THE ELECTRON MICROSCOPY OF REPRECIPITATED COLLAGEN

by

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Almost twenty years ago it was shown by NAGEOTTE[1] that certain forms of tendon could be "dissolved" in very dilute acetic acid to give a water-clear solution from which fibrous material could be precipitated by salting-out. Optical and other properties of this precipitate indicated that it was composed of fibers closely resembling those of the original collagen.

Tendon and other well-oriented forms of connective tissue yield excellent X-ray diffraction fiber diagrams containing many long-spacing reflections. At the time of NAGEOTTE's work, COREY and one of the writers[2] made an X-ray diffraction study of some of this reprecipitated "collagen" to see if it would give the X-ray diagram characteristic of the original tendon. It did give the long spacings of collagen but the diagram was such as to indicate a considerably less perfect degree of fibering.

Very early in the application of the electron microscope, photographs were taken[3,4,5] which brought out the remarkable repetitive fine structure seen in tendon and other white connective tissue. These are repetitions compatible with the spacings measured on the X-ray photographs. We resumed the examination of NAGEOTTE's reconstituted "collagen" through the more direct examination that electron microscopy permits in order to determine if the fibers thus formed in the laboratory show all the elaboration of fine structure that is observed in native collagen. This study, of obvious importance for a knowledge of the circumstances under which collagenous tissues are laid down, is being carried out in a number of laboratories. Thus BAHR[6] has very recently published a preliminary note showing striated reconstituted collagen fibrils. At the Fall 1950 Meeting of the Electron Microscope Society of America, J. GROSS also presented electron micrographs of such fibers and of fibrils with periodicities several times that seen in native collagen.

Native collagen, irrespective of source, has shown repetitions of banding at intervals that have varied from *c.* 600 A to 675 A depending on the methods used in specimen preparation. A fibril of collagen teased from tendon suggests in appearance a series of discs repeating themselves along the fibril every *c.* 650 A. Variations in fine structure of collagen from different sources are mainly in the fine detail within these discs. Often

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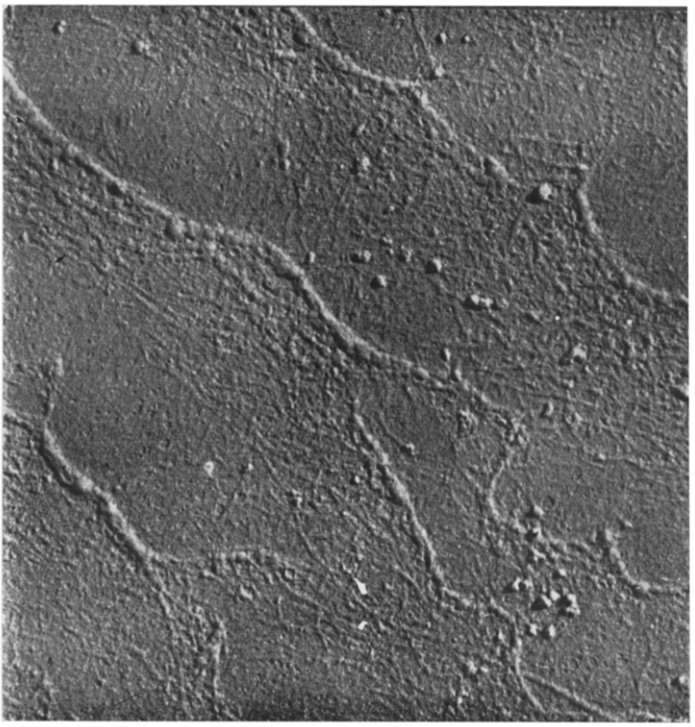

Fig. 1. A dried deposit from a solution of collagen in acetic acid. In a few places the deposit is thin enough so that the filamentous nature of the individual molecular particles can be seen. 21,500 ×

there are series of thin cross-ridges or filaments in place of each disc. Most frequently a pair of cross-ridges has occupied each disc but sometimes they have been more numerous. In some fibers exhibiting pairs of ridges a third cross-filament has been visible in the hollows between the pairs; when this additional thread has been as prominent as the pair, the fiber has taken on the appearance of a continuously cross-striated structure[7] with a repetition of $c. \frac{650\text{ A}}{3} = c.$ 215 A. It has been important to determine not only if reconstituted collagen shows the 650 A repetitions but also if variations in fine structure like those described above could be observed within the laboratory-formed material. Evidently if this is the case and if the conditions under which these variations are produced in the test tube can be determined, this is a new source of information about conditions prevailing in or around the cells that are laying down connective tissue. The experiments described in this paper were made to find out if there are variations in fine structure in reconstituted collagen and to establish within preliminary limits the conditions under which they appear.

