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A CONTRIBUTION TO THE KNOWLEDGE OF THE FINE STRUCTURE AND CHEMICAL PROPERTIES OF ANIMAL CONNECTIVE TISSUE FIBRILS.

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INTRODUCTION.

Collagen is the principal protein constituent of the loose and compact connective tissues. It occurs either as a network of fibrils of approximately one micron thickness and of many microns length associated with almost all tissues in the whole animal series; or as thick bundles forming the tendons (1). Collagen is a fibrous protein, according to the classification of Astbury (2). It is insoluble in water, organic solvents, and dilute solutions of alkalies and acids at ordinary temperatures. The prolonged action of hot water changes collagen into gelatin, a chemical reaction of great importance and from which is derived the term itself (collagen means "glue former"). Another characteristic property of collagen is its swelling by action of dilute acids and alkalies (3). The amino acid composition is roughly the same in the various collagens from different animals, but the relative percentage composition changes from animal to animal (4). The majority of the collagens show a high content of glycine, hydroxyproline, and of aromatic and sulfur containing acids (5, 6). Studies of collagen fibrils by physical methods, such as high and low angle X-ray scattering, electron microscopy, and polarization microscopy have put in evidence the fibrous nature of collagen and have shown the existence of a characteristic period, averaging 640 Å in collagen from the entire animal series (7). The 640 Å period has been observed also in collagen from fossil animals, for instance in the demineralized tusk of a 15,000 year old mammoth (4,7). On

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the other hand, high angle X-ray scattering, which reveals small spacings in the molecular architecture, shows differences in the spacings of the various collagens (8). Of all these methods, electron microscopy provides a special approach to the fine structure of the fibrils and to their arrangement in the cells and tissues by direct visualization. Unfortunately, due to only 10 A resolving power of electron microscopes, details of the architecture of the polypeptide chains within the fibrils cannot yet be observed.

At present there are three major trends of research on collagen fibrils. The first is the consideration of its fine structure, starting from the spatial arrangement of the polypeptide chains forming the fibrils to the organization of the fibrils into the networks of the tissues and tendons. Such studies are being carried out intensively at the moment, but are far from completion (9,10). From such studies serious attempts have been made to reconstruct models of the polypeptide chains in the collagen fibrils. Of these the most promising at the moment are the models of Bear (4), Randall (11), Pauling (12) and Huggins (14), inasmuch as the model of Astbury has been proven unsatisfactory (13). The second trend is the study of the chemical properties of collagen fibrils, particularly with a view to improving the quality of leather (3, 15). Not only is collagen important as the raw material for the manufacture of gelatin and glue, but is extremely important in the manufacture of leather. The hide, or corium, has as its main component (35%) a network of collagen fibrils which give it strength and elasticity. To maintain these properties and to eliminate the disadvantage of putrescence, the collagen fibrils are rendered insoluble by reaction with tannins and chromium salts. These reactions can be controlled by observation with the electron microscope. In the third place, from the biological point of view, collagen is a structural protein intimately associated with the organization of tissues and organs. Therefore knowledge of the normal structure of the collagen fibrils from various tissues and organs, under differing physico-chemical conditions, is the starting point for the understanding of the changes which occur in normal tissues, e.g. histogenesis of the connective tissues and ageing process, as well as pathological changes such as those produced by collagenase in rheumatic fever or gangrene (16, 26).

In the present study we aim to present some electron microscope observations of normal collagen fibrils from various tissues of several animals, principally reptiles; and to show the action of certain chemical and physical factors upon the fibril structure. It has seemed more suitable to present the methods, materials, results and discussion together under each section, rather than as separate chapters.

