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Connective Tissue VII. Changes in Protein and Hexosamine Content of Bone and Cartilage of Rats at Different Ages.* (27574)

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Previously(1,2,3) we reported changes with age in the protein and hexosamine content of several soft tissues of the rat. In general, tissues rich in scleroprotein underwent greater changes in protein and hexosamine composition than did tissues low in scleroprotein. In the former, there was a decrease in nonscleroprotein and a concomitant increase in scleroprotein. Further, we found the hexosamine content to be increased with age in aorta and decreased with age in tendon and skin. Meyer (4) reported that 3 mucopolysaccharides have been isolated from bone, namely, hyaluronic acid, chondroitin sulfate C and keratosulfate. The keratosulfate content of human rib cartilage increased with an increase in age(5). To date, no data have been reported on the relationship between the mucopolysaccharide and the protein of bones and cartilage. If mucopolysaccharide is found in one or more of the several fractions of protein, prepared by standardized methods, it will clearly indicate a very intimate relationship with and a tight binding to the corresponding protein. For these reasons, in the present study hexosamine determinations were carried out for each protein fraction.

Our methods used for fractionation of soft tissue protein are not directly applicable to the fractionation of proteins in bone and cartilage, since the large amount of salts present alter solubility. Desalination is time-consuming and may be associated with a loss of soluble protein and soluble collagen. A combination of the methods of Lowry(6) and Fitch (7) afforded us a satisfactory procedure for fractionation of the various proteins found in the connective tissue of bone and cartilage. The present study is concerned with 1) fractionation of the proteins of bone and cartilage into 6 components and 2) variations with increasing age in the protein and hexosamine content of these moieties.

Method. Bone and cartilage from skull, femur, rib and vertebrae of female rats of Wistar strain, 5 weeks and 2½ years of age, respectively, were freeze dried and ground into fine powder in a Wiley electric mill.† 0.2 to 0.4 g of the powdered bone or cartilage were fractionated according to Schema I into 6 protein fractions, *i.e.*, trichloroacetic acid (TCA) supernatant of base soluble protein, nonscleroprotein, soluble collagen, insoluble

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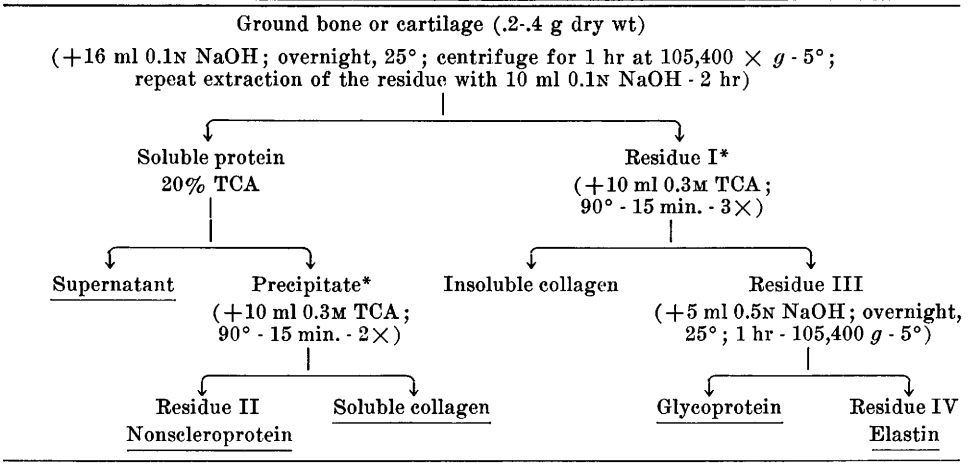
† Purchased from Arthur H. Thomas Co., Philadelphia.

SCHEMA I. Tissue Fractionation.



