OBSERVATIONS WITH THE ELECTRON MICROSCOPE ON THE SOLVATION AND RECONSTITUTION OF COLLAGEN

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PLATES 22 TO 24

(Received for publication, May 28, 1951)

Electron microscope studies of collagen have dealt, for the most part, with the morphology of the mature or completely formed fibers.[1] It has been shown that fibers from a variety of sources have the same characteristic periodic structure which consists of regularly spaced bands of greater density to electrons, arranged crosswise to the long axis of the fibers. The combination of a dense (A band) and a light region (B band), which constitutes a single macroperiod, measures about 640 A (1, 2). Metal shadowing reveals that, in the dried fiber, the dense bands represent elevations in the contour and therefore regions containing greater amounts of non-volatile material, whereas the light bands coincide with depressions (3). Studies at higher resolution reveal further complexity. The more opaque band is found to have two component striae and the more transparent band or depression, a single striation. The number of these intraperiod striations is usually greater in fibers "stained" with phosphotungstic acid but it may be that these are phases or regions within the organization that specifically fix the tungsten compound (4). In any case the over-all picture is one of extremely precise and complicated organization and this poses such questions of morphogenesis as (*a*) what is the form of the fundamental, possibly macromolecular, units which make up the fibers, and (*b*) how do these units combine to give the now familiar, striated fiber.

Some information relative to these problems was obtained from a study of the natural formation of collagen as it occurs in cultures of collagen-containing tissues (5). It was observed that the smaller natural fibers start out with bands of more or less uniform size, spaced at 210 to 270 A, and that only as these fibers grow to greater diameters do their striae acquire the inequalities which account for the macroperiod of 640 A. However, the conditions of this study did not lend themselves to a demonstration of the means by which the fibers grew in size. While there was some indication that the narrower fibers fused to

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[1] For a complete bibliography see Borasky, R., Guide to the Literature on Collagen, Philadelphia, U. S. Department of Agriculture, Eastern Regional Research Laboratory, 1950.

form wider ones, there was no clear-cut demonstration that the smaller, unit fibers were formed by the lateral and longitudinal association of shorter and more slender tactoid-like units as in the case of the transformation of fibrinogen into fibrin (6, 7). Instead, the unit fibers of collagen seemed to have grown from very slender protofibrils, the smallest identifiable units, by accretion of material from the culture medium. Since the possibilities for varying the conditions under which these fibers formed were greatly limited by the presence of living cells in the preparations, it has seemed of interest to investigate other means of fiber formation, *e.g.*, the reconstitution of collagen from solution.

There is evidence from the work of previous investigators that certain types of collagen can be dissolved and reconstituted without apparent harm to the protein. Nageotte (8, 9) demonstrated that when rat tail tendon (collagen A) was treated with a dilute acid, some of the tendon dissolved and could be reprecipitated in a fibrous form by the addition of sodium chloride. Nageotte considered that this reconstituted material was very similar to natural collagen, since the fibers retained the characteristic staining properties of collagen.[2] Wyckoff and Corey (10) demonstrated that the x-ray diffraction pattern of such reconstituted material was like that of the original tendon. Schmitt, Hall, and Jakus (1), Schmitt and Gross (4), and more recently Bahr (11) have examined reconstituted collagen with the electron microscope and have found that it retains its characteristic striated appearance.

In the present study an attempt has been made to examine in some detail the form of the unit of collagen in solution in acetic acid and the sequences in its reaggregation to form fibers under various "controlled" conditions.

Materials and Methods

Solutions of collagen were prepared as follows: Rat tail tendon was treated with a 0.1 per cent solution of trypsin in balanced salt solution[3] (pH 7.8) for 24 hours at 38°C. to remove foreign proteins. To guard against extensive digestion of the collagen itself, care was taken not to tease the fibers more than was necessary for their removal from the animal. The resulting shiny, white fibers were washed in running tap water for 1 hour and then placed in approximately 500 volumes of 0.01 per cent acetic acid, pH 3.8–4.0, at 8°C. for 24 hours. The viscous solution which formed was filtered through surgical gauze and thence through a sintered glass filter of medium porosity.[4] The concentration of the dissolved material was determined by drying an aliquot over phosphorous pentoxide. The filtrate was thereafter diluted with more 0.01 per cent acetic acid to a range of concentration (0.02 to 0.06 per cent) determined by experience to be suitable for the reprecipitation experiments. Samples of this material were examined with the electron microscope to assure that they were free of collagen fibers before being used in the experiments described below.

[2] Nageotte noted that the reconstituted collagen also became "argyrophilic" like its supposed precursor reticulin, but felt that the silver impregnation techniques were dependent on the physical conditions of the fibers, notably, size and conformation, rather than on their chemical composition. Gross (12) has recently examined reticulin with the electron microscope and finds that the individual fibers are simply small strands of collagen.

[3] Earle's solution (13).

[4] The average pore size is described as 14 μ.

Specimens of the collagen solution for studying the form of the contained "macromolecular" units were prepared either (*a*) by drying thin continuous films of the solution on formvar films or (*b*) by forming fine drop patterns or discontinuous films by spraying the solution on formvar-coated screens (14). For both procedures the solution was diluted from 0.04 per cent to 0.0004 per cent with 0.01 per cent acetic acid. A common De Vilbiss atomizer was used to produce the spray. This latter technique was adopted to obtain preparations showing multiple margins between collagen-coated and uncoated film, and to obtain rapid drying, which presumably would reduce the chances for the collagen to polymerize as the concentration increased. These and all other specimens were shadowed with a thin layer of chromium at an angle of 12° (chromium 4 mg., distance 15 cm.).