PERIOD AND FINE STRUCTURE OF FIBRILS

We have studied collagen fibrils associated with the myofibrils of the following skeletal muscles: external oblique of the toad, *Bufo paracnemis*, of the caiman, *Caiman latirostris* (Daudin, 1802), of the snakes *Constrictor constrictor amarali* Stull, 1932 and *Liophis miliaris merremi* (Wied, 1821), of the pigeon and the human. From the reptilian series (caiman, snakes and the lizard, *Tupinambis teguixin* (L., 1758)) were used also collagens from the following muscles: hyoglossus, temporalis, retractor caudae, tip of tongue and heart. Observations were also made on collagen fibrils from veins and the deferent canal of the lizard and snakes. In addition we used tedons from the front feet, dorsum and tail of the albino rat, and tendon from the posterior poison gland of *Bothrops alternata* D.B. et D., 1854 and its homologue in *Constrictor*. The fibrils were prepared for electron microscopy by the technique previously described for preparation of myofibrils (35). Observations were made in both the Siemens, 100b, and the RCA, EMU, electron microscopes. Measurements were obtained from enlarged electron micrographs taken in the RCA, electron microscope whose magnification was determined within 5% error (17).

In all cases the collagen fibrils appear as single filaments, at times in a network or in bundles, presenting a characteristic cross striation formed by the alternation of dark and light bands, which have been called respectively *A* and *B* bands by Schmitt (18) and *D* and *H* bands by Wolpers (19). The dark bands are regions of high electron scattering power, meaning that they have a greater density or more atoms per unit volume than have the light bands. In the metal shadowed preparations the bands are clearly evident when the direction of shadowing is parallel or nearly parallel to the long axis of the fibril (Figure 1). In those preparations where the shadowing is at right angles to the long axis the bands are not clearly seen in the fibrils but the fibril contour is visible in the shadow. In unshadowed preparations the bands can be seen only when the fibrils are quite clean, i.e. when the ground substance layer, sometimes attached to the fibrils, does not cover and obscure the bands. It may be seen clearly in Figure 1 that the *A* band is higher than the *B*, thus giving to the fibril the aspect of a string of beads. However, this appearance is in reality due to the loss of water from the fibril, since it has been shown by means of replicas of wet collagen fibrils that the surface is uniformly smooth. It thus seems reasonable to assume that band *B* has a higher water content than band *A* (20). By selective staining with the so-called electron stains, i.e. osmic acid, phosphotugstic acid or uranyl acetate, a finer structure can

be evidenced in nonshadowed preparations. Four dark lines appear in the *A* band (a, b, c, d,) and a single line in the *B* band (e). Under certain conditions the "b" and "e" lines may appear to be split into two lines each, thus giving a total of seven lines (23). In shadowed fibrils not all the lines become visible. However, due to the fact that the "b" and "c" lines are of large diameter, they become prominent in clean fibrils that are shadowed parallel to their long axis (Figure 1,2), giving the appearance of an extra cross striation in the *A* band, having a period of 270 Å, the so-called intraperiod of shadowed preparations (27). The sum of the lengths of the *A* plus *B* bands adds up to an average of 640 Å, which agrees thus with the characteristic spacing of the dry collagen fibrils as revealed by small angle X-ray scattering (4). In all of our experiments we used metal shadowed specimens, hence variations of the sub-lines were not considered in this study.

Most of the previous work in the electron microscopy of collagen has involved formalin fixation since formalin does not change the intrinsic double refraction of collagen, hence does not change the arrangement of the polypeptide chains in the fibrils (28). Inasmuch as our studies include a variety of collagens it was deemed advisable to determine the influence of several fixatives before entering the definitive experiment. Thus the period and diameter of fibrils was determined after the use of formalin, Zenker's and Bouin's solutions, as well as unfixed material. The results obtained with the tendon from the salivary gland of *Constrictor* are presented in Table 1, where it will be seen that the average period is the same in the unfixed and the fixed materials, but that the range of diameters varies with the fixative. The aspects of the fibrils after such treatment is shown in Figures 1 and 2. On the basis of these results we used either unfixed material, or fixation with 5% formalin in the definitive experiments.

