 4.43 (19)	27.41 ± 4.85 (22)	N.S.
Hexos-amine (femur + humerus)	3	3.21 ± 0.44 (27)	3.19 ± 0.58 (28)	N.S.
	5	3.03 ± 0.28 (22)	2.99 ± 0.10 (21)	N.S.
	7	2.77 ± 0.19 (11)	2.54 ± 0.30 (9)	N.S.
	12	2.78 ± 0.34 (19)	2.39 ± 0.23 (15)	<.001
	22	2.62 ± 0.30 (19)	2.29 ± 0.36 (22)	<.01
Uronic acids (femur + humerus)	3	2.70 ± 0.79 (27)	1.86 ± 0.84 (28)	<.001
	5	1.50 ± 0.20 (22)	1.48 ± 0.26 (22)	N.S.
	12	1.81 ± 0.21 (19)	1.46 ± 0.12 (15)	<.001
	22	1.49 ± 0.65 (19)	1.19 ± 0.36 (21)	<.10
Calcium (femur + humerus)	3	249.68 ± 16.25 (22)	250.23 ± 17.76 (22)	N.S.
	5	262.72 ± 7.96 (22)	258.00 ± 6.17 (22)	N.S.
	7	240.75 ± 22.17 (11)	248.43 ± 21.94 (9)	N.S.
	12	289.90 ± 40.25 (19)	294.20 ± 27.73 (15)	N.S.

Note Mean ± SD and number of observations are given together with Student's t-test

TABLE 4. Effects of 3 to 12 weeks' intensive training (III) on the consentrations of DNA, RNA-ribose, nitrogen, hydroxyproline, hexosamines, uronic acids, and calcium in the long bones

Variable (µg/mg DW) and tissue	Training time (weeks)	Animals		p
		Trained	Control	
DNA	3	4.89 ± 0.65 (16)	5.60 ± 0.59 (7)	<.001
(femur)	7	4.35 ± 0.90 (12)	4.75 ± 0.94 (16)	N.S.
	12	5.65 ± 0.62 (11)	5.45 ± 0.45 (14)	N.S.
RNA-ribose	3	6.04 ± 1.40 (16)	6.45 ± 0.87 (7)	N.S.
(femur)	7	4.74 ± 1.80 (12)	3.91 ± 0.83 (16)	N.S.
	12	4.08 ± 0.57 (11)	4.04 ± 0.61 (14)	N.S.
Nitrogen	3	53.10 ± 7.46 (21)	47.00 ± 4.60 (15)	<.01
(femur +	5	53.71 ± 3.59 (21)	50.90 ± 3.09 (21)	<.01
humerus)	7	54.86 ± 3.63 (10)	49.74 ± 3.23 (10)	<.01
	12	66.48 ± 8.76 (11)	55.70 ± 12.64 (13)	<.05
Hydroxy-	3	26.70 ± 1.62 (22)	27.10 ± 2.41 (15)	N.S.
proline	5	27.99 ± 1.62 (21)	28.31 ± 0.89 (21)	N.S.
(femur +	7	30.94 ± 0.86 (10)	29.69 ± 1.42 (10)	N.S.
humerus)	12	27.87 ± 1.83 (11)	28.25 ± 2.18 (13)	N.S.
Hexos-	3	3.16 ± 0.32 (13)	3.16 ± 0.50 (15)	N.S.
amine	5	2.93 ± 0.24 (21)	2.89 ± 0.32 (21)	N.S.
(femur +	7	3.64 ± 0.15 (10)	3.69 ± 0.28 (11)	N.S.
humerus)	12	2.56 ± 0.16 (11)	2.34 ± 0.15 (13)	<.01
Uronic	3	1.44 ± 0.41 (13)	1.44 ± 0.40 (15)	N.S.
acids	5	1.38 ± 0.18 (21)	1.38 ± 0.18 (21)	N.S.
(femur + humerus)	12	0.99 ± 0.09 (11)	1.18 ± 0.14 (13)	<.01
Calcium	5	262.40 ± 6.50 (21)	260.24 ± 5.71 (21)	N.S.
(femur +	7	222.71 ± 20.62 (14)	217.24 ± 14.37 (10)	N.S.
humerus)				

Note Mean ± SD and number of observations are given together with Student's t-test