* Fitch's method of extraction(7).

collagen, glycoprotein and elastin. Each of the 6 protein-containing fractions was hydrolyzed with 4N HCl at 100° for 5 hours in a sealed tube. Nitrogen determinations were made by micro Kjeldahl digestion and Conway diffusion technics(8). Hexosamine was determined by the method of Rondle and Morgan(9). Proteins were calculated as 6.25 × (total N-hexosamine N).

Results. The changes in protein and hexosamine content of the TCA supernatant fraction of bone and cartilage are shown in Table I. In skull, neither protein nor hexosamine was changed with increasing age. Between 5 weeks and 2½ years of age, a significant decrease to approximately one-half in both protein and hexosamine was shown for femur, rib, vertebra, vertebral cartilage and rib cartilage. The TCA supernatants of both costal and vertebral cartilages contained approximately 10 times as much hexosamine as similar supernatants from the bones studied.

The TCA supernatants of femur and rib were dialyzed against water for 5 hours with 3 changes of water. In femur 70% of hexosamine and 60% of non-hexosamine nitrogen was non-dialyzable. In rib cartilage 90% of hexosamine and 50% of non-hexosamine nitrogen was non-dialyzable.

The changes in protein and hexosamine content of the nonscleroprotein fractions of bone and cartilage are shown in Table II. A significant decrease in protein and hexosamine with increasing age was observed in 5 of the 6 tissues studied. This decrease is greatest in the rib. There is no change with age in either the nonscleroprotein or hexosamine content of vertebral cartilage.

In Table III are shown the changes with age in soluble collagen and hexosamine in the

TABLE I. Protein* and Hexosamine in TCA Supernatant Fraction of Base Extract of Bone and Cartilage of Rat.
(g/100 g of dried tissue)

Tissue	Protein 5 wk	Protein 2.5 yr	P	Hexosamine 5 wk	Hexosamine 2.5 yr	P
Skull	2.23 ± .29†	2.17 ± .50		.043 ± .012	.039 ± .007	
Femur	5.86 ± 1.20	3.72 ± .36	<.01	.200 ± .050	.066 ± .009	<.001
Rib	5.58 ± 1.14	3.48 ± .45	<.01	.116 ± .040	.056 ± .016	<.02
Vertebrae	5.42 ± .98	4.14 ± .85		.134 ± .048	.068 ± .013	<.05
Vertebral cartilage	7.02 ± 1.68	4.62 ± .60	<.05	1.517 ± .146	.848 ± .141	<.001
Rib "	7.10 ± .68	3.88 ± .02	<.001	1.527 ± .302	.738 ± .024	<.001

* (Total N—Hexosamine N) x 6.25.
† Figures are mean ± standard deviation, calculated from 4-8 determinations of each tissue studied.

TABLE II. Protein* and Hexosamine in the "Nonscleroprotein Fraction" of Bone and Cartilage of Rat.
(g/100 g of dried tissue)

Tissue	Nonscleroprotein 5 wk	Nonscleroprotein 2.5 yr	P	Hexosamine 5 wk	Hexosamine 2.5 yr	P
Skull	2.1 ± .3†	.9 ± .4	<.001	.020 ± .002	.010 ± .003	<.001
Femur	5.0 ± .4	2.0 ± .5	<.001	.036 ± .008	.018 ± .005	<.001
Rib	4.5 ± .6	1.4 ± .3	<.001	.029 ± .008	.012 ± .003	<.01
Vertebrae	7.0 ± .7	3.5 ± 1.6	<.001	.049 ± .009	.025 ± .006	<.001
Vertebral cartilage	4.9 ± 1.4	4.0 ± 1.1		.105 ± .011	.114 ± .021	
Costal "	10.5 ± .4	4.6 ± .6	<.001	.112 ± .024	.073 ± .014	<.01

* (Total N—hexosamine N) x 6.25.
† Figures are mean ± standard deviation, calculated from 4 determinations each of 5-wk-old skull and ribs; 3 determinations of 5-wk-old rib cartilage and 6 determinations of all other tissues studied.