Preliminary experiments on fiber precipitation demonstrated that if duplicate preparations were to be obtained it would be essential to reproduce carefully not only the concentration of collagen, hydrogen ions, etc., but also any physical aspects of the technique that would tend to disturb greatly the fiber formations during and after their development. To this end the following procedure was uniformly used:—

A thin wet film of the collagen solution was applied to a formvar-coated slide (1/2 × 3 inches) by simply dipping the slide in the solution. This was immediately immersed in the saline or buffer solution expected to induce precipitation of the fibers. All reactions were carried out at room temperature. The slide was left in solution without mechanical mixing for a measured length of time, rinsed by dipping once in distilled water, and then fixed, either in a 1 per cent solution of osmium tetroxide, or over vapors of a 2 per cent solution of osmium tetroxide. The preparations were then washed in distilled water. Transference to the microscope support or grid was accomplished either by peeling and cutting the plastic membrane under water and placing it on an electron microscope screen, or by floating the formvar membranes off on water and picking them up on screens.

The concentrations of NaCl used were 0.5 per cent, 1.0 per cent, 5.0 per cent, and 10.0 per cent. The buffer solutions were composed of 1 volume of McIlvaine's standard 0.1 M citric acid and 0.2 M disodium phosphate mixtures diluted with 2 volumes of H_2O. Solutions with pH values of 4.8, 5.8, 6.8, and 7.8 were employed.

The Native Collagen Fiber

The structure of dried collagen fibers from the rat tail tendon is shown in Figs. 1 and 1 *a*. These fibers were prepared without the "cleansing" aid of tryptic digestion so that they might represent more closely the form of the native fiber.[5] They show the typical double bands or striations separated by depressions that are repeated every 640 A. These depressions in the contour of the fiber, which develop when the fiber is dried (3), are clearly more pronounced in the larger fibers in which they constitute about 5 per cent of the diameter of the fiber.

Solvation of Collagen Fibers

When collagen fibers of this type are treated with dilute acetic acid (0.01 per cent in H_2O, pH 3.8) they swell very noticeably, lose their white glistening appearance, and become more or less transparent. Fibers treated with 0.002 per cent HCl, pH 3.3, behave similarly. Though gelatinous looking they keep

[5] Sizer (15) has shown that while native collagen is relatively resistant to the action of trypsin, digestion does take place if the fibers are mechanically fragmented.

a longitudinal organization which makes them strongly birefringent. As dissolution continues, this is lost.

When dried for electron microscopic examination, such swollen, gelatinous looking fibers flatten down to a small fraction of their previous thickness (Fig. 2). The longitudinal organization of the fiber components is largely retained as shown by the persistence of the striations (arrow *A*). However, the lateral connections which give the fiber its rigid cylindrical form must be weakened or lost so that the whole structure flattens out. The micrograph gives the impression that there is in the structure a longitudinal filamentous unit (between arrows *B*) and that these filaments possess nodosities or density variations which might give the dry fiber its regular metameric structure.

During the time that the fibers are taking the form shown in Fig. 2, *i.e.* during the 24 hours in 0.01 per cent acetic acid, certain portions of the original collagen go into solution. Under the conditions defined these solutions contained between 0.04 per cent and 0.06 per cent of dissolved material as measured by dry weight determination. The residual collagen fibers present after 24 hours can be filtered off and placed in fresh acetic acid and will again yield a collagen solution of similar concentration. This can be done repeatedly until the original fibers almost completely dissolve. The resulting solution is clear, viscous, and shows some birefringence of flow. The viscosity of the solution when considered in relation to the low concentration of dissolved material suggests the existence of an extremely long, tenuous component.[6]

This account of the dissolved collagen unit is supported by electron micrographs of dried material (Fig. 3). A film of it has the appearance of a felt of fine filaments which vary in diameter from the limits of resolution (30 to 50 A) up to 200 A.

With such preparations it is often difficult to decide what portion of the image is due to artifact, what is collagen, and what supporting membrane, and consequently, what the form and dimensions of the unit structure really are. In order to make these distinctions easier, some of a solution in acetic acid was diluted one hundred times (0.0004 per cent dissolved collagen) and sprayed on to a supporting membrane (as described above) to give discontinuous spreads of the material. Images could then be seen of what are probably some of the smaller fibrous components (Fig. 4). They appear irregular in contour, length, and diameter. They rarely exceed 400 A in diameter and 400 mμ in length. There is no clear evidence of striations or metameric structure. The size and form variation suggests either that the process of dissociation in the acetic acid had not yielded a basic unit or that some reassociation had occurred during the preparation procedures. Various procedural changes failed to give finer or more uniform filaments.

[6] A calculated relative viscosity, *N*, was 2.28. This, while representative, is not constant but varies slightly during measurement and to a greater degree from preparation to preparation and over periods of storage.

Reconstitution of Collagen Fibers

As indicated above and demonstrated earlier by Nageotte, when NaCl is added to a viscous acetic acid solution of collagen prepared as described, or when the solution is neutralized, collagen fibers reform. It is reasonable to expect that by a study of this formation and the conditions influencing it, one may learn something about the fundamental unit of collagen and how it polymerizes to give the precise organization of the mature fiber.

Effect of Sodium Chloride

(*a*) *Variation with Time of Contact.*—The sequences of fiber formation resulting from the addition of NaCl can be followed by stopping the reaction with osmic acid after various intervals of time.

The form of the starting material is shown in Fig. 3 which pictures a layer of collagen obtained by dipping a slide into a 0.02 per cent solution of collagen in acetic acid. This preparation was treated by dipping it into distilled water instead of saline and fixing with OsO_4. There is no evidence of polymerization.

When such a film of dissolved collagen was treated with a 1 per cent solution of sodium chloride for 1 minute and the reaction stopped by fixing in a 1 per cent solution of osmium tetroxide in distilled water, some polymerization did take place (Figs. 5 and 5 *a*). In addition to the background felt of filaments similar to that shown in Fig. 3, a number of wider and longer fibers are present. These fibers are needle-like crystals or tactoids. They are 5μ or less in length, and up to 1000 A in width; larger fibers were occasionally encountered. These tactoids showed a periodic cross-banding with striations spaced at about 210 A. In some there are regions where two out of each group of three regularly spaced striae are slightly more prominent. This produces, though not strikingly, the macroperiod of collagen measuring around 640 A in length. It is clearly shown only in the broader of the large fibers.