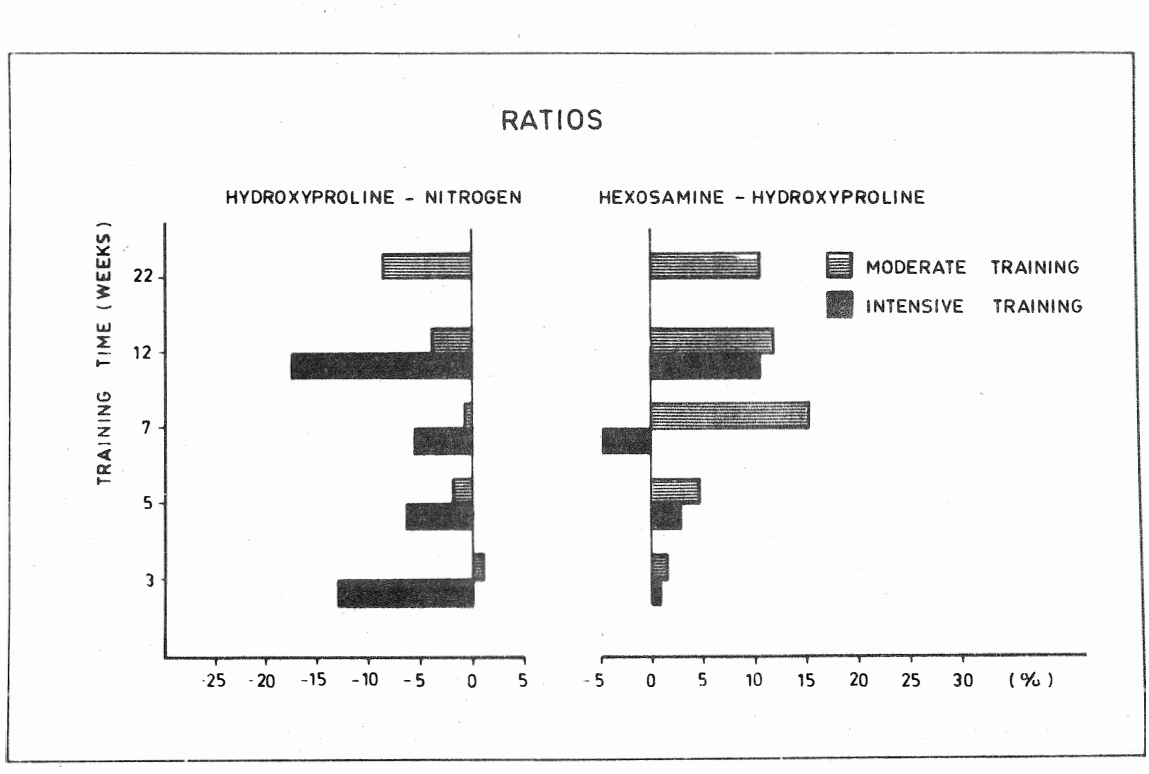

FIGURE 1. Ratios of hydroxyproline-nitrogen and hexosamine-hydroxyproline in percentage between the trained and control mice

REFERENCES

1. ABRAMSON, A.S. Atrophy of disease. Arch. Phys. Med. 29: 562-570, 1948.

2. ANDERSON, J.J.B., L. MILIN AND W.C. CARCKEL. Effect of exercise on mineral and organic bone turnover in swine. J. appl. Physiol. 30: 810-813, 1971.

3. ASBOE-HANSEN, G. Connective tissue. Ann. rew. Physiol. 25: 41-60, 1963a.

4. ASBOE-HANSEN, G. The hormonal control of connective tissue. Int. Tiss. Res. 1: 29-61, 1963b.

5. BITTER, T. AND H. MUIR. A modified uronic acid carbazole reaction. Analyt. Biochem. 4: 330-334, 1962.

6. BLIX, G. The determination of hexosamines according to Elson and Morgan. Acta chem. scand. 2: 467-473, 1948.

7. BOAS, N.F. Method for the determination of hexosamines in tissues. J. Biol. Chem. 204: 553-563, 1953.

8. BURTON, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315-323, 1956.

9. CASUCCIO, C. An introduction to the study of osteoperosis (Biochemical and biophysical research in bone ageing) Proc. roy. Soc. Med. 55: 663-668, 1962.

10. CERIOTTI, G.J. Determination of nucleic acids in animal tissues. J. Biol. Chem. 214: 59-70, 1955.

11. CHVAPIL, M., D. BARTOS AND F. BARTOS. Effect of long-term physical stress on collagen growth in the lung, heart and femur of young and adult rats. Gerontologia 19: 263-270, 1973.

12. DALÉN, N. AND K.E. OLSSON. Bone mineral content and physical activity. Acta orthop. scand. 45: 170-174, 1974.

13. HEIKKINEN, E., H. SUOMINEN, T. VIHERSAARI, I. VUORI AND A. KIISKINEN. Effect of physical training on enzyme activities of bones, tendons and sceletal muscle in mice. In: Metabolic Adaptation to Prolonged Physical Exercise. Proceedings of the 2nd International Symposium on Biochemistry of Exercise, Magglingen 1973, edited by H. Howald and J.R. Poortmans, Basel: Birkhäuser, 1975, p. 448-450.

