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AGE-RELATED CHANGES IN THE COLLAGEN OF BOVINE CORIUM

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SUMMARY

Bovine collagen of increasing biological age shows greater resistance to the action of acids, as measured by extractable collagen. Citrate-soluble content of corium from fetal skin was 30.9% while that of 3 to 6-week old calf was 6.4% and of 18-month steer was 3.2%. Similar trends were also noted in neutral salt-soluble (NSC) and pepsin-treated, acid-soluble (PSC) fractions. The greater resistance to degradation in biologically older bovine collagen is thought to be directly related to increased cross-link formation.

The ultrastructure of bovine corium was shown to become more highly ordered and have less inter-fibrillar matrix material, as the animal age increased. The average diameter of the fibrils also increased. These conditions would be expected to provide an environment favorable to further inter-molecular cross-link formation.

INTRODUCTION

Collagen constitutes the major protein component of skin, bone, tendon and other forms of connective tissue. It is a high molecular weight, relatively insoluble fibrous protein. The basic molecular unit of collagen is a triple-stranded coil, rod-like structure of about 14 Å in diameter, 2800 Å in length and a weight average molecular weight of 300,000 Daltons (1).

Because of the unique physical and chemical properties of this fibrous protein, considerable attention has been focused on collagen from both a medical and industrial viewpoint. The traditional commercial uses have been in the leather, gelatin and glue industry. More recently, however, collagen has found use in such diverse applications as absorbable sutures (2) and regenerated edible sausage casings (3,4). The success of these new applications depend on the ability of collagen, which has been disintegrated from its native fiber assembly, to repolymerize and reform again into a fibrous state.

Commercial processes for producing regenerated collagen structures are largely based on the utilization of the corium layer of hide obtained from bovine animals. These processes are highly dependent upon a uniform and consistent quality of the starting raw material. Unfortunately, the molecular properties of this collagen tissue, particularly as they relate to the age of the animal are poorly understood. While there are reports in the literature on age-related changes in collagen, most of this work has been carried out on very pure forms of collagen such as rat-tail tendon. This study was performed in order to better define some of the chemical and physical changes which occur in bovine collagen during the course of the animals development.

MATERIALS AND METHODS

Bovine skin from freshly slaughtered Holstein cattle was used. Four age groups were included: (1) fetal, (2) 3 to 6 weeks, (3) 18 months, and (4) 40 months. Prior to use in testing, the skin pieces were split with a meat slicer to remove the hair and grain layer.

The effect of acid, neutral salt and enzyme on the extractability, solubility and molecular size distribution was investigated using the fractionation scheme illustrated in Fig. 1. The chromatographic procedure utilized a Waters Associate Model ALC/GPC-224 Liquid Chromatograph equipped with a UV-absorbance detector and a differential refractometer. In initial studies, several column types were evaluated for their ability to separate the collagen fractions under study. The best separation was obtained with Waters Associates μ-Bondagel column (4 mm o.d. x 30 cm), which has an exclusion volume of 1.5 ml and a total volume of 3 ml.

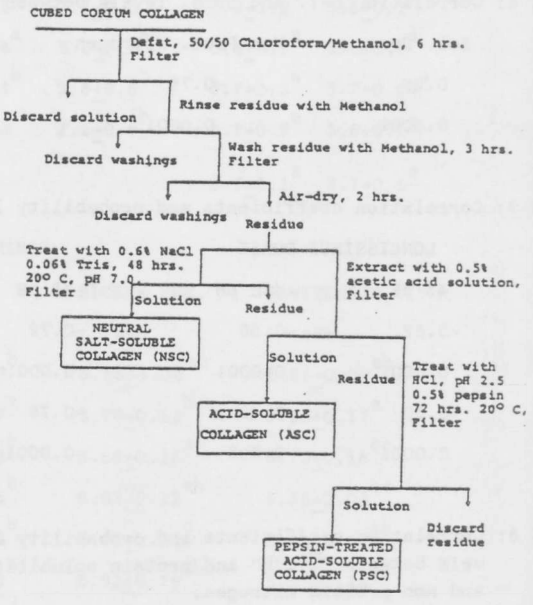

Figure 1. Fractionation of collagen for analysis by Gel Permeation Chromatography.

The packing material is a controlled porosity silica with a permanently bonded ether phase which creates a hydrophilic surface. A known molecular weight collagen Type III, obtained from Sigma Chemical Co. was used as a standard. The soluble collagen fraction of each sample at a retention time of 4.3 minutes was calculated by reference to the standard using the following expression:

$$\% \text{ soluble collagen} = \frac{A}{B} \times \frac{0.05}{C} \times 100$$

where A = Peak height of sample at retention time 4.3, B = Peak height of collagen Type III at retention time 4.3 and C = Dry weight of sample in 100 ml solution.