Preparations made from films that were immersed in saline for 5 minutes show only a few background filaments and small early fibers (Fig. 6). Instead, the predominant type consists of much enlarged, fairly straight fibers with tapering ends. These show various degrees of union, the tapered end of one fiber blending with the body of another to form a larger (broader and longer) fiber (arrow). The striae are in phase in such cases. The striations are not prominent, are spaced at 210 A, and show only slight evidence of differentiation to form the broad striation of the macroperiod.

Treatment of the film of collagen solution for 15 minutes in saline (Fig. 7) brought about the formation of appreciably thicker fibers and the simultaneous development of a more obvious and precise banding. The micrographs show fibers which vary considerably in diameter but have a greater average size than those in the preceding preparations. With respect to fine structure (Fig. 7 *a*), each major period is composed of three distinct striae, two of which seemed generally to possess greater prominence in the dried fiber than the third. There

is some evidence in the micrograph (Fig. 7 *a*) that these striae have a particulate component, but the surface of the fiber is so similar in its granular appearance to the surrounding surfaces that it is difficult to decide whether the particulates are an integral part of the fiber. In none of the fibers formed by salting out with 1 per cent NaCl was the large double striation of the major period as prominent as in the native fiber. Similar fibers were precipitated by equivalent concentrations of lithium chloride and potassium chloride.

(*b*) *Salt Concentration.*—In order to obtain a better understanding of the phenomena involved in salt precipitation and to determine whether fibers of more nearly native type would form, the effect of various concentrations of salt was examined when allowed to act for a uniform length of time. It was first of all noted by gross study of the collagen-salt mixtures that the rate of fiber formation increased directly with the salt concentration. With a 0.5 per cent concentration of NaCl, fiber formation, estimated in terms of solution opalescence, continued over several hours, whereas at a concentration of 10 per cent NaCl the whole process was complete in a few minutes.

The detailed structure of fibers formed under these varied conditions of salt concentration is shown in Figs. 8 to 10. The electron microscope preparations were obtained as before by dipping formvar-coated slides briefly into a solution of collagen (in this series 0.04 per cent) and thereafter for 30 minutes in the salt solutions. Under these conditions the 0.5 per cent NaCl induced the development of long, tortuous, smooth fibers (Fig. 8). These fibers frequently blended with one another (arrows) and showed large variations in diameters. The average width was around 700 A and they rarely exceeded 1000 A. These fibrils and more especially the smaller ones, which ranged in diameter from 300 to 400 A, were as lacking in structure as the filaments described in the control spray preparation (Figs. 3 and 4). Even those as broad as the fibers of Fig. 5, which were produced with 1 per cent saline, failed to show striations. When the height of the dry fiber was examined relative to its width, it was found that the fiber had flattened considerably in drying.

The result of using a 1 per cent solution of sodium chloride on a similar preparation for 30 minutes is depicted in Fig. 9. Instead of a felt of tortuous fibers, long, straight, crystal-like tactoids were formed which have essentially the appearance of those that developed after 15 minutes in the time series (Fig. 7). In contrast with the fibers produced by 0.5 per cent NaCl, these "1 per cent" fibers were definitely striated and had the form characteristic of salt-precipitated fibers. The larger fibers showed some evidence of being compound (arrows), and even where the fusion was not complete the striae were in phase. The background material contained what are probably the earliest stages in fiber formation, here adsorbed to the formvar film. There were all gradations in size from the larger discrete units down to tiny beaded filaments almost at the limits of resolution. Though not as clearly shown here as in the

preceding micrographs it is probable that in fiber formation these finest filaments coalesce laterally to form larger fibrils which, through a series of similar associations, ultimately form the macroscopic fibers.

The fibers resulting from the use of 5 per cent saline (Fig. 10) under identical conditions of time, etc., resembled very closely those formed with 0.5 per cent sodium chloride. They composed a felt of intertwining fibrils. The fibers appear, however, to be smoother and more compact, as though less hydrated. The ratio of fiber width to shadow width is less by half that derived from similar measurements made on the 0.5 per cent salt-induced fibers (compare Figs. 10 *a* and 8 *a*). In other words, the fibers in the 5 per cent preparation (Fig. 10) flatten less in drying and might be described as less flaccid. It is possible to see, in occasional instances, that the larger fibers are made up of several smaller fibers rigidly twisted together. They have diameters ranging up to 1000 A. In these fibers and in the background filaments a faint beading is again evident which is similar to that shown in Fig. 9 in which the focus was better, but in which they (filaments) seemed to be less abundant.

The fibers formed with 10 per cent sodium chloride were essentially the same as those formed with 5 per cent saline.

It is evident from these experiments that 1 per cent saline for 30 minutes or less brought about a precipitation of well formed fibers which, however, in none of the material studied were exact duplicates of the native collagen. The character of the fiber varied with the concentration of NaCl. It appears from the micrographs that the larger fibers were formed through a lateral fusion of similar but smaller fibrils which in an earlier sequence were similarly formed.

Effect of Hydrogen Ion Concentration

Collagen may also be reconstituted from an acetic acid solution by an appropriate adjustment of pH. The details of this phenomenon were observed during the variation of two parameters (*a*) time over which the reconstitution was permitted to proceed at a given pH and (*b*) increasing pH's for a standard time.

(*a*) *Time.*—For examining the sequences in fiber development arising from an increase in pH, films of dissolved collagen (0.06 per cent in 0.0001 M acetic acid) were immersed in McIlvaine's buffer adjusted to pH 5.8.

The fiber development which took place under these conditions during 1 minute is shown in Fig. 11. It appears that the fibers have just begun to form. They are poorly defined filaments, the largest of which are around 800 A in diameter. They lie at random and seem to branch and blend freely. There is no evidence of cross-striations.

The fibers shown in Fig. 12 developed during 10 minutes. In addition to the long tenuous units, such as those shown in Fig. 11, the material contains a few straight, double-pointed fibers. These show the 640 A periodicity typical of

collagen, especially in the thicker parts of the fiber. In places it can be seen that slender fibrils are combined in groups as though coalescing to form a larger fiber (arrow). A common component of the background is a small fusiform fibril like those in Fig. 9. A cursory inspection of these, especially as they show in larger fields than in Fig. 12, provided the impression that many were of the same length. Measurements were therefore made and these demonstrated that in terms of length the fibrils fall into groups with mean lengths which are small, whole-number multiples of 640 A.