EXPERIMENTAL

Collagen solutions were made from the foot and tail tendons of albino rats. Preliminary experiments with young and old adult rats failed to reveal significant differences in reconstituted collagen

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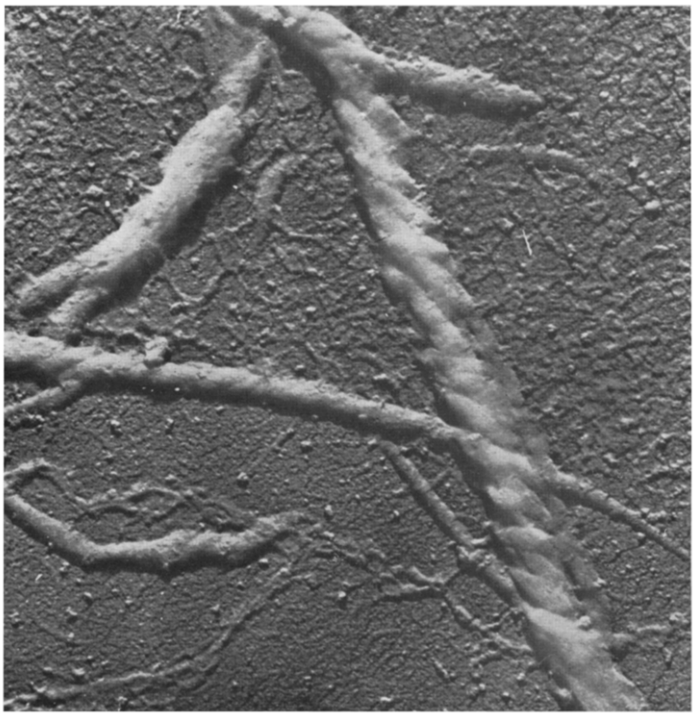

Fig. 2. Non-striated fibrils deposited from a collagen solution by the addition of several volumes of 0.1 *M* NaCl. The thread-like material in the background presumably is collagen which could not be incorporated into the big fibrils. 21,500 ×

that could be attributed to the age or the sex of the rat. A collagen solution has always been made with tendon from one rat only, but in view of the foregoing preliminary observations the ages of the rats have varied from three to ten weeks.

In most cases the collagen solutions have been made by soaking pieces of freshly excised tendon in distilled water to which has been added about one part in 10,000 of glacial acetic acid. Satisfactory solutions have also been obtained with half, and with several times, this amount of acetic acid. Dilute solutions of several other acids have been tried. Citric and hydrochloric acids have yielded useful collagen solutions but phosphoric acid has not dissolved appreciable amounts of tendon. Of the acids tried, acetic acid has been by far the most satisfactory and has been used for the experiments that follow.

A piece of tendon placed in the dilute acid swells but does not completely dissolve even after prolonged soaking. The collagen solutions have been obtained by removing the swollen residues after 24 hours and clearing by low-speed centrifugation or filtration through filter paper, or both. A diluted droplet of one of these water-clear, viscous solutions dried for examination on the usual substrate has shown masses of extremely delicate filaments (Fig. 1) but no large fibers or remnants of collagen. In one set of experiments such a cleared collagen solution was ultracentrifuged for one hour in 6 cubic centimeter tubes in a field of 40,000 times gravity. Some impurities, but no fibrous deposit, formed a pellet which was discarded. The top and bottom thirds of the supernatant gave two solutions which were examined for their relative abilities to yield reconstituted collagen. Both gave excellent striated fibrils that did not differ significantly amongst themselves or from fibrils from non-ultracentrifuged solutions. This experiment was carried out primarily to get a collagen solution that would be free of any minute fragments of the original tendon and coarser aggregates of molecular particles that might be imagined to be present. Electron micrographs of air-dried droplets of the non-centrifuged solutions (Fig. 1) have not shown such fragments, but the fact that well-striated collagen precipitates

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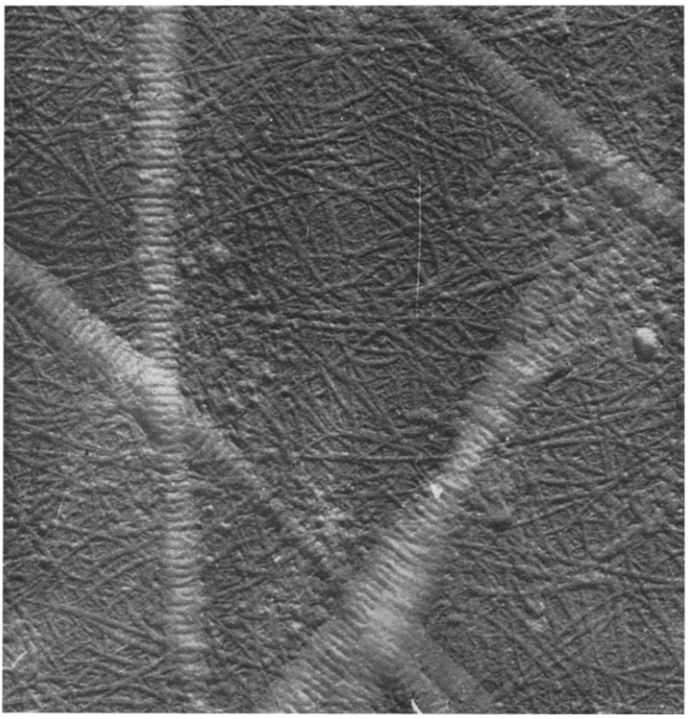