The final observations on the normal collagen fibrils included measurements of the period and diameter of a number of fibrils taken from different regions of various animals, and studies on the form of the fibril in general. In Table 2 are presented measurements of the length of period and diameter of fibrils from various organs and animals. It can be seen that although the period varies slightly, both from organs of the same animal or from animal to animal, it remains uniformly around 640 A, confirming Bear's observations with low angle scattering (8). The diameters presented greater variability. In the fibrils from certain organs, e.g. tongue of the lizard, the diameters are uniform. In others, however, as for example from the tail of the caiman, Figure 3, the range of diameters may be extensive, in this case from 640 to 1400 Å. The constancy of period plus the variation in diameter of the fibril has been taken

by Bear (4) as the basis of his model of the collagen fibril to support the view that the fibril is composed of units, the protofibrils, whose diameter is many times smaller than that of the fibril and whose composition is that of few polypeptide chains, the whole aligned in such manner as to give constant period.

It is believed that the collagen fibril is composed of a series of hollow rings (27) and indeed electron micrographs of cross sections of tendons (9) have been presented showing the hollow appearance. These observations in no way disagree with the observations made with the polarizing microscope (28) indicating that collagen fibrils have both form and intrinsic birefringence, inasmuch as the protofibrils are located only in the periphery. Randall (9) disagrees with the idea of the hollowness of the fibril, claiming that if true the fibril should show less density than in fact is the case. As a result of the present studies we believe that the fibril is hollow, and understand as "hol lowness" the absence of protein or other solid material and the presence of a liquid phase in the core of the fibril. The liquid phase disappears during the drying process in the preparation of the fibril for the microscope, leaving a partially collapsed fibril of beaded appearance. In ultrathin sections the fibrils appear hollow due to the preservation of the tridimensional structure and the loss of water during fixation. The general form of the collagen fibril is well ilustrated in a series of photographs of the tendons of the posterior poison gland of *Bothrops* (Figures 5 and 6). The tendon was left in physiological saline at 4-5 degrees C for 6 weeks for maceration, then washed and blended in distilled water, dried and shadowed with chromium. It will be seen that some of the fibrils split lengthwise showing in the region of the split a series of incomplete rings(5A, 6A). In others the orientation of the section of the fibril is such that one can observe a hollow, broken end (5A). And in still others the extremities of the fibrils are flattened out (5B) or frayed (6B), suggesting that the inner material has been drawn off during the drying process. If, as we believe on the basis of these preparations, the fibril has a liquid, or semi-liquid core, then Randall's comments are no longer valid. Further evidence of the ring structure of the fibril is the extensibility. Under the action of tension along the length of the single fibril (the whole tendon cannot be greatly extended as it breaks too easily) the fibril increases in length, principally by change of length of the light and with little or no change in the dark band. If the tension is sufficient the rings appear to separate, breaking the fibril into units comparable to the sarcomeres of myofibrils. This process can be best observed in fibrils under tension caused by breakage of the supporting grid film in the electron microscope (as in Figure 4 showing fibrils of unfixed tail tendon being deformed by longitudinal tension due to film breakage), inasmuch as stretch of the single fibril by other means is exceedingly difficult. Such observations

play an important role in the visualization of the disposition of the amino acids in the polypeptide chains inasmuch as in collagen the alpha-beta keratin chain model cannot be applied (4, 29).

ACTION OF HOT WATER ON FIBRILS

It is well known that, under the prolonged action of hot water, collagen dissolves and turns into gelatin. For tendon there is a characteristic shrinkage temperature which depends upon the medium and the previous treatment of the tendon (3). For fresh tendon in water this temperature is from 60 — 65 degrees C. Fresh tendon, at this temperature, first suffers a sudden shortening accompanied by increase in diameter and decrease in period. Upon further heating the tendon is dissolved slowly and is changed into an amorphous structure, as indicated by low angle X-ray scattering (3, 4). We have carried these observations further by noting the changes in single fibrils by means of the electron microscope, using rat tail tendon. The single fibrils after two minutes in distilled water at 60 degrees C shorten in length, decrease in period and increase in diameter, thus indicating that the macroscopical changes mentioned above are the result of changes at the fibrillar level (Figure 10A). At 40 degrees C no change in the structure of the fibrils could be observed (Figure 10B). At 60 degrees C after two minutes the extremities of the fibrils begin to lose their shape. After a longer exposure the striations begin to disappear, showing that the loss of the characteristic long spacing (revealed by low angle X-ray scattering) is accompanied by the loss of the 640 Å period, thus indicating that the two are actually the same. After further exposure at this temperature the fibrils dissolve completely. At 100°C the changes occur with great rapidity. Within two minutes the fibrils are changed completely into gelatin.