The larger fibers displayed in these preparations increased in size and number with time. After 20 minutes, as shown in Figs. 13 and 13 *a*, the fiber mats contain units of great size range. There are long tenuous fibrils as in Figs. 11 and 12, thicker striated units of intermediate size as in Fig. 12 (arrow *A*), and a number of extremely large fibers running in size up to 2600 A (Fig. 13 *a*). The striae of these larger fibers more closely resemble those in native collagen than the shallow striae in the saline-precipitated units (Figs. 5, 6, 7, and 9). The arrows (*B*, Fig. 13) point to a few places providing evidence that the larger fibers result from the coalescence of smaller units.

(*b*) *Hydrogen Ion Concentration.*—Polymerization of the collagen at different pH values was investigated by subjecting thin layers of the solution for 30 minutes to McIlvaine's buffer prepared at pH's of 4.8, 5.8, 6.8, and 7.8 (Figs. 14, 15, 16, and 17 respectively). Within this range the fibers form most rapidly at the pH's nearest the reported isoelectric point of collagen[7] but under none of these conditions did they form as rapidly as in the saline series.

At pH 4.8, which is close to that at which collagen goes into solution, relatively few fibers were formed with the 30 minute treatment. Those obtained were large, showed a well developed 640 A periodicity, and resembled natural collagen (Fig. 14). In the background of the micrograph there are large numbers of blunt, relatively short and stout fibrils. These have widths of between 400 and 500 A and lengths which, like those in the material shown in Fig. 12, are small multiples of 640 A. These small units are probably comparable also to the more slender background fibrils noted in the saline series (Fig. 9).

At pH 5.8 (Fig. 15) the large range in fiber widths, which was encountered in the time series (Fig. 13), was again evident and was greater than in any of the lower concentrations of H ion examined. The larger, broader fibers are markedly striated and in a form which, though blanketed here by unpolymerized material, was similar to that shown in Figs. 1, 13, and 14.

[7] While purified collagen is known to be isoelectric between pH's 6.0 to 7.5, the isoelectric point of raw unpurified collagen is reported by Cassel and Kanagy (16) as pH 5.8. According to the same authors, trypsin reduces the isoelectric point to pH 5.5. Since purification procedures employed in these experiments were limited to digestion with trypsin and solvation in dilute acetic acid, it has been assumed that the isoelectric point of this material lies between pH 5.5 and pH 6.0.

Figs. 16 and 17 depict fiber development which took place at pH's 6.8 and 7.8 respectively. There was generally a significant decrease in the average width of the fibers relative to length as the pH went up. Typical collagen periodicity is present in the larger fibers of the pH 6.8 preparation, but none is clearly evident in the fibers of the pH 7.8 precipitated material.

DISCUSSION

These observations teach little concerning the solvation of collagen fibers by dilute acetic acid. The pronounced swelling of the fibers and the water-like clarity of the ultimate solution indicate that the component units have become greatly hydrated. Furthermore, since the swelling fiber loses its cross-sectional organization before its longitudinal, it would seem that the lateral bondings are the more susceptible to the influence of the acid. However, the nature of this influence is not clear. Presumably it is the effect of the dissociated H^+ ion, since a dilute solution of hydrochloric acid is as effective as acetic acid. On this assumption, the separation and solvation of the fibrous particles would be referable to the electrokinetic potentials developing between them at pH values far removed from the isoelectric point.

The reconstitution of fibers from the units of the acetic acid solution can be accomplished either by increasing the pH or by adding various concentrations of NaCl. In agreement with the x-ray diffraction evidence (10) and earlier electron microscope observations (1, 4, 11), the fibers that are formed have the major morphological features of native collagen. Certain minor differences are mentioned below.

The sequences in fiber formation are well defined and appear to be essentially the same whether salt or neutralization of acidity is used. The small protofibrils present in the acid solution fuse to form larger units of the same shape which in turn repeat this process to form larger units and subsequently fibers. It is the same pattern of events which occurs in the development of fibrin strands in mixtures of fibrinogen and thrombin (6, 7).

What similarities, if any, exist between this method of fiber development and that which occurs *in vivo* is difficult to say because so little is known about the latter. In the case of skin collagen (5) the adult fiber appears to start out as a very slender fibril which increases in size by accretion to itself of unpolymerized collagen. No sequence of tactoid formation and alignment has been noted. Possibly, even *in vivo*, skin and tendon collagen are formed differently; but it seems probable that dissimilar conditions of the natural and experimental fiber formation account for the major part of the difference. For example, in no conceivable *in vivo* situation would there exist the concentration of dissolved collagen present in the acetic acid solutions used in the present work or the conditions employed to induce a sudden condensation into fibers.

A 1 per cent solution of NaCl incorporated in the acetic acid solution of

collagen obviously leads in time to the development of precisely organized fibers of collagen. In our experience, these saline-precipitated fibers never develop as pronounced elevations in the banding as are found in native fibers of a similar size, but no explanation for this is apparent.

The role of sodium chloride in reconstituting the fibers is not evident from these experiments. Presumably at a low (0.5 per cent) concentration there is a specific ion effect as well as possibly some lyotropic action. Other neutral salts of the Hoffmeister series, such as potassium and lithium chlorides, also induce the formation of fibers, but no differences in rate or subsequent fiber structure were noted. The lower concentrations of NaCl may bring the collagen out of solution to only a limited degree, and the individual fibrils flatten in drying as though hydrated and hence more flaccid at the time of fixation. Presumably if the time allowed for reaggregation had been sufficiently long in the presence of this low concentration of saline, typical collagen fibers would ultimately have been obtained. High concentrations of salt (5 per cent and 10 per cent), on the other hand, force the collagen out of solution much more rapidly, as if it had not had time to aggregate to larger well organized fibers (such as those formed with 1 per cent NaCl) before all was in solid form. The resulting fibrils, on comparison with the ordinary, seem firm and less hydrated, and suggest a pronounced lyotropic action.