Fig. 3. Collagen deposited by addition of citrate buffer at a p_H of 4.6. At a lower p_H only thin fibers like those in the background are produced. Few of the long striated fibrils are uniform in diameter and many taper much more than any shown here. 22,000 ×

from the top fraction of the ultracentrifuged supernatant is further evidence that the reconstituted fibers develop from the dissolved collagen.

Preparations of reconstituted collagen for electron microscopic examination were made by mixing small volumes of a clarified solution, prepared in the way just indicated, with several volumes of salt solutions or buffers of various compositions and p_H values. Droplets of the resulting suspensions were placed on the usual formvar-covered screens. After standing for a couple of minutes to allow fibrils of the reconstituted collagen to settle out on the formvar, the remaining liquid was withdrawn and the formvar washed with several changes of distilled water to remove adhering salts. After drying, the preparation thus obtained was shadowed, usually with palladium, and examined under an RCA type EMU electron microscope.

RESULTS

In a first series of experiments a 0.1 *M* solution of NaCl was used as precipitant. Definite fibers were obtained in this way but they proved to be for the most part non-striated. Relatively thick fibrils tapering to a point on one or both ends (Fig. 2) were frequent. In some photographs these seemed to be built of helically wound threads; the fibrils themselves have often been intertwined to form ropes such as that appearing in Fig. 2. It is striking how much these salt-precipitated fibrils of collagen resemble the particles of many greases[8,9] as seen under the electron microscope.

The mean size of these fibrils has varied with the concentration of the precipitating

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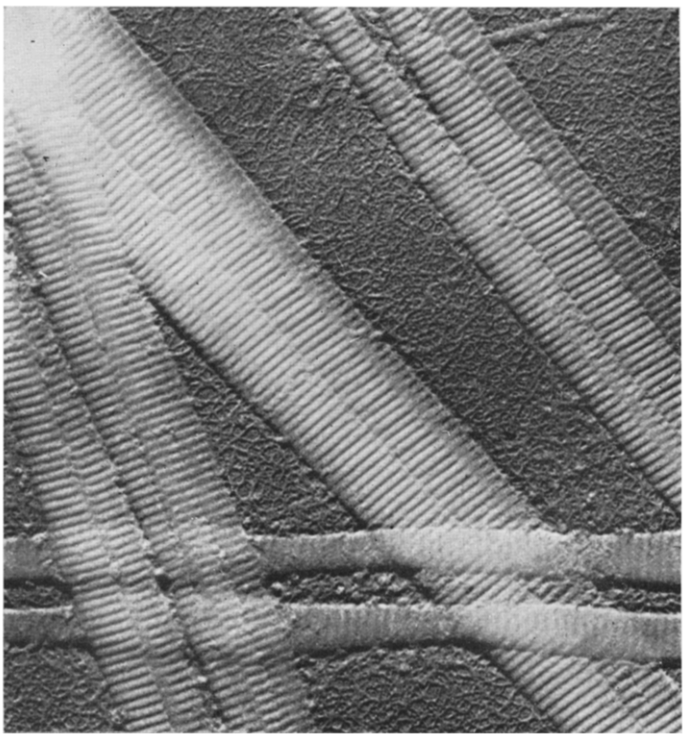

Fig. 4. Striated fibrils formed at p_H 5.0. The striations here are raised ridges which do not exhibit an internal fine structure. The well defined filaments in the background presumably are the relatively few particles which are not incorporated into the long fibrils. 22,000 ×

salt solution. They have been smaller the more concentrated the salt, and the more dilute the collagen solution. Though NaCl-precipitation has resulted in a preponderance of non-striated fibrils, definitely striated fibrils have occasionally been formed.