TABLE III. Protein* and Hexosamine in "Soluble Collagen Fraction" of Bone and Cartilage of Rat.
(g/100 g of dried tissue)

Tissue	Soluble collagen 5 wk	Soluble collagen 2.5 yr	P	Hexosamine 5 wk	Hexosamine 2.5 yr	P
Skull	.51 ± .10†	.28 ± .07	<.01	.009 ± .003	.003 ± .001	<.01
Femur	.70 ± .05	.36 ± .07	<.001	.010 ± .002	.004 ± .002	<.01
Rib	.64 ± .10	.35 ± .06	<.001	.010 ± .003	.004 ± .002	<.01
Vertebrae	.90 ± .09	.56 ± .11	<.001	.010 ± .001	.005 ± .001	<.001
Vertebral cartilage	1.28 ± .27	.86 ± .05	<.01	.032 ± .005	.033 ± .010	
Costal "	1.41 ± .10	.71 ± .18	<.01	.023 ± .004	.017 ± .003	<.05

* See Table I.
† See Table I.

TABLE IV. Protein* and Hexosamine in "Insoluble Collagen Fraction" of Bone and Cartilage of Rat.
(g/100 g of dried tissue)

Tissue	Insoluble collagen 5 wk	Insoluble collagen 2.5 yr	P	Hexosamine 5 wk	Hexosamine 2.5 yr	P
Skull	18.4 ± 1.7†	17.8 ± .8		.078 ± .007	.061 ± .012	<.05
Femur	12.4 ± .7	12.0 ± .6		.099 ± .014	.071 ± .023	<.001
Rib	15.7 ± 1.0	16.3 ± 1.5		.071 ± .006	.053 ± .010	<.01
Vertebrae	14.0 ± .9	15.8 ± .8	<.01	.094 ± .010	.072 ± .017	<.05
Vertebral cartilage	25.4 ± 2.4	22.1 ± 1.8	<.05	.183 ± .024	.173 ± .035	
Costal "	22.3 ± 2.5	14.7 ± 3.5	<.02	.196 ± .036	.434 ± .077	<.01

* See Table I.
† See Table I.

"soluble collagen fraction" of bone and cartilage. Both soluble collagen and hexosamine were decreased by approximately 50% in old age in 5 of 6 tissues studied. The decrease was least marked in vertebral cartilage. There was no change in hexosamine content of the "soluble collagen fraction" of vertebral cartilage.

Age is not associated with any change in the amount of insoluble collagen in the skull, femur or rib (Table IV). On the other hand, there is an increase in insoluble collagen in the vertebrae and a decrease in rib and vertebral cartilage. In old age, the hexosamine content of "the insoluble collagen fractions" of all of the bones studied was decreased, and that of rib cartilage increased.

After extraction of insoluble collagen we were left with a mixture histologically still staining for both glycoprotein and elastin. Treatment, as above mentioned in the Schema with 0.5N NaOH, removed the glycoprotein,

TABLE V. Glycoprotein* of Bone and Cartilage of Rat and Its Hexosamine Content. (g/100 g of dried tissue)

Tissue	Glycoprotein			Hexosamine		
	5 wk	2.5 yr	P	5 wk	2.5 yr	P
Skull	1.71 ± .13†	1.20 ± .05	<.001	.052 ± .005	.038 ± .002	<.001
Femur	1.90 ± .25	1.48 ± .07	<.01	.068 ± .007	.054 ± .002	<.001
Rib	1.73 ± .15	1.20 ± .07	<.001	.050 ± .007	.037 ± .003	<.01
Vertebrae	2.21 ± .28	1.35 ± .09	<.001	.064 ± .008	.048 ± .006	<.01
Vertebral cartilage	2.13 ± .42	1.95 ± .36		.061 ± .008	.070 ± .011	
Costal "	3.01 ± .59	5.94 ± .27	<.001	.076 ± .015	.206 ± .019	<.001

* N x 6.25.
† See Table I.

analyses for which are contained in Table V. It should be emphasized here that no such material was ever found in our fractionation of the connective tissue of soft parts. This glycoprotein could not be extracted even when the concentration of NaOH was increased to 0.5N for the initial extraction.