It is evident that just as decreasing the pH causes collagen to dissociate into filaments, increasing the pH causes the filaments to reaggregate or reassociate. Supposedly the reduction in net charge on the particles, as one approaches and goes through the isoelectric point, permits this reassociation. However in this case, unlike the saline series, the component filaments appear to fit together more accurately, leading to the earlier emergence (in terms of fiber size) of striations as the fibers develop. It can be noted that a decrease in average fiber width parallels the increase in pH. This change in the width-length ratio of the fibers suggests that lateral association of the fundamental units takes place with greater frequency at the lower pH's and end-to-end association more frequently at the higher pH's. Presumably this could mean a difference between the sides and ends of particles in relation to the type of concentration of reactive (bonding) groups. Further evidence for this explanation of the phenomenon can be extracted from a comparison of the form of the background protofibrils in Fig. 14 with the form of those in Fig. 12. Under conditions of lower pH's these protofibrils are thicker and shorter and in some cases are obviously composed of only two or three 640 A lengths. It appears as though the fundamental units had in this case shown a greater tendency to bundle together laterally.

These observations admittedly fail to provide any significantly better understanding of the form of the collagen molecule. They do, however, require some discussion in this connection. All the evidence, viscometric and microscopic,

indicates that the majority of the components of the acetic acid solution are fibrous even beyond the resolution of the electron microscope. The size range of the fibrils suggests that the dissolution of the collagen fibers was not complete in the material examined. In reaggregation, the smallest units resolvable are again fibrous. At pH 4.8 these protofibrils are segmented at 640 A lengths as though units of this length had been bundled together. The inference is that, at least in acetic acid solutions, the basic unit is a very slender molecule 640 A long.

SUMMARY

The course of events in the solvation and reconstitution of collagen obtained from rat tail tendon is described as seen with the electron microscope. Under the influence of 0.01 per cent acetic acid the collagen fibers swell and dissociate into submicroscopic filaments, the smallest of which are probably beyond the resolution of the electron microscope. These filaments can be made to reconstitute into fibers either by the addition of neutral salts to the acid solution or by raising the pH. The structural form of the resulting fibers is influenced by the concentration of the salt and by the pH of the solution employed. Saline concentrations around 1 per cent and pH's ranging from 4.8 to 6.8 lead to the formation of needle-shaped crystals, or tactoids, showing the striated structure characteristic of collagen. Saline concentrations outside of this range (0.5 per cent and 5.0 per cent) lead to the formation of long fibrils without evidence of striations. pH's on the alkaline side of 6.8 bring about the formation of long slender fibers. Some of the possible reasons for these different fiber forms are discussed.

Apparently fibers are formed *in vitro* by the lateral and longitudinal association of the filaments seen in the original solutions. Some of the fibers thus formed may in turn associate laterally and longitudinally to form the larger fibers.

The formation of the needle-shaped crystals appears to be an orderly process since it leads to the formation of a periodicity in the fibers. The striations in the smaller fibers are uniform and regularly spaced at around 210 A. With continued growth of these fibers there is a simultaneous development of a more obvious and precise banding. It is evident that two out of each group of three regularly spaced striae are more prominent. This produces the macroperiod of collagen measuring around 640 A in length.

The authors wish to express their gratitude to Dr. Gertrude F. Perlmann for making certain corrections in the manuscript.

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PLATES

EXPLANATION OF PLATES*

PLATE 22

FIG. 1. Electron micrograph of native collagen fibers derived from rat tail tendon. In the preparation of the specimen a small strand of tendon was placed in balanced salt solution and teased with needles until a suspension of fine fibers was obtained. This was centrifuged and resuspended in three successive changes of the salt solution, and small portions of the final sediment (or suspension) were placed on plastic-coated electron microscope screens and dried sufficiently to make the material adhere to the screens. The preparations were then fixed in 1 per cent osmium tetroxide, washed in distilled water, dried over phosphorous pentoxide, and shadowed with chromium. × 19,300.

FIG. 1 *a*. Enlargement of a portion of Fig. 1.

The wider fibers show the characteristic banded pattern of collagen consisting of two prominent cross-bands followed by a depression, the entire unit measuring around 640 A. It is to be noted that in the narrow fibers (arrow) this macroperiodicity is replaced by a finer banding. × 32,000.

FIG. 2. Electron micrograph of rat tail tendon partially dissolved with acetic acid. The tendon fibers were "cleansed" with a 0.1 per cent solution of trypsin for 24 hours at 37°C., washed in running tap water, and suspended in 0.01 per cent acetic acid. After 24 hours at 8°C. some of the residual gelatinous looking fibers were placed on electron microscope screens, dried, and shadowed with chromium.

The swollen fibers have flattened to a small fraction of their previous thickness on drying. In spite of the swelling the fibers have retained considerable longitudinal organization as is shown by the persistence of striations in the flattened fibers (arrow *A*). In some areas (arrow *B*) the fibers appear to be composed of fine longitudinal filamentous units. × 18,200.