In order to get some indication of the conditions favorable to the development of the striated fibrils a series of experiments was next made in which the precipitant was a buffer. This first series of buffers consisted of mixed sodium citrate and phosphate (0.02 *M*). The results obtained with it showed very clearly the sharp relation that exists between buffer p_H and the structure of the precipitated collagen. Best-striated fibrils were obtained with buffers of p_H between 4.5 and about 5.5. Sporadically, however, well-striated preparations resulted from the use of a buffer lying outside this p_H range and further experiments were therefore made. These have indicated that there is a better correlation between the final p_H of the suspension and the structure of the precipitate; they also brought out other factors important in determining the conditions under which striated fibrils form. Chief among these have been concentration of the original collagen solution, and both the concentration and the chemical nature of the buffer. Thus better results were obtained with a citrate than with either a phosphate or with the original mixed citrate-phosphate buffer. A more extensive series of experiments was therefore next made with citrate buffers.

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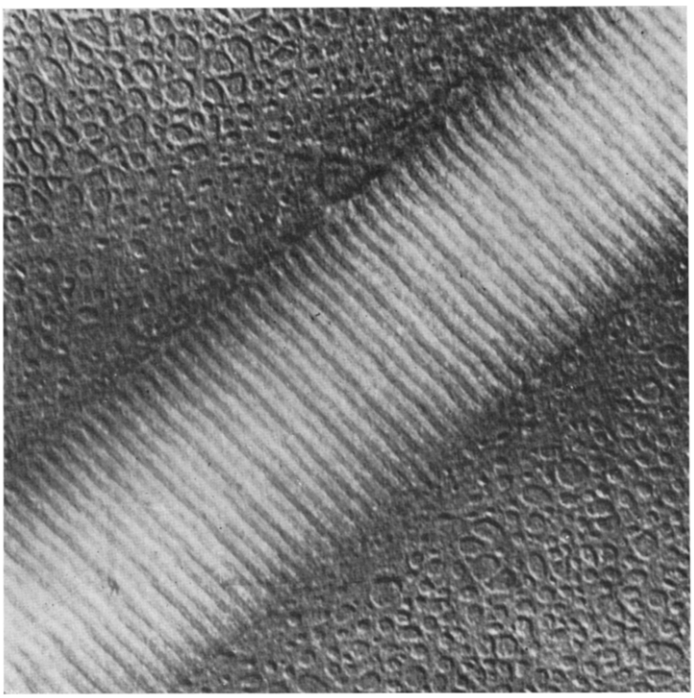

Fig. 5. A single striated fibril from a mass precipitated at p_H 5.0. Each striation here has a central thread-like filament and a subordinate thread on either side. 39,000 ×

Using a 0.02 *M* sodium citrate buffer, preparations containing many striated fibers were obtained when the final p_H lay between *c.* 4.5 and *c.* 5.0. Below *c.* p_H 4.5 few if any striated fibrils were produced, the aggregates consisting of the thin non-striated fibrils that provide the matted background to Fig. 3. As this figure illustrates, the precipitate formed at p_H 4.5–4.6 is a mixture of these thin filaments with some large striated fibrils; the latter frequently and quite characteristically are not the indefinitely long rods produced at higher p_H's but instead terminate at one or both ends as cones tapering to a point. In the region between p_H *c.* 4.7 and *c.* 5.0 practically all the material of the collagen solution may be incorporated into a mass of beautifully striated large rod-shaped fibers (Fig. 4 and 5). At values of p_H immediately above 5.0 the striations are usually less distinct (Fig. 6). Close inspection of the photographs shows that this is due in part to the accumulation of an exceedingly fine-textured material on the surface of the fibers. With further increase in alkalinity of the buffer, all traces of striation disappear and the mean diameter of the fibers is greatly reduced (Fig. 7).

Experiments were next made with collagen solutions diluted up to 100-fold and with buffers of various concentrations. These have shown that the production of well-formed, striated fibrils like those of Fig. 4 and 5 is favored by a high concentration of the collagen solution and a low concentration of the buffer. Thus, while striated fibrils

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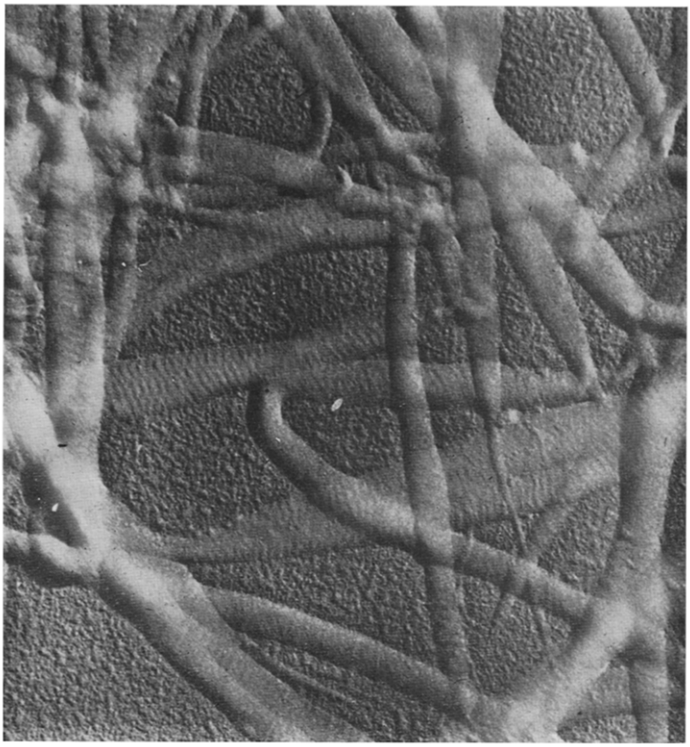

