A NEW PARTICLE TYPE IN CERTAIN CONNECTIVE TISSUE EXTRACTS*

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Chemical and electron microscopic studies of the in vitro reconstitution of fibrils from acid solutions of collagen have revealed that the form of the axial repeating structure in the fibril is markedly influenced by the physical chemical environment. This paper describes a new structure precipitated from collagen solutions, the organization of which is regarded as having fundamental significance for collagen structure.

Early studies1–6 patterned after the classic Nageotte experiment6 demonstrated that the addition of NaCl to dilute acid solutions of certain forms
of collagen, such as rat tail tendon or ichthyocol, precipitate fibrils which, when viewed in the electron microscope, exhibit a very regular axial repeating period. Neutralization or addition of 0.1–0.2 M solutions of salts with monovalent cations precipitated fibrils with the axial period and detailed intraperiod fine structure characteristic of native collagen fibrils. At somewhat higher ionic strength the precipitated striated fibrils had periods of about one-third the normal, or 220 Å. At still higher salt concentration the fibrils formed were structureless.5,8

![Frequency distribution curve of lengths of segments produced from phosphate extracts of calf corium.](image)

FIGURE 1

Following the procedure for preparing “procollagen” described by Tustanovsky and Orekhovich, et al., whereby skin and other tissues are extracted with acid citrate buffer and the extract dialyzed against water, the present authors found that the precipitate which forms on dialysis contains, besides typical collagen fibrils, a new fibril type.11 This fibril, having an axial period ranging from 1800 to 3000 Å, was called the “long-spacing” or “LS” form. To distinguish between this structure and the new struc-
ture to be described below, we shall refer to it as "fibrous long-spacing" or "FLS."

FLS could be produced also from acetic acid filtrates of fish swim bladder collagen (ichthyocol) by the addition of plasma α-1 acid glycoprotein followed by dialysis. The acid filtrates contain about 0.1% collagen. At glycoprotein concentrations of about 0.02% or higher, FLS are formed almost exclusively. At lower concentrations (ca. 0.001%) of glycoprotein, chiefly collagen-type fibrils are reconstituted. At intermediate concent-

![Frequency distribution curve of lengths of segments obtained from phosphate extracts of carp swim bladder tunic.](image)

trations both types are formed. FLS can be converted into the collagen type by dissolving in 0.05% acetic acid and dialyzing against 1.0% NaCl.

Although glycoprotein is highly efficient in producing FLS and collagen, it is not unique in this property; a number of other substances will accomplish the same result. It was therefore concluded that the components necessary for the formation of cross-striated fibrils, whether of the collagen or FLS type, are contained in the collagen extracted from the connective tissue. The precipitating substances seem to be relatively non-specific under the conditions of the experiment.
Detailed intraperiod structure may be observed in LS fibrils. At least 14 bands of characteristic position and density have been observed in preparations stained with phosphotungstic acid (PTA). The pattern of banding (Fig. 5) is strikingly different from that of collagen (Fig. 6) not only in the relative positions of the bands but also in being non-polarized or symmetrical in the axial direction.

It is generally assumed that collagen is composed of protofibrils or polypeptide chains with sequences of amino acids running in the same direction in adjacent chains and with discontinuities in register, thus forming a cross-

striated fibril with polarized structure. It is possible that the symmetry of FLS structure is produced by a packing of polarized chains oriented in both directions (antiparallel array).

Using 70,000 as the molecular weight of procollagen and 600 the number of amino acid residues, Bresler, et al., concluded that procollagen must be a highly coiled chain since they deduced that the length of the molecule is only 380 A. If the helix were uncoiled the molecule would have a length of the order of 2000 A. The suggestion has been made that in LS fibrils the chains are uncoiled, being essentially in a β state. For this there is as yet no direct evidence. Although high-angle x-ray patterns of FLS prep-
arations displayed the features characteristic of normal collagen, more conclusive evidence is required to prove or refute this point of view.