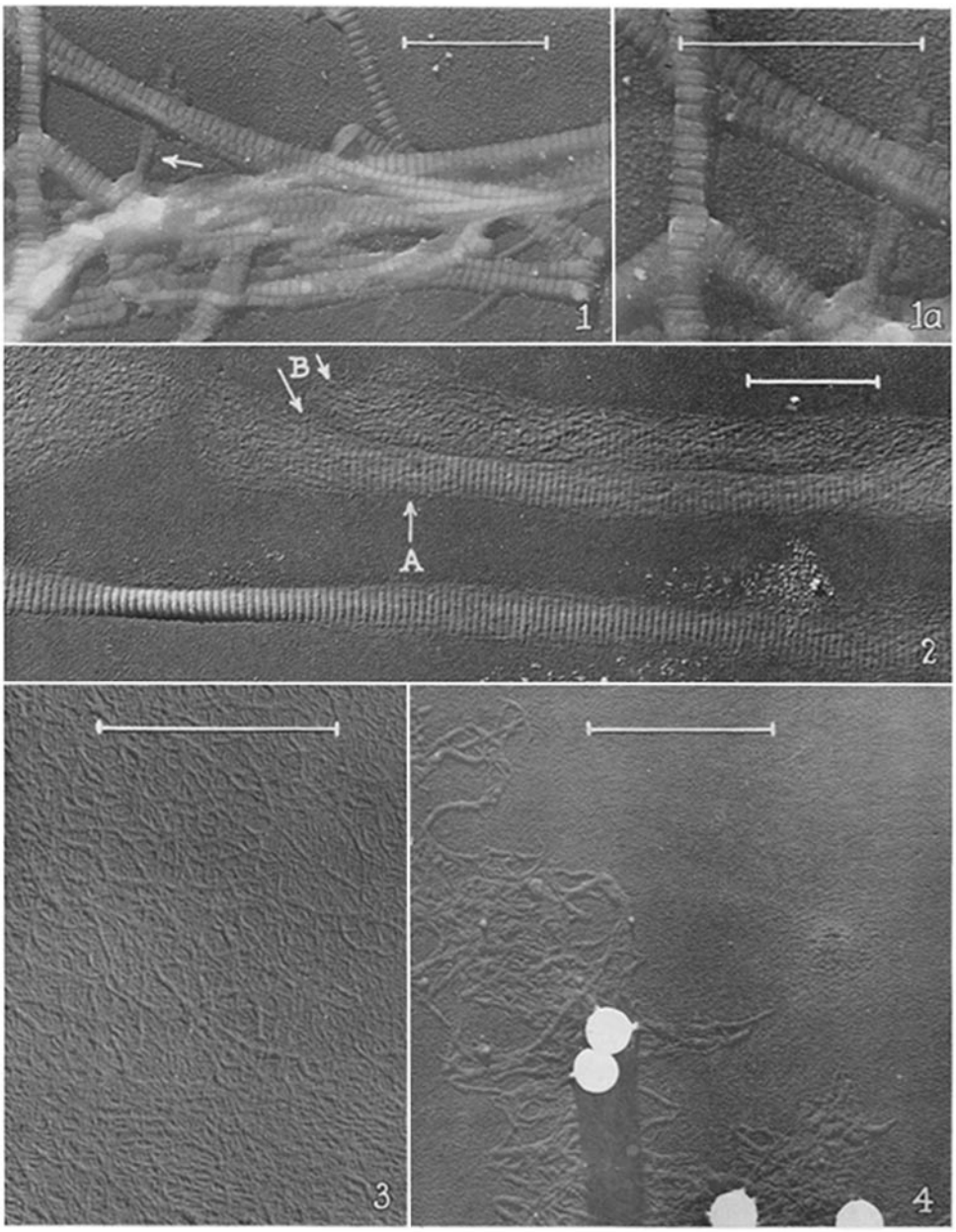
FIG. 3. Electron micrograph of a dried film of the viscous collagen solution. A uniform layer of the material was formed by dipping a plastic-coated slide into a 0.02 per cent solution. The slide was then immersed in distilled water for 5 minutes, fixed in a 1 per cent solution of osmium tetroxide for 5 minutes, and finally washed briefly in distilled water. After drying, the plastic membrane supporting the collagen was floated off on water, and picked up on electron microscope screens. Chromium-shadowed.

The collagen is in the form of a felt of fine filaments which vary in diameter from around 50 A up to 200 A. × 32,000.

FIG. 4. Electron micrograph of the content of a droplet of collagen solution, taken to show the size and configuration of the contained collagen filaments. A 0.0004 per cent solution of collagen in 0.01 per cent acetic acid was sprayed onto membrane-covered electron micrograph screens with an atomizer. The tiny droplets dried immediately.

The filaments rarely exceed 400 A in diameter and some are much finer. The spheres are latex particles included for size reference and to make it easier to locate the droplets. Shadowed with chromium. × 25,000.

* The line on each micrograph represents the size of 1 micron.



(Vanamee and Porter: Reconstitution of collagen)

FIG. 5. Electron micrograph of a preparation made the same as that pictured in Fig. 2, with the exception that it was immersed in 1 per cent saline for 1 minute, instead of in distilled water. In addition to the background mat of fibers a number of needle-like bodies (tactoids) are present. They are 5 μ or less in length and the central portions may achieve diameters up to 1000 A. Shadowed with chromium. × 14,300.

FIG. 5 *a*. Enlargement of a portion of Fig. 3 to show fine structure. The wider fibers show a fine cross-banding spaced at 270 A and regularly spaced differences in the size of these bands produce a secondary macroperiodicity measuring about 640 A. × 32,000.

FIG. 6. Electron micrograph showing fiber development in the collagen films after 5 minutes of reaction with the 1 per cent saline. The background filaments have apparently all gone to form large fibers 1000–1500 A wide and several microns long. In certain places one unit can be seen to blend perfectly with the body of another to form a wider fiber (arrow). Other fibers have not completed such a union, but have lined up with their striae in phase. The finer striae are spaced at 270 A, but in some of the wider fibers a larger 640 A period is faintly evident (Fig. 6 *a*). Shadowed with chromium. × 14,300.

FIG. 6 *a*. Enlargement of Fig. 6 to show fine structure. × 32,000.

FIG. 7. Electron micrograph showing the result of a 15 minute treatment with 1 per cent sodium chloride. The fibers have increased in width and are slightly rounder. The macroperiods (640 A) are more prominent than in the preceding micrographs, and each clearly has three component striae which are beaded (Fig. 7 *a*). None of these precipitated fibers ever showed the prominently striated form of the native fiber (Fig. 1). Shadowed with chromium. × 14,300.

FIG. 7 *a*. Enlargement of a portion of Fig. 7 to show fine structure. × 32,000.