Fig. 6. Fibrils precipitated at p_H 5.5 with potassium citrate. The striae are much less well-marked than in the two preceding photographs. 21,500 ×

were produced from a five-fold dilution of the original collagen solution of one experiment, only small non-striated filaments resembling those of Fig. 7 were obtained after dilutions of 25 times, or more. The survey of the effect of buffer-concentration on fiber-formation indicated that the best-striated fibers were obtained with sodium citrate buffers of concentrations between *c.* 0.02 and *c.* 0.1 *M*. With 0.2 *M* buffer many small non-striated filaments accompanied the striated fibers in precipitates formed even at the optimum p_H. These thin filaments have resembled those obtained from dilute collagen solutions and from the use of more dilute alkaline buffers (Fig. 7). Often these thin filaments precipitated by strong buffer have been wound about one another to produce ropes like that of Fig. 2. Usually the ropes have been tightly twisted but sometimes they have been loosely wound, as in Fig. 8. With a buffer strength as great as 0.5 *M*, the thin filaments have predominated over the thicker striated ones. There has been some, though not conclusive, evidence that increase in buffer strength somewhat restricts the optimal p_H range within which striated fibers form.

A study also was made of the effect of salt concentration when NaCl solutions were used as precipitants. This demonstrated that the collagen solutions had to be at least 0.001 *M* with respect to NaCl before preparations were obtained significantly different from those given by the salt-free solutions themselves (Fig. 1). A few of the tapered

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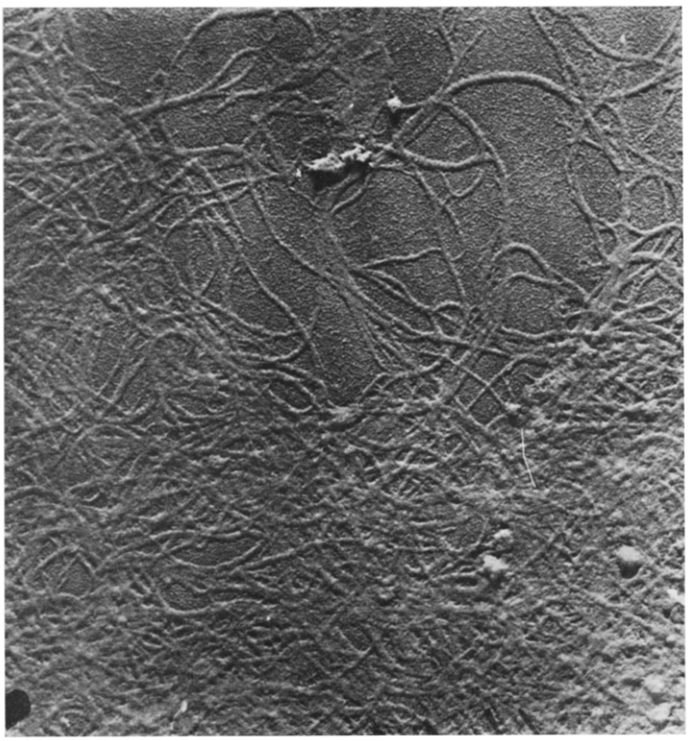

Fig. 7. At greater alkalinity (p_H 8.0) the precipitate is a mass of fine unstriated fibrils. 21,500 ×

needles of Fig. 2 were seen in the 0.001 *M* preparations and they were much more numerous when 0.01 *M* NaCl was employed. They were most numerous in precipitates from *c.* 0.1 *M* NaCl and it was with this salt concentration that striated fibrils were sometimes observed. Still more concentrated salt (1.0 *M* NaCl) yielded mats of thin, uniform, unstriated rods.

A few observations have been made with buffers having lithium and potassium as cation in place of sodium. Precipitates with both these (0.03 *M*) in the neighborhood of p_H 5.0 have contained many striated filaments. Sodium is therefore not essential to the formation of striated collagen. In the preparations made with potassium and lithium citrates more non-striated material has been present and the striations have often been less well developed; this may, however, simply be due to the fact that with them somewhat different optimal conditions are required for the production of striated material.

Phosphate buffers also give striated fibers but the optimum conditions for their production have seemed more restricted. In our limited experiments best striations have been seen in preparations obtained by precipitation with 0.1 *M* buffer at p_H 5.0.