Relationship to histological and ultrastructure changes was observed using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Corium samples were fixed in a solution of 4.0 percent paraformaldehyde and 1.0 percent glutaraldehyde in cacodylate buffer at pH 7.2 for 18 hours at 26°C. Following rinsing in buffer and then in water, the samples were fixed further in 1.0 percent aqueous osmium tetroxide for 2 hours at 26°C and rinsed in dionized water. Dehydration was accomplished in a graded series of ethanol solutions. Impregnation and embedding was accomplished in Epon 812 epoxy resin. Diamond knife

ultramicrotomy provided thin sections which were post stained using lead citrate and uranyl acetate to enhance contrast (5). Thin sectioning was carried out in both longitudinal and cross directions. The resulting stained, thin sections were examined, and single electron images were recorded at electron optical magnifications of 2,600X, 6,600X and 14,000X while employing an RCA EMU4 transmission electron microscope. The images were enlarged for printing to a total magnification of 10,000X, 25,000X and 50,000X.

For SEM examinations, samples were fixed as above, but without the use of secondary osmium tetroxide treatment. These samples were critical-point dried to retain the anticipated three dimensional morphology of the hide components. The cut sample edges were sputter coated with a conductive layer of gold-palladium. Electron images were recorded at magnifications of 3,000X, 5,000X, 10,000X and 25,000X, using JEOL JSM-U3 scanning electron microscope.

RESULTS AND DISCUSSION

Soluble collagen fractions

Typical elution patterns for acetic acid extracts of corium samples from fetal, 3 to 6-week and 18-month animals are shown in Fig. 2. The major peak at elution time 4.3 is considered to be the tropocollagen monomer in the 300,000-350,000 Dalton molecular weight range. The peak height of this fraction was used in determining quantitative estimates for comparison between age groups. Results show decreasing levels of soluble collagen at elution time 4.3 with increasing age. The data on acid-soluble collagen from fetal corium (7.6%) was significantly higher ($P \leq 0.001$) than that of the 18-month (3.4%) age group and significantly higher ($P \leq 0.05$) than that of the 40-month (2.7%) age group. Similarly, the soluble collagen content of the 3 to 6-week age group was significantly higher than that of the 18-month ($P \leq 0.01$) and the 40-month ($P \leq 0.05$) age groups. The 18-month age group also contained significantly higher acid soluble collagen ($P \leq 0.05$) than that of the 40-month age group. When the calculations for acid-soluble collagen were based on peak area measurements, the same trend was observed.

Typical elution patterns for sodium chloride extracts of corium samples from fetal, 3 to 6-week and 18-month animals are shown in Fig. 3. In fetal skin extracts, three major peaks were evident at elution time: 4.1, 4.3 and 4.5. Comparative estimates of total NaCl extractables were calculated from peak areas. While the significance of these values are in question, the trend appears to show a reduction in solubles extracted with increasing animal age.

The effects of pepsin on the solubilization of collagen from corium of varying ages can be seen in Fig. 4. The fetal sample was most susceptible to enzyme degradation, followed next by the 3 to 6-week sample, then the 18-month sample. No differences in the elution profiles were seen between 18-month and 40-month samples.

The results of the present study are in agreement with that of Nishihara and Miyata (6) who found that the collagen fiber of mature steer hide was resistant to the action of pepsin while that of young calf was more readily solubilized. Etherington (7) concluded that the number and location of intermolecular cross-links were important factors in the degree of resistance of tendon collagen to enzymatic dissolution. The new data support the view that chemical cross-links form within and between the monomolecular collagen to render it more resistant to enzymatic degradation. The authors have reported elsewhere on the work carried out to measure stress/strain behavior of denatured collagen as an indication of cross-link density (8). While considerable information now exists on the possible mechanisms involved in collagen cross-link formation, the complete chemical identity of these stabilizing forces in insoluble mature tissue remains to be elucidated.

ELECTRON MICROSCOPY

Typical TEM micrographs obtained in each of the four animal age groups studied are shown in Fig. 5 (cross-sectional views at 50,000X). The average fibril diameter reported (Table 1) is based on the measurement of 200 individual fibrils. The average fibril diameter increased from 1050 Å at fetal age to 1552 Å at 40 months. In fetal skin, 98 percent of the fibril diameter measurements were below 1600 Å while in the 40-month animals only 40 percent were below this size.