14. HEIKKINEN, E. AND I. VUORI. Effect of physical activity on the metabolism of collagen in aged mice. Acta physiol. scand. 84: 543-549, 1972.

15. HEIKKINEN, E., T. VIHERSAARI AND R. PENTTINEN. Effect of previous exercise on fracture healing: A biochemical study with mice. Acta orthop. scand. 45: 481-489, 1974.

16. INGELMARK, B.E. Morpho-physiological aspects of gymnastic exercise. Bull. Fed. int. d'Educ. phys. 27: 37-41, 1957.

17. ISSEKUTZ, B. Jr., J.J. BLIZZARD, N.C. BIRKHEAD AND K. ROHDAHL. Effect of prolonged bed rest on urinary calcium output. J. appl. Physiol. 21: 1013-20, 1966.

18. KAO, K.-Y.T., W.E. HITT, R.L. DAWSON AND T.H. MCGAVACK. Conncective Tissue VII. Changes in protein and hexosamine content of bone and cartilage of rats at different ages. Proc. Soc. exp. Biol. (N.Y.). 110: 538-543, 1962.

19. KIISKINEN, A. AND E. HEIKKINEN. Effect of prolonged physical training on the development of connective tissues in growing mice. In: Metabolic Adaptation to Prolonged Physical Exercise. Proceedings of the 2nd International Symposium on Biochemistry of Exercise, Magglingen 1973, edited by H. Howald and J.R. Poortmans, Basel: Birkhäuser, 1975, p. 253-261.

20. KIISKINEN, A. Physical training and connective tissues in young mice Physical properties of Achilles tendons and long bones. Department of Public Health Publications No 28, University of Jyväskylä, 1976.

21. KIISKINEN, A. AND H. SUOMINEN. Blood circulation of long bones in trained growing rats and mice. Europ. J. appl. Physiol. 34: 303-309, 1975.

22. KING, D.W. AND R.G. PENGELLY. Effect of running on the density of rat tibias. Med. Sci. Sports 5:68, 1973.

23. MINARI, O. AND D.B. ZILVERSMITH. Use of KCN for stabilization of color in direct nesslerization of Kjeldahl digests. Analyt. Biochem. 6: 320-327, 1963.

24. MUNRO, N. AND A. FLECK. The determination of nucleic acids. In: Methods of Biochemical Analysis, edited by D. Glick, New York: Wiley Interscience, 1966, p. 113-176.

25. NILSSON, B.E. AND N.E. WESTLIN. Bone density in athletes. Clin. orthop. 77: 179-182, 1971.

26. PYBUS, J., F.J. FELDMAN AND G.N. BOWERS Jr. Measurement of total calcium in serum by atomic absorption spectrophotometry with use of a strontium internal reference. Clin. Chem. 16: 998-1007, 1970.

27. RENNIE, M.J. AND R.H. JOHNSON. Alteration of metabolic and hormonal responce to exercise by physical training. Europ. J. appl. Physiol. 33: 215-226, 1974.

28. ROSENFELD, R., A. ROSENFELDOVA, J. STEIGLOVA AND I. KRAPILOVA. Effect of exercise or acceleration on calcium metabolism in rats. Physiol. Bohemoslov. 22: 195-199, 1973.

29. SAVILLE, P.D. AND R. SMITH. Bone density, breaking force and leg muscle mass as functions of weight in bipedal rats. Amer. J. Phys. Antrop. 25: 35-39, 1966.

30. SAVILLE, P.D. AND M.P. WHYTE. Muscle and bone hypertrophy. Positive effect of running exercise in the rat. Clin. orthop. 65: 81-88, 1969.

31. SOBEL, H., J. MARMORSTON AND F.J. MOORE. Collagen and hexosamine content of femurs of rats. Proc. Soc. exp. Biol. 87: 346-349, 1954.

32. SMITH, E.L. AND S.W. BABCOCK. Effects of physical activity on bone loss in the aged. Med. Sci. Sports 5: 68, 1973.

33. TIPTON, C.M., R.D. MATTHES AND J.A. MAYNARD. Influence of chronic exercise on rat bone. Med. Sci. Sports 4: 55, 1972.

34. WOESSNER, J.F. Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys. 93: 440-447, 1961.

PART 2. BIOCHEMISTRY OF ACHILLES TENDONS

ABSTRACT. Effects of physical training on collagen, ground substance and nucleic acid concentrations in Achilles tendons were studied in male mice of NMRI-strain. The mice to be trained and their controls were about 2 weeks old at the beginning of the training, which took place on a 5^{o} inclined treadmill 5 days a week for 3 to 22 weeks. The daily duration of exercise was increased progressively over 3 weeks. The final daily exercise bouts were 50 and 80 minutes for moderate programs and 180 minutes for the intensive program at a speed of 30 cm/sec.