The striated fibrils referred to in the preceding paragraphs have shown the characteristic 650 A repetitions of collagen. In a very few instances we have obtained, instead,

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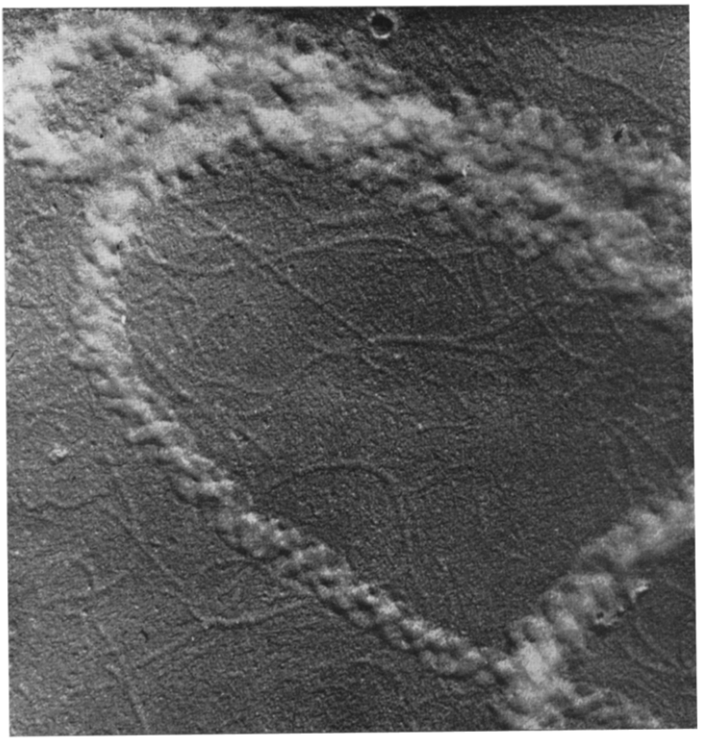

Fig. 8. Sometimes these thin fibrils become inter-twined to form ropes like those of this picture. Precipitated at p_H 6.5. 21,500 ×

fibrils continuously striated like some seen in the animal body (Fig. 7 of Reference 7). They have been produced so rarely that we are not yet in a position to define the conditions under which they form. A good example was furnished by precipitating a hydrochloric acid (p_H 3.8) extract of tendon with 0.03 *M* potassium citrate of p_H 5.0 (Fig. 9). Other continuously striated fibrils have been observed after precipitation of a similar extract with 0.1 *M* NaCl.

A striking feature of some well-striated preparations obtained under optimal conditions of p_H and ionic strength has been wave-like thickenings along parts of individual fibers (Fig. 10 and 11). Sometimes these thicker regions run nearly transverse to the fiber but usually they take a diagonal direction. The intervals between the bands of these "superperiods" also vary from fiber to fiber, being as few as two of the 650 A striae and as many as five or six. Often they occur in fibers whose composite character is attested by the fact that the 650 A striae do not have a constant direction across the entire fiber (Fig. 11 and 12). Evidently they arise as the fiber develops but an adequate understanding of them must wait for a clearer picture than we now have of the details of this development.

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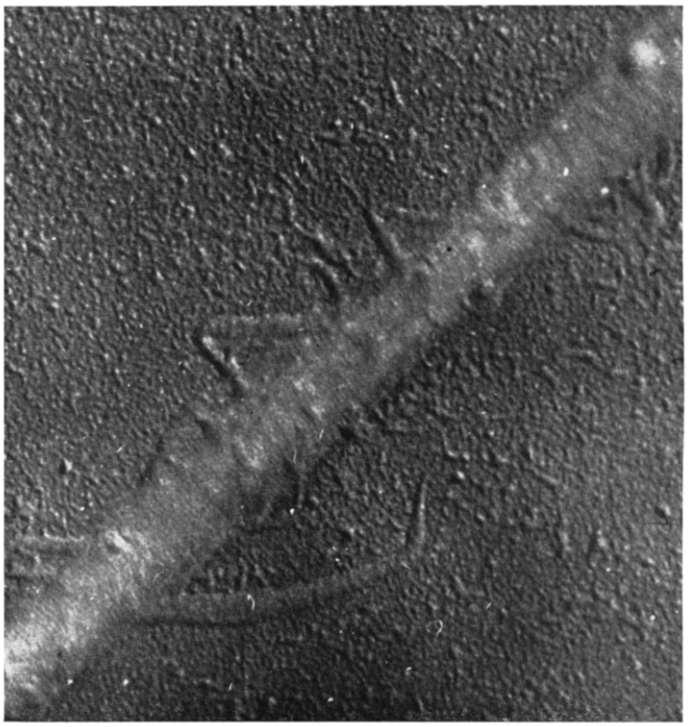

Fig. 9. A continuously cross-striated fiber (interstria distance = *c.* 220 A) in which there is no indication of the usual 650 A ridges. Precipitated from an HCl solution at p_H 5.1 with 0.03 *M* potassium citrate. 36,500 ×

DISCUSSION

Experiments such as the foregoing demonstrate that collagen fibers showing the periodicities of tendon are produced in the test tube from dissolved collagen. Though there are secondary variations in the details of the order in structure shown in these fibers depending on the conditions of precipitation, the fundamental 650 A repetition of collagen is preserved in fibers showing periodic order.