As in the case with fibrils, the fiber, composed of hundreds of thousands of fibrils, increased in diameter with increasing age. In fetal skin (Fig. 6), collagen fibers are shown in cross-section of relatively small diameter (4×10^4 to 8×10^4 Å) which are separated by relatively wide areas of interfiber matrix. This interfiber matrix is also quite extensive in corium from 3 to 6-week calf, but is considerably reduced in the 18-month sample and a very minor fraction in the 40-month sample. A corresponding decrease in the volume fraction of interfibrillar matrix between fibrils is also evident with increasing age. Each collagen fibril within a bundle is surrounded by a cellular substance of low electron density. As the collagen fibrils increase in diameter with age, the enlargement appears to be accompanied by a reduction in the relative amount of interfibrillar material which invests each fibril.

Scanning electron images from 3,000X to 25,000X magnification show a more three-dimensional morphology than that obtained with TEM. It is also evident that with increased age, the structure was more dense and compact. For example, the micrograph of fetal corium (Fig. 7) reveals a rather loose arrangement of fibers and fiber bundles with considerable interfiber matrix. This is in striking contrast to that of the 18-month (Fig. 8) sample which shows massive, tightly-packed fiber bundles.

The observations in both TEM and SEM studies that show an ultrastructure of bovine corium becoming more dense and compact with increasing age is in agreement with studies on other collagen tissues. Rundall (9) reported a decrease in matrix volume with age in chick tendon. Studies carried out by Torp et al. (10) on rat-tail tendon also showed a decrease in the cellular material between fibrils with increasing age. Such conditions would be expected to provide an environment favorable to further intermolecular cross-link formation on the basis of shorter distance between molecules in the packing arrangement. This is also compatible with the hypothesis that the more crystalline portions of the molecule have the greater frequency of cross - links.

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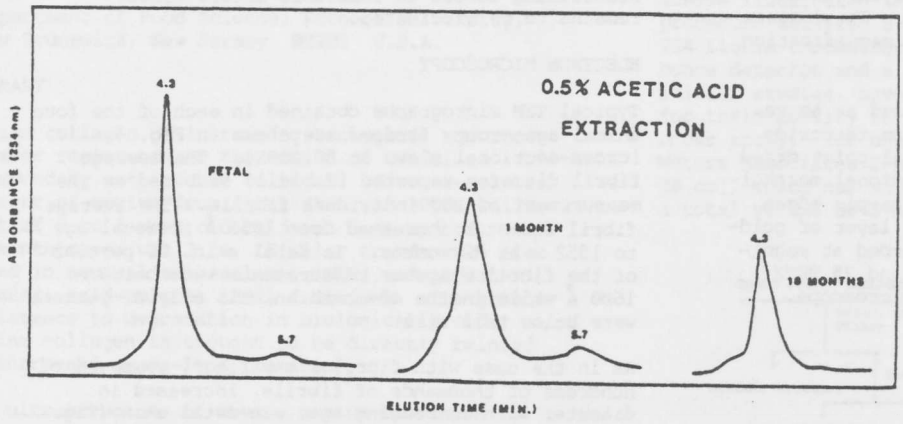


Figure 2. Elution patterns of 0.5% acetic acid extracts from corium samples of fetal, 1 month and 18 month animals.

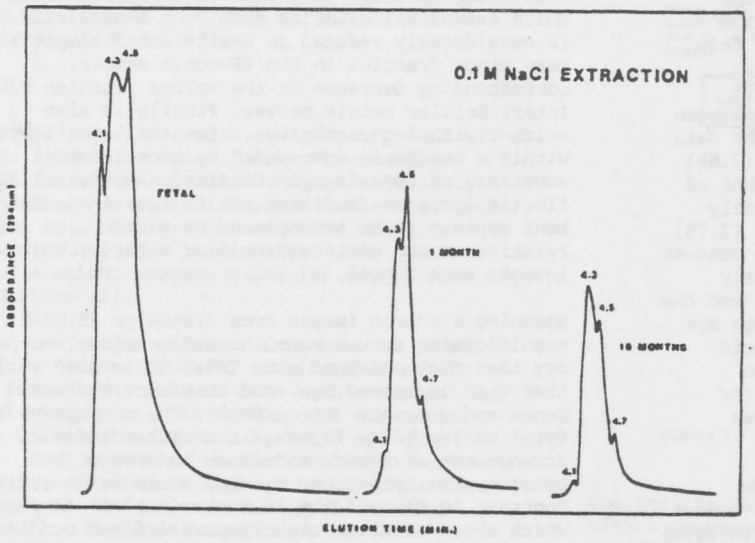


Figure 3. Elution patterns of 0.1M NaCl extracts from corium samples of fetal, 1-month, and 18-month animals.