FIG. 8. Electron micrograph of a fiber mat formed by the action of 0.5 per cent NaCl in water during 30 minutes. In its preparation a formvar-coated slide was dipped in a 0.04 per cent solution of collagen and then placed in the saline for 30 minutes. It was thereafter fixed for 45 minutes over vapors of 2 per cent osmium tetroxide, washed in distilled water for 15 minutes, and then transferred by the formvar film to electron microscope screens.

The fibers formed under these conditions are long, tortuous, smooth fibers which frequently blend with each other. There is no clear evidence of striations. Shadowed with chromium. × 14,300.

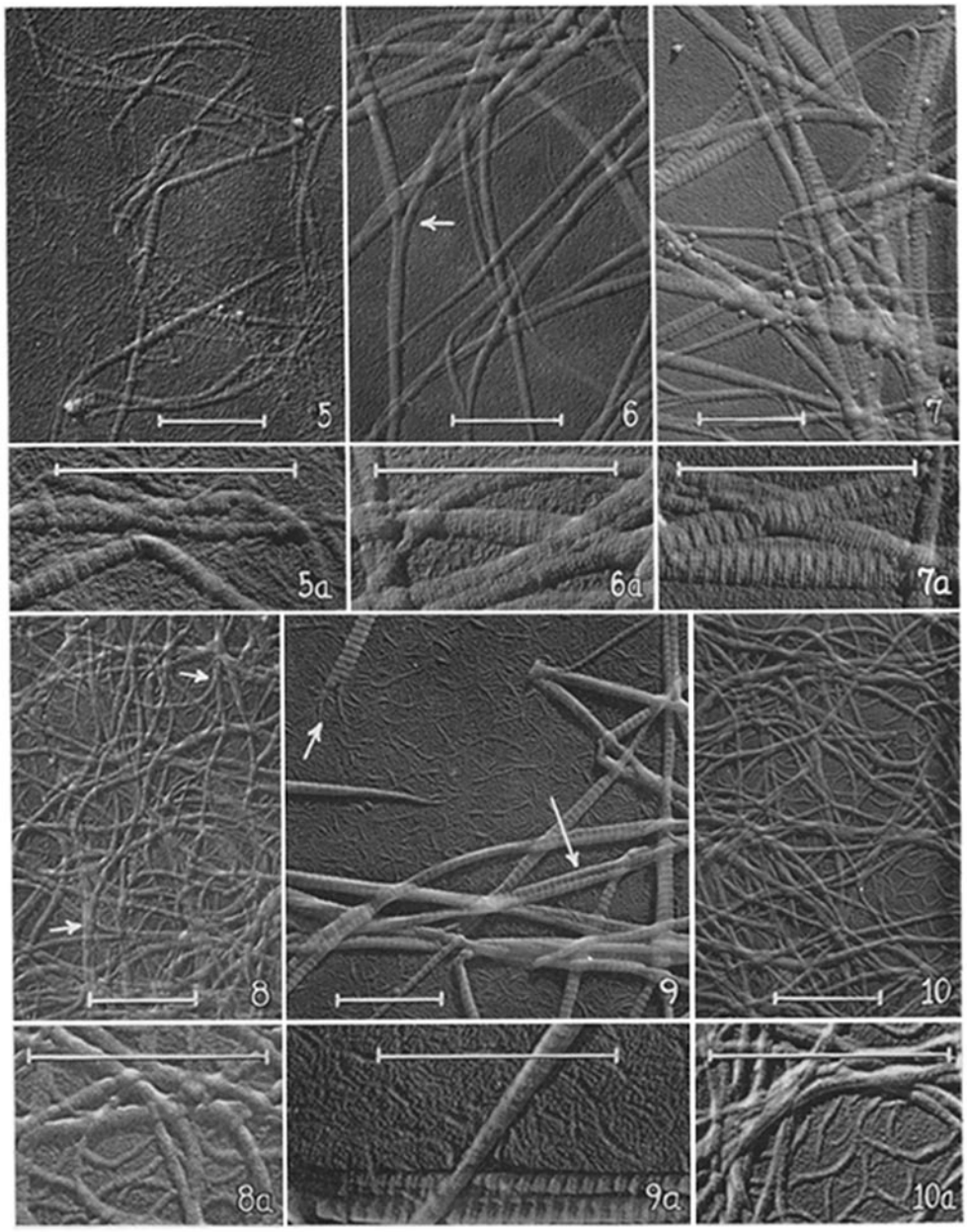
FIG. 8 *a*. Enlargement of a portion of Fig. 8 to show fine structure. × 32,000.

FIG. 9. Electron micrograph of a preparation of reconstituted collagen made in the same manner as for Fig. 8, with the exception that a 1 per cent solution of sodium chloride was used. The fibers resemble those described in Fig. 7, with the exception that they seem to have flattened less in drying. Also, the supporting membrane shows numerous filaments still present. These differences are probably due to the increased reaction time and to the higher concentration of collagen used in this series of preparations. The arrows point to evidence of the compound form of the larger fibers. Shadowed with chromium. × 14,300.

FIG. 9 *a*. Enlargement of a portion of Fig. 9. × 32,000.

FIG. 10. Electron micrograph of a fiber mat formed by the action of a 5 per cent solution of NaCl. Preparation procedures were otherwise the same as for material depicted in Figs. 8 and 9. The fibers appear much the same as those formed by the action of 0.5 per cent saline. However, they appear more rigid and less flaccid than comparable filaments. Some of the fibers are beaded as though beginning to show a faint periodicity. Shadowed with chromium. × 14,300.

FIG. 10 *a*. Enlargement of a portion of Fig. 10. × 32,000.



(Vanamee and Porter: Reconstitution of collagen)

PLATE 24

FIG. 11. Electron micrograph of a preparation made to show the effect of increase in pH on a solution of collagen in acetic acid. A formvar-coated slide was dipped in a 0.05 per cent collagen solution and then into McIlvaine's citric acid-disodium phosphate buffer (diluted with two volumes of water) at pH 5.8. In this case the reaction was stopped after 1 minute by fixation with a 1 per cent solution of osmium tetroxide.

The fibers have just begun to form and consist of a felt of poorly defined filaments up to 800 A in diameter and of indefinite length. They show no evidence of fine structure. × 14,300.