The laboratory production of these collagen fibers gives especial point to the question of how they develop through the orderly arrangement of the molecular particles present in a collagen solution. Differences in fine detail of this order both in laboratory-made and in native collagen make it especially desirable to try to define the conditions that yield these differences. This is important for the light it may throw on the physico-chemical conditions that prevail in the animal body as connective tissue is laid down and also because we may in this way get a clew to the altered conditions in a diseased animal responsible for the changed structure that sometimes has been observed.

The present experiments show some but not all the steps whereby striated fibers of collagen form from their solution. If precipitation takes place from too strong a salt solution or at unfavorable p_H values, non-striated fibers result. When conditions are

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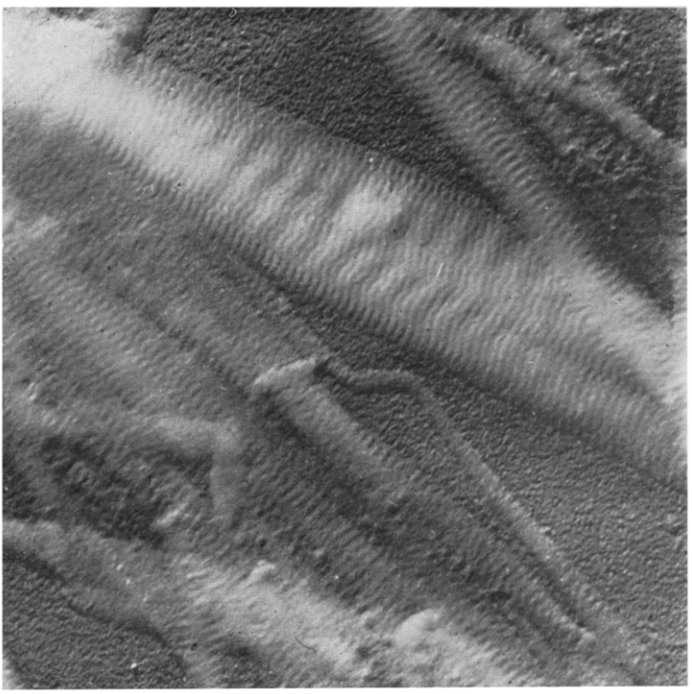

Fig. 10. Fibrils precipitated at p_H 5.0 (0.03 *M* citrate) which show periodic thickenings. Note that in practically none of the fibers of this photograph are the 650 A striae rectilinear. This is due to the composite nature of the big fibers. 21,500 ×

favorable for the formation of striations these may be a system of thick ridges (Fig. 4) or of cross threads which usually but not necessarily are in pairs. The striated fibers themselves may appear thick and rod-like or so flattened (Fig. 11) after drying as to be almost sheet-like. The cross striations may traverse large fibers uninterruptedly, as in Fig. 4 and 5, or they may zigzag, as in Fig. 10, 11 and 12, in such a fashion as to indicate that the fiber is a composite of narrower ones twisted about and displaced with respect to one another. This composite nature of many of the larger fibers is a factor contributing to the "superperiods" they sometimes show.

SUMMARY

Collagen reprecipitated from its solutions in dilute acid may show all the fine details of structure which the electron microscope has revealed in native collagen. A study has been made of the conditions of ionic strength, p_H and concentration which lead to striated reprecipitated collagen. Citrate buffers (0.02–0.1 *M*) have given best results. At values of p_H between 4.6 and *c.* 5.0, nearly all the reprecipitated collagen has been well-formed and striated. Above and below this region the fibrils have been thinner and usually unstriated. Precipitation with NaCl has given non-striated fibrils whose dimensions and appearance have depended on the concentrations of the reactants.