After removing the above fraction, our residue stained as though it were pure elastin. However, in analysis, trace amounts of hexosamine, as shown in Table VI, were still present. With increasing age, both this glycoprotein of bone and its hexosamine decreased to approximately ⅔ the value noted in the young animal. In rib cartilage, there was a doubling of the values for this glycoprotein and its hexosamine between 5 weeks and 2½ years of age. With age, there was a decrease in both elastin and the associated hexosamine in all the bones studied. No change occurred in the elastin or hexosamine of costal and vertebral cartilage.

In Table VII we have summarized the changes occurring during aging in the protein and hexosamine in bone and cartilage of rat as determined by 1) adding the corresponding figures from Tables I through VI, and as determined by 2) direct analysis of specimens without fractionation. The latter set of determinations were from specimens obtained from a separate set of subjects not involved in any of the fractionation procedures. The correlation between the two sets of data is quite close, except for the figures representing total protein in the femurs of young animals. Perhaps one might expect variations of this degree with determinations upon animals of several lots sacrificed at different times.

Discussion. The percentage of hexosamine found in several bones examined never exceeds 4% of the total protein (Table I), whereas, in both vertebral and rib cartilage this figure approximates 20% in the young animal and 10% in the older one. Furthermore, from the data on the dialysis of the TCA supernatants obtained during the extraction of femur and rib cartilage, it may be logical to assume that a glycoprotein (less than 4% hexosamine) exists in the femur, whereas, mucoprotein exists in rib cartilage. If this is the case, this latter material should contain the sodium chondroitin sulfate-protein complex as described in rat rib cartilage by Gross *et al.*(10).

TABLE VI. Protein* and Hexosamine in "Elastin Fraction" of Bone and Cartilage of Rat. (g/100 g of dried tissue)

Tissue	Elastin			Hexosamine		
	5 wk	2.5 yr	P	5 wk	2.5 yr	P
Skull	.181 ± .053†	.099 ± .036	<.02	.008 ± .002	.004 ± .002	<.01
Femur	.175 ± .044	.112 ± .036	<.001	.007 ± .002	.005 ± .001	<.05
Rib	.162 ± .026	.078 ± .020	<.05	.006 ± .001	.004 ± .002	<.001
Vertebrae	.229 ± .046	.127 ± .040	<.01	.008 ± .002	.006 ± .002	
Vertebral cartilage	.167 ± .032	.157 ± .026		.007 ± .001	.006 ± .001	
Costal "	.177 ± .060	.256 ± .140		.006 ± .002	.008 ± .004	

* See Table I.
† See Table I.

TABLE VII. Summary of Changes in Protein and Hexosamine in Bone and Cartilage of Rat. (g/100 g of dried tissue)

Tissue	Protein		Hexosamine	
	5 wk	2.5 yr	5 wk	2.5 yr
Skull—Sum of Data (Tab. I-VI)	25.13	22.45	.210	.155
Direct Determination without Fractionation	23.98(2)*	20.89(2)	.180(2)	.141(2)
Femur—Sum of Data (Tab. I-VI)	26.04	19.67	.420	.158
Direct Determination without Fractionation	31.91(3)	17.97(5)	.463(3)	.189(5)
Rib Cartilage—Sum of Data (Tab. I-VI)	44.50	30.09	1.940	1.476
Direct Determination without Fractionation	42.37(3)	31.09(5)	2.000(3)	1.420(5)

* Represents No. of animals corresponding to No. of determinations from which the mean is derived.