We found increased concentrations of DNA , nitrogen, hexosamines, and to a lesser degree, uronic acids in the Achilles tendons of the trained mice vis a vis the controls. No difference in the concentrations of hydroxyproline and RNA-ribose was observed between the groups. The ratio of glycosaminoglycans, especially hexosamines, to hydroxyproline increased and hydroxyproline to nitrogen decreased after prolonged training.

The results show that physical training during growth affects the chemical composition of Achilles tendons. Prolonged training may contribute to maintaining a high glycosaminoglycan concentration in matured tendons.

ACHILLES TENDONS, PHYSICAL TRAINING, COLLAGEN, GROUND SUBSTANCE, NUCLEIC ACIDS, GROWTH

INTRODUCTION

Our knowledge of the effects of physical training on tendons and ligaments is largely based on research concerning physical and morphological parameters (cf. Tipton et al., 24); only few publications have also covered some chemical variables.

Microscopic studies indicate an increase in the cross-sectional area of the primary bundles and, to a lesser degree, of the cell number as reasons for an increased volume of tendons in growing trained rabbits (12, 13). Contradictory results are published as to the collagen concentration of tendons and ligaments after training. Viidik (25) observed non-significant changes in the collagen concentration of tendons in trained rabbits and Tipton et al. (23) in the ligaments of trained rats whereas Tipton et al. (22) reported an increase in collagen concentration and no change in hexosamine concentration in the ligaments of trained dogs. Heikkinen and Vuori (11) demonstrated an accelerated turn-over of hydroxyproline and nitrogen accompanied by an increased hydroxyproline-nitrogen ratio in the tendons of trained aged mice. On the other hand prolonged immobilization of adult dogs brought about a nearly uniform reduction in the concentration of water, hexosamines and uronic acids and no changes in the hydroxyproline concentration in tendons capsules, collateral ligaments and cruciate ligaments after 10 to 12 weeks inactivity (1).

According to our preliminary studies (15) physical training seemed to cause changes in the chemical composition of Achilles tendons. Therefore a series of studies was carried out to obtain a better understanding of the biochemical reactions occuring in the Achilles tendons of male mice trained either during growth only or until after reaching maturity. Attention was focused on possible changes in the concentrations of nitrogen, hydroxyproline, hexosamines, uronic acids, and nucleic acids in the Achilles tendons of trained and control mice. We also investigated and have reported separately on chemical changes taking place during physical training in the connective tissues of long bones, skin and heart.

MATERIAL AND METHODS

Animals and physical training. A detailed description of experimental animals and training procedures is being published elsewhere (16). Two weeks old (14 ± 2 days) male mice of NMRI-strain were randomly assigned to test and control groups. The mice to be trained were gradually adapted to running on a treadmill. The training took place on a 5^{o} inclined treadmill operating at a speed of 30 cm/s five days a week for 3 to 22 weeks. The duration of daily exercise as well as running speed were increased progressively during the first 3 weeks of training.

The final daily exercise bouts were 50 and 80 minutes for moderate training programs I and II and 180 minutes for the intensive training program III. The daily exercise was divided into two sessions, one in the morning and the other in the afternoon.

Preparation of samples

The mice were killed under ether anestesia by decapitation, the limbs were cut off with scissors at room temperature and immediately chilled in ice in sealed plastic tubes and stored deep-frozen (at -20°C) until preparation. For the preparation the limbs were allowed to thaw at room temperature. Immediately after thawing the Achilles tendons were manually separated as carefully as possible using scalped and forceps from muscle and bone tissue, dried (at 95°C overnight) and weighed (Mettler H 20T, Switzerland). The Achilles tendons used for nucleic acid analyses were prepared at + 4°C, freezed and dried for one day in a lyophilizer (Heto, Danmark), and weighed, each sample containing both Achilles tendons from four animals.

Chemical methods

Achilles tendons were hydrolyzed overnight at 103°C in 2 N HCl for the analyses of glycosaminoglycans. Aliquots were taken to determine the concentrations of hexosamines (10) and uronic acids (3). For the determination of nitrogen and hydroxyproline the hydrolyzing of the previous hydrolysate was continued in 6 N HCl at 130°C for 3 hours and thereafter evaporated completely. The residues were dissolved in distilled water and filtered. Aliquots were taken to determine the concentration of hydroxyproline by Stegeman's colour reaction as modified by Woessner (27). After combustion of the samples in 8 N sulphuric acid the concentration of nitrogen was determined by the method introduced by Minari and Zilversmith (17). The DNA and RNA fractions were separated by Schmidt and Thannhäuser's method as modified by Munro and Fleck (18). The concentration of DNA was determined by Burton's colour reaction (4) and RNA-ribose concentration by Geriotti's colour reaction (6). The variation coefficients for the chemical methods in this experiment were: nitrogen 7.1 per cent, hydroxyproline 3.1 per cent, hexosamines 4.6 per cent, uronic acids 4.2 per cent, DNA 1.6 per cent, and RNA-ribose 3.2 per cent. The results are given in relation to the dry weight of samples.