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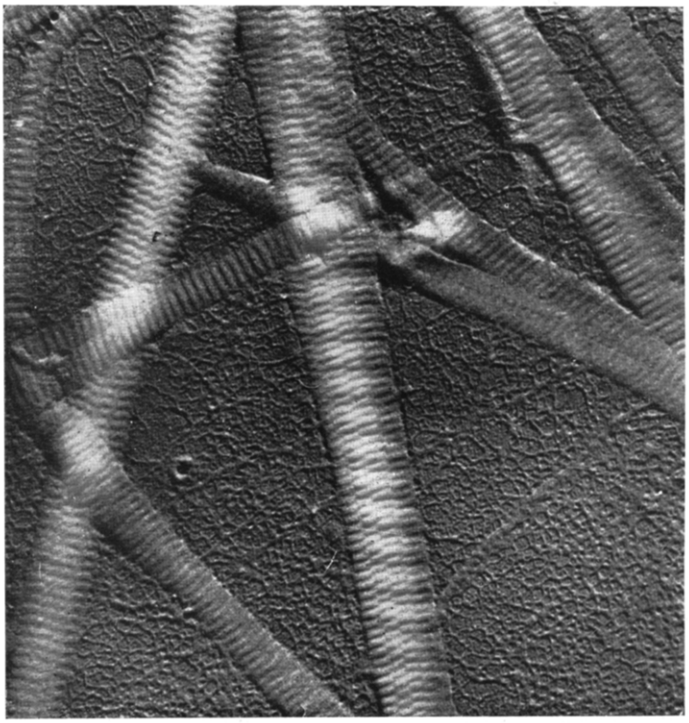

Fig. 11. Striated collagen precipitated by 0.1 *M* citrate buffer (p_H 4.85). The broad fiber running centrally from top to bottom is obviously composite and shows a well defined superperiod. There is well-marked zigzagging of the striae on the fiber to the left of it. The fibers at the right are especially flat. 22,000 ×

RÉSUMÉ

Le collagène reprécipité de ses solutions dans l'acide dilué peut montrer tous les fins détails de structure que le microscope électronique a révélé dans le collagène natif. Nous avons étudié les conditions de force ionique, de p_H et de concentration ayant donné lieu a l'apparition de collagène reprécipité strié. Les tampons au citrate (0.02–0.1 *M*) donnèrent les meilleurs résultats. A des valeurs de p_H comprises entre 4.6 et environ 5.0, presque tout le collagène reprécipité était bien formé et strié. En dehors de ce domaine les fibrilles étaient généralement plus minces et non-striées. La précipitation au NaCl donnait lieu à l'apparition de fibrilles non-striées dont les dimensions et l'apparence dépendaient des concentrations des substances en réaction.

ZUSAMMENFASSUNG

Aus Lösungen in verdünnter Säure gefälltes Kollagen kann dieselben strukturellen Einzelheiten aufweisen, welche das Elektronenmikroskop in nativem Kollagen sichtbar gemacht hat. Ionenstärke-, p_H- und Konzentrationsbedingungen, bei welchen Kollagen in gestreifter Form, gefällt wurde, wurden untersucht. Die besten Ergebnisse wurden mit Citratpuffer (0.02–0.1 *M*) erzielt. Bei p_H-Werten von 4.6 bis zirka 5.0 war beinahe die Gesamtheit des gefällten Kollagens wohlgeformt und gestreift. Bei höheren und bei niedrigeren p_H-Werten waren die Fibrillen meistens dünner und nicht gestreift. Fällung mit NaCl gab nicht gestreifte Fibrillen deren Grösse und Aussehen von der Konzentration der reagierenden Substanzen abhing.

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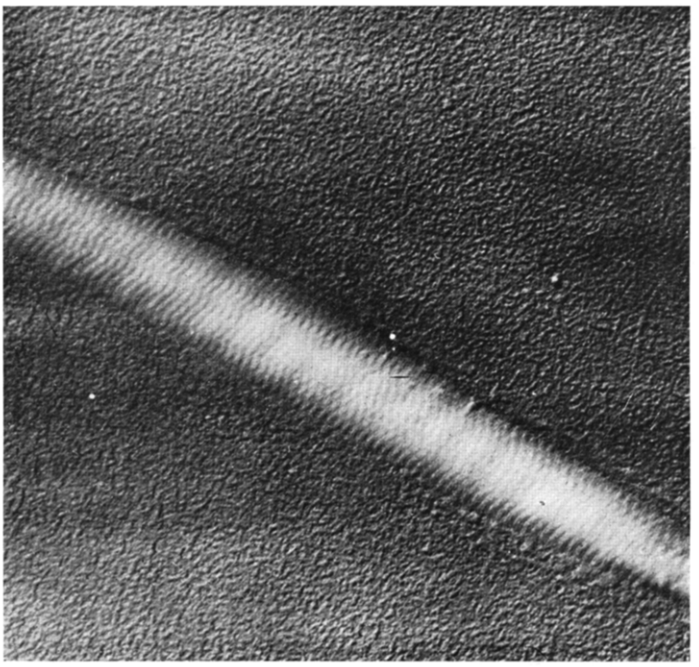

Fig. 12. A composite fiber precipitated at p_H 5.0 which shows both a superperiod and a well marked criss-crossing of its 650 A ridges. 21,500 ×

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