These data also show that, with increasing age, the nonscleroprotein of bone and cartilage decreases in a fashion similar to that observed for the same material in soft tissues, which are rich in connective tissue(1,2). Our data on the decrease of insoluble collagen in the costal cartilage of rats are in close agreement with the results obtained in the guinea pig by Exer and Lagier(11). The glycoprotein fraction (containing 3-4% hexosamine) is a specific protein fraction, found for the first time, appearing only in bone and cartilage and not in the soft tissues studied. This particular protein contains a higher percentage of hexosamine than the nonscleroprotein of the corresponding bone and cartilage. It is decreased in bone and increased in costal cartilage with increases in age. Meyer(5) reported that the amount of keratosulfate in human costal cartilage increased directly with age. Such an increase may account for our finding more hexosamine in the glycoprotein fraction of costal cartilage in the older animal than in the younger. Inasmuch as the specific glycoprotein above referred to also increased with age, we are led to believe that the hexosamine fraction is probably an integral part of this glycoprotein. The exact nature of the glycoprotein found in these experiments and not previously isolated from bone and cartilage is now being investigated.

TCA is considered to be a good solvent for extraction of mucopolysaccharides(12,13). Therefore, in this study, some of the hexosamine in the collagen fraction is probably extracted from the glycoprotein fraction and the glycoprotein fraction may contain more hexosamine than is now shown in Table IV.

Summary. 1. Proteins of bone and cartilage from skull, femur, rib and vertebrae of female rats, Wistar strain, age 5 weeks and 2½ years, respectively, were fractionated into TCA supernatant of base soluble protein, nonscleroprotein, soluble collagen, insoluble collagen, glycoprotein and elastin. 2. With an increase in age, there was a decrease of protein and hexosamine in TCA supernatant of base soluble protein, nonscleroprotein and soluble collagen of both bone and cartilage. 3. The amount of insoluble collagen in skull, femur and rib did not change with age; in vertebrae it increased and in cartilage, it decreased. 4. Certain glycoproteins, not present in the connective tissues studied, other than bone and cartilage, decreased in bone and increased in costal cartilage wtih increasing age.

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Recovery of PPLO of Atypical Pneumonia on Artificial Agar Medium.* (27575)

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Epidemiologic and volunteer studies have strongly suggested that the Eaton agent is etiologically associated with the syndrome of cold agglutinin positive atypical pneumonia of man(1,2,3). After its recognition in 1944 this filterable agent was classified as a virus, although it is of large size (200-250 mμ) and sensitive to streptomycin and tetracycline(4, 5,6). Recently the organism was cultivated on an artificial agar medium and shown to be a PPLO, *i.e.*, a member of the genus *Mycoplasma*(7). Growth on agar was achieved with a strain of Eaton agent (FH) which had been passaged many times in eggs and subsequently adapted to monkey kidney tissue culture(8,9). The present study was initiated to determine if naturally occurring strains of Eaton PPLO resembled the laboratory adapted strain as regards growth on an agar medium. In addition it was important to learn if PPLO agar was a sensitive medium for the recovery of strains from infected individuals.

Materials and methods. Agar medium. The complete medium consisted of 7 parts Difco PPLO agar, 2 parts uninactivated horse serum and 1 part 25% yeast extract. The extract was prepared from active baker's yeast by boiling with distilled water, followed by clarification through filter paper. This material was then autoclaved and stored at −20°C until used. The agar was prepared in 70 ml quantities and sterilized by autoclaving. After cooling to approximately 45°C the agar was supplemented with the yeast extract and horse serum. The final medium contained 500 units of penicillin and 5 μg of amphotericin per ml and thallium acetate at a final concentration of 1:2000. Approximately 6 to 7 ml of the agar medium was added to a plastic plate which measured 5 cm in diameter.

Cultivation. One-tenth ml of throat swab fluid was inoculated onto the agar and spread by streaking with a bacteriologic loop. The inoculated plates were incubated at 35°. One series of plates was incubated in a desiccator jar; a lighted candle was placed in the jar before the lid was secured in order to produce an increased CO_2 tension. Other inoculated plates were incubated aerobically without further manipulation.

* The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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