Substantially all of the collagen in acid solutions of ichthyocoll may be converted to FLS if the concentration of acid glycoprotein, added before dialysis, is sufficiently high. This suggests the possibility that thin particles having the length of the FLS period, i.e., about 2000 Å, may occur in

![Figure 4](image)

**FIGURE 4**

Ultraviolet absorption spectra of: 1. Phosphate extract of calf corium; 2. Phosphate extract of carp swim bladder tunic.

the collagen solutions or may be dissociated from a higher polymer. The fact that segments of the order of 2000 Å in length were occasionally observed in "procollagen" solutions (Fig. 7) lends support to this view. Accordingly, efforts were made to isolate the material of which such elongate particles are composed.

Earlier experiments indicated that FLS could be produced from mildly
alkaline phosphate extracts of skin and other connective tissues. Dialysis of such extracts produces an amorphous precipitate; extraction of this precipitate with citrate buffer (pH = 3.8, μ = 0.2), followed by dialysis, leads to the formation of FLS together with collagen-type fibrils.

A slight variation of this procedure led to the discovery of an entirely different and new long-spacing type. This involved the dialysis of a phosphate extract (pH = 8, μ = 0.4) against citrate buffer (pH = 4, μ = 0.2)—rather than the citrate extraction of the dialyzed precipitate of a phosphate extract. Shortly after the beginning of dialysis a white copious precipitate begins to form. In the electron microscope this material was seen to contain (besides variable amounts of unstructured material and thin filaments) a new component in the form of segments having a characteristic pattern of internal band structure (Fig. 8).

In figures 1 and 2 are shown the distribution curves of lengths of segments obtained from phosphate extracts of calf corium and carp swim bladder tunic. The curves differ with regard to the mean length and dispersion. The reasons for the differences are not yet clear; however, they serve at least to establish an order of magnitude for the segment length. The segment lengths clearly fall within the range of periodicity of the fibrous long spacings. These new structures will be called “segment long spacings” or “SLS.”

Further characterization of the material precipitated as SLS required considerable purification. It proved very difficult to separate SLS from contaminants in skin extracts, either before or after precipitation. Far more satisfactory as starting material is the tunic of the carp swim bladder after preliminary extraction with dilute acetic acid or acid citrate buffer. In such preparations the finely filamentous contaminant is present only in

FIGURES 5–10 (P. 485)

Fig. 5. Long spacing fibril (FLS) produced by dialysis of a mixture of α-1 acid glycoprotein and ichthyocol filtrate. Note the detailed symmetrical intraperiod fine structure. PTA stained. Mag. 44,500X.

Fig. 6. Collagen fibril of cow hide showing characteristic polarized intraperiod banding. Stained with PTA. Mag. 150,000X.

Fig. 7. Segments of LS produced early in the dialysis of a citrate extract of newborn rat skin against water. Note similarity of intraperiod fine structure with that of FLS (Fig. 1). Mag. 24,000X.

Figs. 8 and 8A. Segment long spacings obtained by dialysis of a phosphate extract of calf corium against citrate. Mag. 23,000X. Note detailed asymmetric fine structure particularly in the enlarged pair of segments joined end to end in 8A. Mag. 44,000X.

Figs. 9 and 9A. Segments obtained from phosphate extracts of carp swim bladder tunic which had been previously extracted with dilute acetic acid. PTA stained and lightly shadowed with chromium. Mag. 18,000X. More detailed fine structure is illustrated in 9A. (PTA alone). Mag. 80,000X.

Fig. 10. Segments, produced by the addition of ATP to an ichthyocol filtrate. PTA stained and lightly shadowed with chromium. Mag. 24,000X. 10A is a higher magnification. Mag. 49,000X.
very small amounts. An electron micrograph of purified SLS produced from a phosphate extract is shown in figure 9.