In order to ensure the reliable comparison of the results between the trained and control animals special care was taken to analyse the respective

samples of the trained and control mice within the same testseries together with the appropriate standards. The comparability of the results between different training programs and age groups is somewhat impaired by relatively large variation observed in the initial and final body weight of mice taken from different litters (Tables 1 and 2) and by some variations in chemical methods over the three year experimental period.

Statistical methods

The results were statistically evaluated by Student's t-test for non-correlating means (two sided test).

RESULTS

The dry weight of Achilles tendons analysed chemically increased significantly in the trained animals compared to the controls only after 3 weeks' moderate training and decreased after 7 weeks' intensive training (Tables 1 and 2). The chemical results are presented in Tables 3 and 4. The concentration of nitrogen, hexosamines, DNA and, to a lesser degree, of uronic acids were on average higher in the Achilles tendons of trained mice vis a vis the controls after both training programs when continued beyond 3 weeks. No difference was observed between the groups in the concentration of hydroxyproline and RNA-ribose. A systematic reduction in the hydroxyproline nitrogen ratio was found after the two prolonged training programs (Fig. 1).

DISCUSSION

The results show that Achilles tendons respond chemically to physical training after a relatively short adaptation period both in cells and ground substance. The increased DNA concentration in the Achilles tendons of trained mice may be due either to an increased number of cells as observed by Ingelmark (13) or to an increased polyploidisation in tendon cell nuclei (2).

The collagen concentration in the Achilles tendons was not changed by training. Similar results have been found by Viidik (24) in the peroneus brevis tendon of rabbit after prolonged training and by Tipton et al. (23) in the knee ligaments of trained rats. On the other hand, Tipton et al. (22)

have reported an increased hydroxyproline concentration in the tendons of trained dogs. However, the age of the dogs could not be ascertained, hence, it is possible that the increase in hydroxyproline concentration was connected with ageing and not evoked by training (23).

The concentration of non-collagen proteins were significantly increased contrary to the findings of Tipton et al (22) as judged from the increased nitrogen concentration and decreased hydroxyproline-nitrogen ratio after prolonged training (Tables 3 and 4 and Figure 1). A part of this increase is explained by the increased concentration of glycosaminoglycans possibly indicating and activated synthesis of proteoglycans and glycoproteins in the fibroblasts of the Achilles tendons in trained animals. A relative increase in hexosamines and uronic acids in relation to hydroxyproline has been considered as an indicator of biologically young connective tissues (21, 20, 14, 7, 8, 9). Since physical inactivity seems to reduce (1) and physical activity to maintain (22) or even to increase the glycosaminoglycan-hydroxyproline ratio, this might indicate that the rate of maturation and ageing is influenced by training. The assumption is also supported by the observations on thermal reactivity (26, 5). Viidik demonstrated that distal tendons of the external digitorum longus muscle in the limbs of trained rabbits shrank less at 62° in a saline solution than those of the controls. Byrd (5) observed similar changes in rat tail tendons after training. Viidik concluded that the tendons of trained animals are molecularly less stable i.e. "younger", also indicating an accelerated metabolic turn-over, which agrees with the observations of Heikkinen and Vuori (11) reporting the encreased metabolism of collagen and total proteins in the Achilles tendons of aged mice after training.

More detailed analysis of different glycosaminoglycan components is needed to clarify the metabolic effects induced by physical training. Adaptation mechanisms also await further elucidation. Especially effects of growth, sex and other hormones should be studied in concomintant rheological and chemical investigations of tendons and ligaments.

ACKNOWLEDGEMENTS

We wish to thank Mrs. Kirsti Ojala and Mr. Erkki Helkala for skilful technical assistance and the Ministry of Education (Research Council for Physical Culture and Sport. Finland) for financial support.

TABLE 1. Effects of 3 to 22 weeks' moderate training (I+II) on the gain of body weight and dry weight of Achilles tendons. Mean ± SD and number of observations are given together with Student's t-test.