Our results with the rat tendon thus agree with those of Lelli (30) for human dermis.

The action of the hot water can be interpreted as follows (4). The protofibrils are first changed from the orderly state, normally occuring in the intact collagen fibril, into a disordered state. With continued exposure the hot water changes the collagen protofibrils into free gelatin molecules.

ACTION OF DILUTE ACETIC ACID ON TENDON

Years ago Nageotte (31) showed that rat tail tendon could be dissolved in dilute acetic acid (10^{-4} molar) producing a clear and very viscous solution.

This observation has been extended to collagens of different animal origins (cf. Bear for references (4)). The collagen can be reprecipitated from the viscous solution by changing the salt concentration by dialysis, or by diluting with suitable buffers of definite pH (32, 34). The reprecipitated collagen presents the same structure as the original, as shown by X-ray diffraction (33), i. e. the 640 A spacing, but a less orderly internal arrangement. The understanding of the reprecipitation of collagen from solution, as well as the reaction of collagen solutions with mucoproteins (34), polysacharides, glucoproteins and nucleic acid derivatives (7), which also produce precipitates formed of banded fibrils, is of the utmost importance for obtaining information on the mechanism of fibrogenesis in tissues.

In view of the fact that descriptions in the literature of the exact conditions for dissolution and reprecipitation of collagen are incomplete (32) we have repeated these experiments with several types of collagen from several animals, varying the temperature, molarity of acetic acid, pH, and ionic concentration. We have thus determined the definite conditions necessary for precipitation of certain collagens, and have discovered that certain others enter only with difficulty, or not at all, into solution, and that temperature plays an important part in the process.

A. — Rat Tail Tendon — If rat tendon is placed, under sterile conditions, in any volume of 10^{-4} molar acetic in stoppered flasks at 4-5°, or at 37°C, only a very small fraction of the tendon is dissolved. Macroscopical changes, however, can be observed; the changes differing with the temperature. After repeated efforts we succeeded in obtaining better than 95% dissolution by freezing the tendon first with dry ice, cutting with a microtome into sections 30-40 microns thick, then placing the acetic acid at 37° for 6 hours under sterile conditions. The entrance into solution was aided by periodical shaking with glass beads. Repeating the process without the cutting also proved successful. Thus the prefreezing in some way permits easier dissolution, perhaps by acting upon the ground substance that surrounds the collagen. Frozen tendon did not dissolve at 4-5° C. The relative amounts of tendon and acetic acid are not critical. In the final experiments we used a ratio of one part moist tendon to five parts of 10^{-4} molar acetic acid by weight, inasmuch as higher ratios produce solutions too viscous to be filtered through the Seitz filters. The viscous solution of collagen thus produced was filtered through a Seitz filter to eliminate any suspended matter or any undissolved fibrils, and the filtrate examined in the electron microscope. No fibrillar structures could be found in the final solution. The reprecipitation process depended greatly upon the temperature. The best precipitates were produced using one part of collagen

solution to five parts of 0.1 molar citric acid-sodium citrate buffer by volume at pH 4.9. At 4-5° C no visible precipitate in the collagen-buffer mixture can be observed even after one week. Low speed centrifugation (Servall, 5000 rpm, 2 hours) did not separate out any observable fibrillar precipitate. If, however, the collagen solution and the buffer, both previously brought to room temperature, are mixed and let stand at room temperature, a complete precipitation is obtained within two hours. This white, gelatinous precipitate can be easily separated from the supernatant liquid by decantation or by centrifugation. For examination in the electron microscope the salts were washed out by repeated washings with distilled water and recentrifugation. The final precipitate, suspended in distilled water, was then examined in the usual way and found to contain characteristic collagen fibrils of the same period as those in the original fibrils. In general the reprecipitated fibrils showed sharp contours, although the extremities appeared somewhat frayed. In various preparations there appeared several types of fibrils, varying from short, non-striated (Figure 9A), to longer, non-striated (Figure 7B), longer striated (Figure 8) and finally definitive fibrils (Figure 9). It is tempting to suggest that these represent stages in the formation of the cross striated collagen fibril, i.e. that the cross striation occurs after the fibril reaches a certain length and probably a certain tension.