It will be noted that the SLS structure is polarized. This is most easily noticed by observing the interband region of very low density which occurs slightly off-center (Figs. 8A and 9A). As many as 18 bands have been observed in individual segments.

Phosphate extracts both of skin and of acid extracted swim bladder tunic manifest strong absorption at about 2600 A and a minimum at about 2375 and 2450 A (Fig. 4) suggesting the possibility that nucleic acid or nucleotides may play a role in the formation of SLS. It is true that certain solutions such as phosphate extracts of salt-reconstituted ichthyocol, which yield SLS upon dialysis against citrate buffer, show no absorption maximum at 2600 A. Nevertheless it seemed worth while to examine the efficacy of nucleic acid and various nucleotides in forming SLS from collagen solutions.

Yeast ribonucleic acid and DNA dissolved in 0.05 M citrate, pH = 4, and also in water, were added to ichthyocol filtrate to make concentrations ranging from 0.005 to 0.6%. Immediate precipitation of structureless filaments, but no SLS, occurred in all cases.

Adenosine-3-phosphoric acid, adenosine-5-phosphoric acid, uridylic, and guanylic acids in 0.2% concentration produced no precipitate. Hexametaphosphoric acid, sodium pyrophosphate, and the sodium salts of ADP and adenosine triphosphate produced flocculent precipitates containing collagen-type or structureless fibrils immediately after mixing. No SLS or FLS were formed.

However, when adenosine triphosphoric acid (ATP), dissolved in 0.05% acetic acid, was added in 0.1 to 0.25% final concentration to ichthyocol filtrate, precipitation started almost immediately and continued for several hours in the cold. These precipitates are composed exclusively of SLS although some very finely filamentous material is present. Their structure (Figs. 10 and 10A) is essentially identical with that of SLS obtained from phosphate extracts of skin and swim bladder. Figure 3 is a distribution curve of segment length in such a preparation of ATP-induced SLS. There is considerable variability in segment width from one preparation to the next and oftentimes within the same preparation as shown in figures 8

FIGURES 11-14

Fig. 11. ATP-produced segments washed in dilute acetic acid. Fraying indicates the parallel array of filamentous components composing the segment. Mag. 42,000X.

Fig. 12. ATP-produced segments revealing great variability in width and also emphasizing the fibrous nature of the components. Mag. 21,000X.

Fig. 13. ATP-induced SLS showing several standing on end. Calculation from shadow length indicates a height nearly equal to the length of those segments lying flat. Their three dimensional quality is also apparent. Mag. 12,000.

Fig. 14. SLS transformed to collagen after dissolving in NaCl and dialyzing against the same salt. A small amount of amorphous debris is usually present. Mag. 27,000X.
and 12. Frequently one observes segments standing on end, as in figure 13, and it is obvious that these structures are not flat ribbons but have significant thickness. This is also evident from the shadows cast by those segments lying flat. Preliminary study indicates that SLS is very highly hydrated. Application of the freeze drying technique of Williams\textsuperscript{16} may provide interesting information on the shape of these structures.

Washing the precipitate several times with fresh 0.1\% ATP in 0.05\% acetic acid further removed the earlier described contaminant and provided a highly purified preparation of SLS. When such purified SLS is exposed to acetic acid in the same concentration in which they were formed (0.05\%, pH = 3.5) the segments begin to disintegrate. This process is in itself of considerable interest for, when followed with the electron microscope, it lends strong support to the view that the segments are composed of a parallel packing of very thin fibrous particles of uniform length (Fig. 11). It may also be seen that the banded structure is still evident in some of the thin segment frays.

For present purposes it seems reasonable to assume that the chains in the SLS are in parallel array and that ATP molecules tend to bond neighboring chains laterally. More evidence is needed before a definitive suggestion can be made regarding the specific nature of this bonding. Preliminary estimates indicate that SLS preparations which had been washed with acid may contain as much as 12\% of ATP which would correspond to about one mole of ATP per 3278 grams of protein.