Variable	Training time (weeks)	Animals: Trained	Control	p
Initial body	(3)	11.1 ± 1.6 (33)	11.0 ± 1.6 (43)	N.S.
weight (g)	(5)	11.0 ± 1.6 (21)	10.8 ± 1.9 (21)	N.S.
	(7)	12.7 ± 2.2 (28)	9.9 ± 1.8 (24)	<.001
	(12)	10.6 ± 3.2 (51)	10.4 ± 1.9 (22)	N.S.
	(22)	11.9 ± 5.5 (85)	12.4 ± 5.4 (63)	N.S.
Final body	3	29.0 ± 2.4 (21)	28.0 ± 3.3 (21)	N.S.
weight (g)	5	33.9 ± 2.6 (21)	38.9 ± 3.8 (21)	<.001
	7	34.5 ± 2.8 (21)	33.9 ± 2.9 (11)	N.S.
	12	37.1 ± 3.8 (19)	37.9 ± 2.3 (18)	N.S.
	22	41.8 ± 4.6 (20)	42.1 ± 5.2 (22)	N.S.
Dry weight	3	1.54 ± 0.25 (21)	1.32 ± 0.21 (21)	<.05
of Achilles	5	1.64 ± 0.34 (21)	1.63 ± 0.34 (21)	N.S.
tendons (mg)	7	1.83 ± 0.23 (21)	1.73 ± 0.26 (9)	N.S.
	12	1.83 ± 0.19 (19)	1.84 ± 0.16 (18)	N.S.
	22	1.73 ± 0.39 (20)	1.72 ± 0.32 (22)	N.S.

Note Some of the above comparison data are taken from Kiiskinen (16).

TABLE 2. Effects of 5 to 12 weeks' intensive training (III) on the gain of body weight and dry weight of Achilles tendons. Mean ± SD and number of observations are given together with Student's t-test.

Variable	Training time (weeks)	Animals: Trained	Control	p
Initial body	(5)	11.1 ± 1.6 (33)	11.1 ± 1.5 (32)	N.S.
weight (g)	(7)	8.5 ± 1.0 (24)	8.5 ± 1.0 (21)	N.S.
	(12)	11.5 ± 1.1 (30)	11.8 ± 0.9 (20)	N.S.
Final body	5	33.1 ± 2.9 (22)	37.7 ± 2.9 (22)	<.001
weight	7	30.7 ± 2.9 (14)	35.3 ± 3.8 (17)	<.005
	12	40.7 ± 1.7 (11)	42.0 ± 2.7 (14)	N.S.
Dry weight	5	1.51 ± 0.22 (22)	1.61 ± 0.16 (22)	N.S.
of Achilles	7	1.03 ± 0.11 (14)	1.23 ± 0.18 (18)	<.05
tendons (mg)	12	2.16 ± 0.37 (11)	2.29 ± 0.26 (14)	N.S.

Note Some of the above comparison data are taken from Kiiskinen (16).

TABLE 3. Effects of 3 to 22 weeks' moderate training (I+II) on the concentrations of nitrogen, hydroxyproline, DNA, RNA-ribose, hexosamines and uronic acids in the Achilles tendons

Variable (ug/mg DW)	Training time (weeks)	Animals		p
		Trained	Control	
Nitrogen	3	164.47 ± 11.01 (21)	167.02 ±18.16 (21)	N.S.
	5	161.80 ± 19.62 (21)	150.99 ± 26.94 (20)	N.S.
	7	181.91 ± 10.55 (21)	167.19 ± 23.74 (9)	<.05
	12	194.63 ± 23.01 (19)	162.40 ± 17.03 (18)	<.001
	22	177.20 ± 25.51 (20)	167.50 ± 15.60 (22)	N.S.
Hydroxy-	3	114.54 ± 7.50 (21)	113.84 ± 18.15 (21)	N.S.
proline	5	124.58 ± 5.23 (21)	128.92 ± 8.09 (21)	N.S.
	7	118.57 ± 9.92 (21)	109.78 ± 13.73 (9)	N.S.
	12	133.32 ± 21.60 (19)	134.50 ± 10.65 (18)	N.S.
	22	123.65 ± 12.43 (20)	127.18 ± 7.92 (22)	N.S.
DNA	3	2.79 ± 0.16 (3)	2.56 ± 0.12 (3)	N.S.
	7	2.44 ± 0.25 (5)	2.07 ± 0.16 (5)	<.05
RNA-ribose	3	1.61 ± 0.15 (3)	1.55 ± 0.07 (3)	N.S.
	7	1.29 ± 0.11 (5)	1.38 ± 0.11 (5)	N.S.
Hexos- amines	5	3.74 ± 0.44 (10)	2.94 ± 0.48 (10)	<.01
Uronic acids	5	0.98 ± 0.13 (11)	0.91 ± 0.17 (9)	N.S.