FIG. 12. Electron micrograph of a similar preparation of collagen treated for *10* minutes with buffer at pH 5.8. The mat is less dense than in the preceding micrograph and the fibers are somewhat better delineated. This material shows, in addition, a few short tactoid units in which the macroperiod of collagen is evident. Smaller fibers and protofibrils are coalescing to form larger fibers (arrows). Shadowed with chromium. × 14,300.

FIG. 13. Electron micrograph showing the action of buffer similar to that used in Figs. 11 and 12 when the reaction was permitted to continue for *20* minutes. The fibers have become more sharply defined and the larger ones show a fine periodicity measuring around 270 A. In addition to these a number of much broader fibers measuring up to 260 A in diameter are evident. The fine structure of these fibers, unlike the saline series, is almost indistinguishable from that of natural collagen. It consists of two prominent cross-bands followed by a deep depression, the entire band measuring 640 A. Shadowed with chromium. × 14,300.

FIG. 13 *a*. Enlargement of a portion of a different micrograph of the same material to show large fibers and striations. × 32,000.

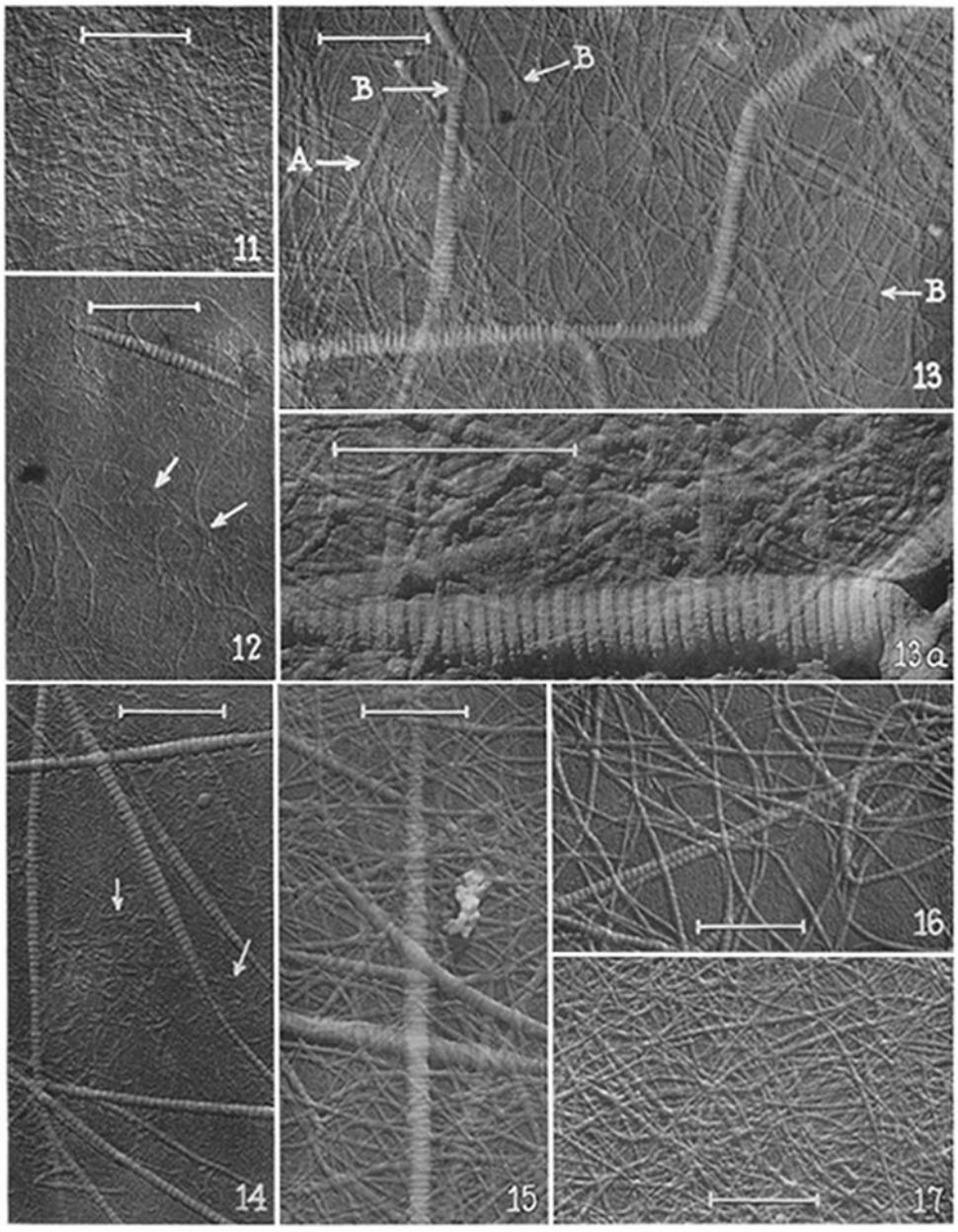
FIG. 14. Electron micrograph of fibers formed from collagen solution by dipping a coated slide into McIlvaine's buffer, at pH 4.8. The reaction was continued for 30 minutes and the preparations were thereafter fixed over vapors of OsO_4 and washed as described above.

Relatively few fibers have formed and these are predominantly of large diameter and show a well developed banding. The protofibrils in the background (arrows) are relatively thick. Compare with Fig. 12. × 14,300.

FIG. 15. Electron micrograph of a preparation of fibers reconstituted at pH 5.8. As in Fig. 13, a mass of slender fibrils is evident along with the relatively large units. The apparent irregularity of the striations in the larger fibers is due to the overlying network of finer fibers. × 14,300.

FIG. 16. Electron micrograph of fiber reconstitution at pH 6.8. Here the average fiber diameter is clearly smaller. They seem, on the whole, rounder in cross-section than the fibers described in the previous two micrographs. Shadowed with chromium. × 14,300.

FIG. 17. Electron micrograph of collagen fibers reconstituted at pH 7.8. A dense mat of long slender fibers has been formed, the largest of which are under 1000 A. × 14,300.



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