Particularly pertinent is the question of the relationship between collagen, fibrous LS, and segment LS. Can they be converted one into the other and, if so, by what means? Experiments outlined below that all of these forms are interconvertible.

Conversion of SLS to Collagen.—When a pure preparation of SLS is dissolved in 0.17 \textit{M} NaCl and dialyzed at room temperature against NaCl, a heavy fibrous precipitate forms which is composed entirely of well-structured collagen fibrils with the characteristic collagen banding and a small amount of granular debris (Fig. 14). The conversion to collagen is accelerated at temperatures up to 37\textdegree C.

Conversion of Collagen to SLS.—Collagen-type fibrils with characteristic banding may be obtained by dialyzing an acid filtrate of ichthyocol against a 1\% NaCl solution. This precipitate of collagen fibrils, after removal by centrifugation, was dialyzed exhaustively in the cold against 0.05\% acetic acid. The precipitate dissolved to form a tenuous gel. This was diluted to the original volume with 0.05\% acetic acid and ATP added to make a concentration of 0.2\%. The precipitate which formed was nearly pure SLS.

Conversion of Fibrous LS to Segment LS.—LS fibrils, produced by dialysis of a mixture of ichthyocol filtrate and acid glycoprotein (procollagen), may
be dissolved in acetic acid. Dialysis of such a solution against 1% NaCl produced collagen fibrils while addition of ATP produced SLS.

These results demonstrate that the various forms of fibrous "collagens" may be freely converted, one into the other. The likelihood that thin fibrous protein particles about 2200 A long, or polymers thereof, exist in various extracts of connective tissue is indicated both by the action of glycoprotein, to form the symmetrical fibrous LS, and by the action of ATP, to form the polarized, asymmetric segment type of LS.

Preliminary chromatographic analyses reveal that the amino acid pattern of SLS protein resembles that of collagen fairly closely. Tyrosine values are low and this amino acid may actually be absent. Hydroxyproline is present in ichthyocol SLS in a concentration approximately 90% of that found in ichthyocol collagen. After allowance is made for the ATP nitrogen, the nitrogen content of the SLS protein was estimated to be 18.2%, in good agreement with that of collagen. Films of SLS give a high-angle x-ray pattern characteristic of collagen.

Neither fibrous nor segmental LS have thus far been observed in tissues. This may be due to the relatively high ionic strength of tissue fluids and to the fact that the long particles may not exist in the uncombined state to any appreciable degree either in the ground substance or in the formed fibrils of the connective tissue. This problem is being investigated.

The possible range of conditions under which SLS may form has not yet been determined. It would be premature to implicate ATP itself or other nucleotides specifically in their formation from native tissue extracts. The presence of purine- or pyrimidine-containing compounds in the phosphate extracts may conceivably be coincidental. It is quite possible that the long protein particles may be capable themselves of aggregating in the manner characteristic of SLS when the physical chemical environment is appropriate. The possible relationship of these particles to collagen precursors is at present a matter for speculation.

Elongate particles or molecules having lengths of the order of 1500 to 3000 A have been deduced to exist in tropomyosin,17 L-myosin,18 paramyosin,19 and keratin.20 It is possible that application of the type of technique described above may permit direct electron microscopic visualization of the elongate particles through their reaction with a compound like ATP which may unite chains of similar length and constitution laterally and form either segmental or fibrous LS structures, as in the case of collagen.

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13 It should be noted, however, that a wide variety of proteins and other high polymers will not reproduce this phenomenon.

HYDROGEN BONDED HELICAL CONFIGURATIONS OF THE POLYPEPTIDE CHAIN

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Pauling and Corey1–3 have formulated two helical configurations for polypeptide chains in which: (1) the residues have the dimensions deduced by consideration of the results of crystal structure studies in amino acids and related compounds,4 (2) all residues are equivalent, and (3) each residue forms an N—H···O hydrogen bond of length about 2.75 Å in