Note Mean ± SD and number of observations are given together with Student's t-test

TABLE 4. Effects of 5 to 12 weeks intensive training (IH) on the concentrations of nitrogen, hydroxyproline, DNA, RNA-ribose, hexosamines and uronic acids in the Achilles tendons

Variable (µg/mg DW)	Training time (weeks)	Animals		p
		Trained	Control	
Nitrogen	5	209.55 ± 27.84 (22)	183.91 ± 27.65 (22)	<.05
	12	186.52 ± 31.78 (9)	153.65 ± 20.05 (9)	<.05
Hydroxy-	5	128.45 ± 7.13 (22)	128.31 ± 10.76 (22)	N.S.
proline	7	129.43 ± 8.19 (12)	124.43 ± 5.31 (14)	N.S.
	12	126.40 ± 16.13 (10)	126.43 ± 15.31 (14)	N.S.
DNA	7	2.45 ± 0.10 (3)	1.93 ± 0.07 (3)	<.001
RNA	7	1.42 ± 0.21 (3)	1.42 ± 0.09 (3)	N.S.
Hexos- amine	5	3.33 ± 0.65 (22)	2.64 ± 0.63 (22)	<.01
Uronic acid	5	1.26 ± 0.46 (21)	1.02 ± 0.25 (22)	<.05

Note Mean ± SD and number of observations are given together with Student's t-test

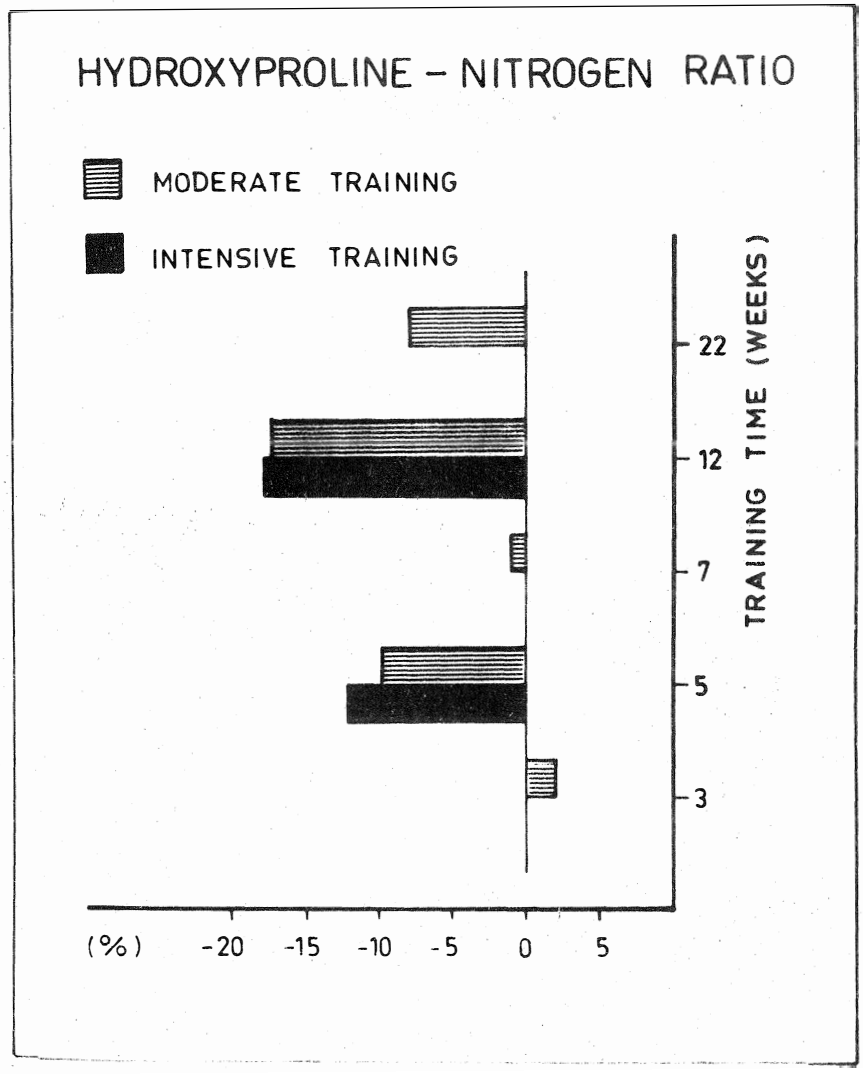

FIGURE 1. Ratio of hydroxyproline to nitrogen in percentage between the trained and control mice