B — *Salivary Gland Tendon* — Attempts to repeat the experiment above with the posterior tendon of the labial glands of *Constrictor* failed, even when longer contact times were used. This difference in behaviour is puzzling inasmuch as both the rat tail, and the gland tendon are pure tendons without other tissue involved. The gland tendon showed a certain degree of swelling after 24 hours in the acetic acid. Fibrils fixed in 5% formalin, blended in distilled water and examined in the usual way in the electron microscope, showed an increase in period of cross striation with decrease in sharpness of outline of striations.

C — *Tendons From Various Parts of Body* — The two previous experiments strongly suggest that the dissolving power of the acetic acid depends upon the animal, upon the region of the body from which the tendon is taken, and upon the preteatment. Without previously freezing the tendon the action of acetic acid appears to be limited to swelling, the amount and type of which depends upon the temperature.

To test this thesis, tendons were taken from the tail, dorsum and paws of albino rats. Pieces of approximately the same size from each region were placed, without prefreezing, in 10^{-4} molar acetic acid under sterile conditions at 4° and 37°C and thus maintained for 24 hours. Subsequently they were

fixed in 5% formalin, blended in distilled water and prepared for electron microscopy. In one set of experiments the acetic acid was brought to the desired temperature before adding the tendon. In a second series the tendons were placed in the acetic acid at room temperature and then brought to the desired temperatures. The results differed considerably, as will be shown below.

At the lower temperature (4°C) the tendons swell amazingly and become transparent; having the appearance of a rigid jelly and the tendons do not lose their shape even after shaking (Figure 11). At 37°C, on the other hand, the tendon swells less, becomes opaque and loses its silken appearance. The fibrils from the tendons brought gradually from 37° to 4°C appeared in the electron microscope as zig-zag fibrils of short and irregular period (Figure 12). This phenomenon principally was seen in the tendons from the tail and dorsum. In some of the zig-zag fibrils the period was maintained, in others it disappeared. No zig-zag fibrils were observed at 37°C.

The meaning of the formation of the irregular, zig-zag type of fibril is not clear from these studies. It seems to be related to the effect of temperature inthat when the tendons are placed directly in acetic acid previously cooled to 4°C no zig-zag formation occurs. Thus it is evident that it is caused by the gradual lowering of the temperature. Noticeable also was the fact that only at the low temperature was the cross striation lost, while at the higher temperature the striations continued observable even when the fibril was apparently disintegrating along the long axis. In fact, the aspect of the fibrils after being treated with acetic acid at 37°C is similar to that after treatment with water at 60°C.

ACTION OF DILUTE ALKALI ON TENDON

It has been held previously that dilute alkali does not attack collagen. However, Gross (16), has shown that 0.1 normal sodium hydroxide causes fragmentation of the collagen fibrils in the transverse direction and eventually causes the reduction of some fibrils to an amorphous mass. Attempts to repeat the experiments of Gross with tendon from the labial glands of *Constrictor* were unsuccessful. The collagen fibrils were isolated as described previously and suspended in 10^{-4} normal sodium hydroxide. Controls were made by suspending tendons in distilled water. Both were then left for 24 hours at 37°C and at 0°C and subsequently examined in the electron microscope. As far as could be determined from the micrographs no changes occurred. Thus these experiments show that either (1) dilute alkali does not have an effect upon

tendon, as previously believed, but that a stronger concentration could have or (2) that, as found in other experiments mentioned above, the effect of an agent upon collagen will depend greatly upon the animal and region from which the tendon is taken. Further experimentation is underway to clarify this point.