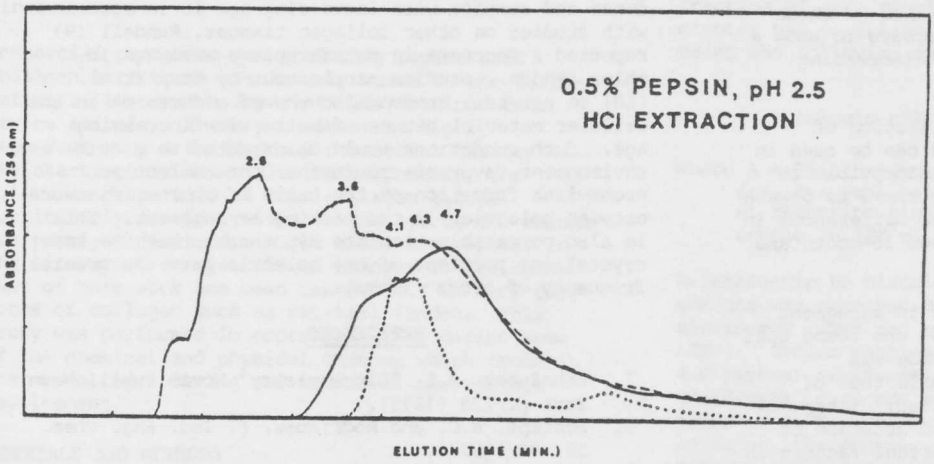
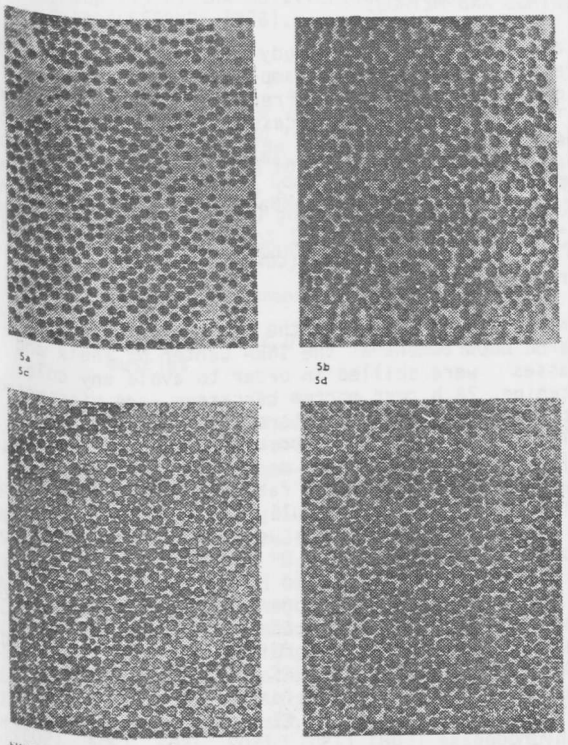


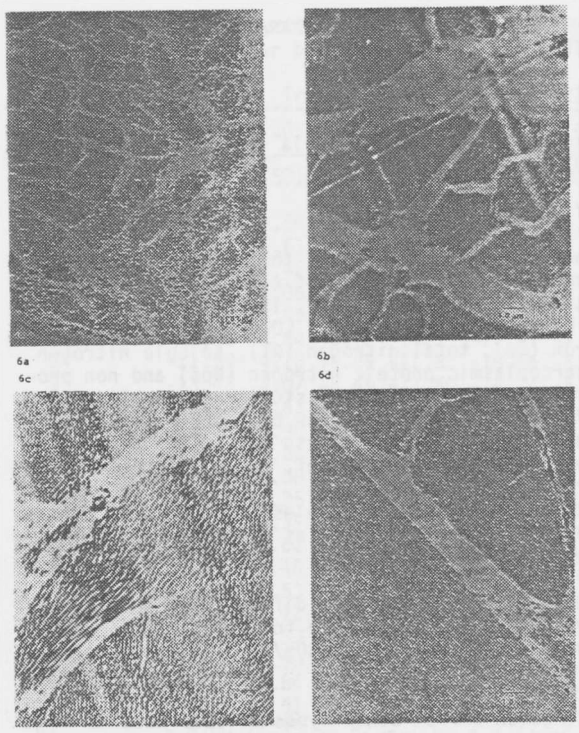
Figure 4. Elution patterns of soluble collagen extracts recovered following treatment of corium with 0.5% pepsin, pH 2.5. The dashed line is from fetal skin, the solid line from 1-month age and the dotted line from 18-month age animals.