REFERENCES

1. AKESON, W. H., D. AMIEL, D. LaVIOLETTE and SECRIST, D. The connective tissue responce to immobility: an accelerated ageing responce? Exp. Geront. 3: 289-301, 1968.
2. BENEKE, G, K. ERVIG and W. SCHMIDT. Polyploidisierung und Heterochromatisierung von Schenenzellekernen in Abhängigkeit von Lebensalter. Beitr. Path. Bd. 141: 19-32, 1970.
3. BITTER, T. and H. MUIR. A modified uronic acid carbazole reaction. Analyt. Biochem. 4: 330-334, 1962.
4. BURTON, K. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62: 315-323, 1956.
5. BYRD, R. J. The effect of controlled, mild exercise on the rate of physiological aging of rats. J. Sports Med. Phys. Fitness 13: 1-3, 1973.
6. CERIOTTI, G. J. Determination of nucleic acids in animal tissues. J. Biol. Chem. 214: 59-70, 1955.
7. CLAUSEN, B. Influence of age on connective tissue. Hexosamine and hydroxyproline in human aorta, myocardium and skin. Lab. Invest. 11: 229-234, 1962a.
8. CLAUSEN, B. Influence of age on connective tissue. Uronic acid and uronic acid-hydroxyproline ratio in human aorta, myocardium, and skin. Lab. Invest. 11: 1340-1345, 1962b.
9. CLAUSEN, B. Influence of age on chondroitin sulfates and collagen of human aorta, myocardium, and skin. Lab. Invest. 12: 538-542, 1963.
10. GATT, R. and E. R. BERMAN. A rapid procedure for the estimation of amino sugars on a micro scale. Analyt. Biochem. 15: 167-171, 1966.
11. HEIKKINEN, E. and I. VUORI. Effect of physical activity on the metabolism of collagen in aged mice. Acta physiol. scand. 84: 543-549, 1972.
12. INGELMARK, B. E. Über den Bau der Sehnen während verschiedener Altersperioden und unter verschiedener funktionellen Bedingungen. Upsala Läk.-Fören. Förh. 50: 357-396, 1945.
13. INGELMARK, B. E. Der Bau der Sehnen während verschiedener Altersperioden und unter wechselnden funktionellen Bedingungen. I. Eine quantitative morphologische Untersuchung an den Achillessehnen weisser Ratten. Acta anat. (Basel) 6: 113-140, 1948.
14. KAO, K. T., D.M. HILKER and T. H. McGAVAK. Connective tissue III. Collagen and hexosamine content of tissues of rats of different ages. Proc. Soc. exp. Biol. 104: 359-361, 1960.

15. KIISKINEN, A. and E. HEIKKINEN. Effect of prolonged physical training on the development of connective tissue in growing mice. In: Metabolic Adaptation to Prolonged Physical Exercise. Proceedings of the 2nd International Symposium on Biochemistry of Exercise, Magglingen 1973, edited by H. Howald and J. R. Poortmans. Basel: Birkhäuser, p. 253-261, 1975.

16. KIISKINEN, A. Physical training and connective tissues in young mice - Physical properties of Achilles tendons and long bones. Department of Public Health Publications No 28. University of Jyväskylä, 1976.

17. MINARI, O. and D. B. ZILVERSMITH. Use of KCN for stabilization of color in direct nesslerization of Kjeldahl digests. Analyt. Biochem. 6: 320-327, 1963.

18. MUNRO, N. and A. FLECK. The determination of nucleic acids. In: Methods of Biochemical Analysis, edited by D. Glick. New York: Wiley Interscience p. 113-176, 1966.

19. RENNIE, M. J. and R. H. JOHNSON. Alteration of metabolic and hormonal responce to exercise by physical training. Europ. J. appl. Physiol. 33: 215-226, 1974.

20. SOBEL, H., S. GABAY, E. T. WRIGHT, I. LICHTENSTEIN and N. H. NELSON. The influence of age upon the hexosamine-collagen ratio of dermal biobsies from men. J. Geront. 13: 128-131, 1958.

21. SOBEL, H. and J. MARMORSTON. The possible role of the gel-fiber ratio of connective tissue in the aging process. J. Geront. 11A: 2-7, 1956.

22. TIPTON, C. M., S. L. JAMES, W. MENGER and T. -K. TCHENG. Influence of exercise on strength of medial collateral knee ligaments of dogs. Amer. J. Physiol. 218: 894-902, 1970.

23. TIPTON, C. M., R. K. MARTIN, R. D. MATTHES and R. A. CAREY. In: Metabolic Adaptation to prolonged Physical Exercise. Proceedings of the 2nd International Symposium on Biochemistry of Exercise. Magglingen 1973, edited by H. Howald and J. R. Poortmans. Basel: Birkhäuser, p. 262-267, 1975..

24. TIPTON, C. M., R. D. MATTHES, J. A. MAYNARD and R. A. CAREY. The influence of physical activity on ligaments and tendons. Med. Sci. Sports. 7: 165-175, 1975.

25. VIIDIK, A. The effect of training on the tensile strength of isolated rabbit tendons. Scand. J. palst. reconstr. Surg. 1:141-147, 1967.

26. VIIDIK, A. Functional properties of collagenous tissues. Int. Rev. Connect. Tissue Res. 6: 127-217, 1973.

27. WOESSNER, J. F. Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. 93: 440-447, 1960.