SUMMARY

Collagen fibrils from various organs of toad, reptiles, pigeon, rat and human have been studied by means of the electron microscope. Observations have been made upon the influence of stretch, fixatives, salts, acids, alkalis and temperature upon the structure of the fibril. Also studied were the conditions necessary for dissolution and reprecipitation of collagen.

The normal fibrils from various organs of different animals showed an identical period, but varied considerably in diameters. The variation was more marked in certain animals and organs than in others, e.g. fibrils from lizard tongue were extremely uniform, whereas those from the tail of the caiman varied considerably.

Fibrils were observed fresh, and fixed in formalin, Bouin's fluid and Zenker's fluid. In all cases the period remained uniform, but the diameter of the fibrils appeared to vary with the fixative. Greatest uniformity was obtained with 5% formalin.

When subjected to tension the fibril increases in length, principally in the light band. With continued tension the fibril breaks into individual units of the length of the two bands only.

Tendons left in physiological saline for long periods of time easily split longitudinally during blending and show considerable flattening when dried on the grids. In many fibrils thus treated may be seen incomplete rings in the periphery of the fibril, or a hollow core at the extremities. We thus believe the fibril to normally have a liquid, or semiliquid, core.

At 40°C the fibrils show no change in water. Within two minutes of exposure to water at 60°C the fibrils begin to lose their shape. On continued exposure the fibrils shorten, increase in diameter with conseqent shortening of period, slowly dissolve and finally become an amorphous gelationous mass. At 100°C these changes occur within 2 minutes. The hot water appears to change the collagen protofibrils into free gelatin molecules, by stages in which the protofibrils are changed from the normal orderly to a disorderly state.

Temperature was found to play an important role in dissolution and reprecipitation of the fibrils. Prefreezing the tendons was necessary to cause their

dissolution in the acetic acid. At 4-5° dissolution is extremely slow; at 37°C quite rapid. Reprecipitation at 4-5° does not occur within one week, whereas it is complete within 2 hours at 37°C.

Fibrils dissolved in acetic acid and reprecipitated in citrate buffer showed several stages of reformation. It is believed that the sequence is: (1) short, non striated fibrils, (2) long, non-striated fibrils, (3) long, striated fibrils. Thus it appears that a certain length of fibrils and certain tension is necessary for the formation of the striation in the reprecipitated fibrils.

Tendons from the tail, dorsum and paw of the white rat showed different responses to the same treatment. Attempts to dissolve and reprecipitate the posterior tendon of the labial glands of *Constrictor* were unsuccessful. Thus it was found that the conditions necessary for dissolution and reprecipitation of collagen fibrils varies with the animal and organ of origin.

Non-prefrozen tendons added to acetic acid precooled to 4°C swell and become transparent but remain linear in form. Those put in acetic acid at 37°C and then lowered do 4°C show a zig-zag form. No zig-zags are formed at 37°C.

Dilute alkali appeared to have no effect upon *Constrictor* tendon at either 4° or 37°C.

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TABLE I

INFLUENCE OF VARIOUS FIXATIVES UPON TENDON OF LABIAL GLAND OF SNAKE.

Fixative	Non-fixed	5% formalin	Bouin	Zenker
Period (Å)	660	660	660	660
Length (Å)	1000-1600	1400-2000	830-2600	1100-1300

TABLE II

PERIODS AND DIAMETERS OF COLLAGEN FIBRILS FROM SEVERAL ANIMAIS

(in Angstrom units)

Animal	Snake	Lizard	Caiman	Toad	Pigeon	Woman
Organ						
External oblique				d=798 p=630	d=798 p=600	d=798 p=570
Heart	d=1100 p=600					
Temporalis	d=630 p=590					
Hyoglossus	d=600 p=600	d=640-900 p=640				
Labial gland tendon	d=1400-2000 p=660					
Vein	d=800 p=630					
Deferent canal	d=630 p=600					
Retractor of hemipenis	d=900 p=600					
Tail			d=640-1400 p=640			
Tongue		d=600 p=590				