5a
5c

5b
5d

FIGURE 5.– Transmission electron micrographs of corium from animals of increasing age: a) cross-sectional view of fetal corium showing individual fibrils with considerable interfibrillar material which surrounds each fibril, b) 1-month animal showing fibrils which appear to be somewhat larger, c) 18-month animal in which fibrils are considerably larger and more densely packed, d) 40-month animal showing less interfibrillar matrix. Cross-sectional views at 50,000X.



6a
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FIGURE 6.– Transmission electron micrographs of corium from animals of increasing age: a) fetal corium showing collagen fibers in cross-section which are separated by relatively wide channels in the matrix, b) corium from 1-month animal showing fibers in cross-section containing more densely packed fibrils, c) corium from 18-month animal with fiber diameters considerably larger than that of the fetal or 1-month animals, d) corium from 40-month animal showing exceedingly large fibers with very little inter-fiber matrix material. The fibrils are very densely packed. 10,000X.

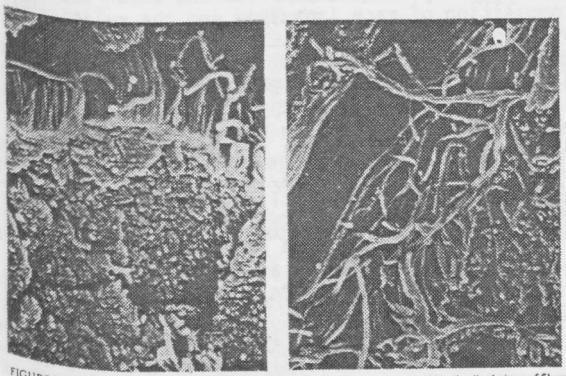

FIGURE 7.– Scanning electron images of fetal corium showing cross-sectional and longitudinal views of fibers and fiber bundles. The fibers are quite regular in form but of a relatively small diameter. The diameter of the individual fibrils in longitudinal section can vary in SEM because of variations in heights of the gross specimens (5,000X).

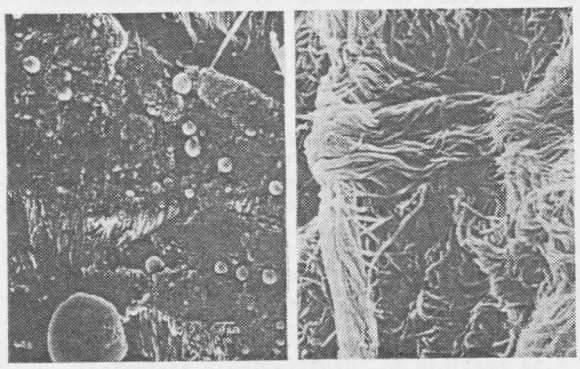

FIGURE 8. – Scanning electron images of corium from 18-month animals showing both cross as well as longitudinal aspects of the fibrils, fibers and fiber bundles. The fibers are closely packed and of considerably larger diameter than that seen in corium of fetal or 1-month samples (Figures 6 and 7). The spherical forms seen at the surface of the razor cut edge of the fiber bundle are likely to be the untravelled ends of several fibrils (5,000X).

Table 1.

Comparison Of Changes In The Fibril And Fiber Diameters

With Age As Estimated From Electron Micrographs[a]

Age of Animal	TEM Plate No.	Fibril Diameter[b] (Å) Average	Fibril Diameter[b] (Å) Range	TEM Plate No.	Fiber Diameter[c] (Å) Range
Fetal	7 & 29	1050	600-1400	63	4×10^4 - 8×10^4
1 month	17	1142	800-1600	20	8×10^4 - 18×10^4
18 months	43	1413	1000-1800	46	10×10^4 - 32×10^4
40 months	48	1552	1000-2200	49	24×10^4 - $<32 \times 10^4$

[a] Measurements made directly from electron micrographs specified, using a 7X hand-held magnifier with reticle graduated in 0.1 mm.

[b] 1 mm @ 50,000X = 200 Å. In each case 200 measurements were taken.

[c] 1 mm @ 10,000X = 4000 Å. All fibers in the field were measured, however they were too few to use in calculating an average.