THE INTERACTION OF MUCOPROTEIN WITH SOLUBLE COLLAGEN; AN ELECTRON MICROSCOPE STUDY*

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Communicated March 25, 1951

The collagen of certain forms of connective tissue, such as rat tail tendon and the fish swim bladder (ichthyocol), dissolve in dilute acid to yield a clear, relatively viscous solution. When NaCl is added to such a solution to a concentration of 0.2–1.0%, or if the solution be neutralized, a fibrous precipitate of collagen is produced.1–3 Electron microscope studies have demonstrated that the reconstituted fibrils show the axial period and intra-period fine structure typical of native collagen fibrils although the acid filtrate contains only very thin filaments.4,5 The process by which the thin filaments in the acid filtrate aggregate laterally to produce the typical collagen structure is of interest not only from the physical chemical point of view but also because a better understanding of the phenomenon may provide clues as to the mechanism of fibrogenesis in vivo. Investigations of the process of fibril reconstitution from acid filtrates of collagen by the addition of salt have been made in these laboratories6 and will be reported in detail elsewhere. For the present it may be noted that the type of fibril structure observed in the electron microscope (axial repeating patterns of about 650 Å, 220 Å, or no apparent pattern) depends upon the concentrations of salt and collagen. The experiments described in this paper suggest that other factors may also be of importance in the process of reconstitution.

Most forms of dense connective tissue, such as the corium of the skin, swell to varying extents in dilute acid but do not appreciably dissolve. The important discovery was made recently by a group of Russian workers7–9 that if mammalian skin corium is extracted with organic acid buffers (chiefly citrate, pH 3.0 – 4.5, Γ/2 = 0.2) the extract, after dialysis against water, yields a "crystalline" fibrous material. This material they have called "procollagen," believing that it may be biochemical precursor of collagen. From physical chemical studies Bresler, et al.,9 deduced that procollagen is a cylindrical macromolecule having a diameter of about 17 Å, a length of 380 Å and a weight of about 70,000. It was suggested that the macromolecule is composed of a coiled polypeptide chain about 2400 Å long and that fiber formation is due to linear and lateral aggregation of procollagen macromolecules.

Electron microscope examination of "procollagen" prepared according
to the directions of Orekhovich, et al., revealed two types of fibrils: crossstriated collagen-type fibrils having an axial period averaging about 650 A, and a new type of fibril having an axial period ranging from 2000 to 3000 A. Until this new type of fiber is characterized chemically it will be referred to as "long-spacing" or, briefly, LS fibril.

LS fibrils were obtained from extracts of rat, calf and steer skins, rat tail tendon and fish swim bladder. Considerable fine structure, consisting of characteristically positioned bands, may be observed within the main period (figure 3). An analysis of this intraperiod structure will be presented subsequently. The period is fairly uniform within individual fibrils but varies considerably in different fibrils in the same preparation. The distribution curves of preparations from various tissues differ somewhat among themselves. In figure 1 is shown the distribution curve of axial long spacings observed in material prepared from rat skin.

No LS fibrils have been observed in the numerous preparations of fragmented native collagen fibers which have been studied in this laboratory over a period of some years. Nor have they been observed thus far in thin sections of connective tissue though it must be admitted that relatively little study of thin sections has been made as yet upon skin and tendon (which are good sources of LS fibrils). Though it is possible that the fragmentation and sectioning techniques may destroy LS fibrils, it seems more probable that this new fibrous form does not pre-exist in the tissue but is produced artificially from precursors present in connective tissue extracts.

Collagen may be purified by the method of Bergmann and Stein, which includes extensive extraction with 10% NaCl followed by extraction with dibasic phosphate and, finally, with ether. Such purified collagen yields
(See opposite page for caption).
very few if any LS fibrils when tested in the usual manner. It would appear that the purifying extractions remove a precursor or precursors of LS fibrils. Of the extractives used only the phosphate is involved in removal of precursor. Phosphate extracts of calf skin, rat skin and rat tail tendon, when dialyzed against water yield a flocculent precipitate, amorphous in the electron microscope. Extraction of this precipitate with citrate buffer (pH 3.8, \( \Gamma/2 = 0.2 \)) and dialysis of the clear solution against water yields typical LS fibrils. Efforts were made, therefore, to determine the nature of the material which, with collagen, is extracted by phosphate and transformed into LS fibrils. Since mucoprotein (MP) has long been considered to be associated with collagen in the genesis of connective tissue and since phosphate is an excellent solvent for MP, the working hypothesis was tentatively adopted that MP may be one of the precursors of LS fibrils.

To test this hypothesis an MP preparation was added to a filtered acid solution of collagen obtained from tissue which had been sufficiently extracted with phosphate to remove precursor of LS fibrils. In relation to the bearing of the hypothesis on the genesis of connective tissue it might be most appropriate to use tissue MP for these tests. One experiment was in fact made with an MP preparation obtained from calf skin by a modification of the method of Weimer, et al.,\(^{12}\) with results essentially similar to those described below. However, for most of the experiments plasma MP was used.

Bovine mucoprotein was isolated from beef plasma by the procedure described by Weimer, et al.,\(^{12}\) When added to an acetic acid filtrate of ichthyocol or rat tail tendon\(^{13}\) in a ratio of 1 part of MP to about 10 parts of colla-

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**PLATE 1**

Figure 3. LS fibrils from dialyzed citrate extract of skin from 3 day old rat. Stained with 1% phosphotungstic acid (PTA) and lightly shadowed with chromium. Magnification 74,000 \( \times \).

Figure 4. LS fibrils precipitated by dialysis from a mixture of plasma mucoprotein (Schmid preparation) and acetic acid solution of ichthyocol. Stained with PTA. Magnification 45,000 \( \times \).

Figure 5. LS fibril precipitated by dialysis from a mixture of plasma mucoprotein (Schmid preparation, total concentration 0.03%) and acetic acid solution of ichthyocol. Note longitudinal fibrillation. Shadowed with chromium. Magnification 68,000 \( \times \).

Figure 6. Collagen-type fibrils (and one LS fibril) precipitated by dialysis from a mixture of plasma mucoprotein (total concentration 0.01%) and acetic acid solution of ichthyocol. Shadowed with chromium. Magnification 44,000 \( \times \).

Figure 7. Fibrous gel formed by dialysis of acetic acid solution of ichthyocol. Note absence of striated fibrils. Stained with PTA. Magnification 13,000 \( \times \).

Figure 8. LS fibrils precipitated by dialysis of a mixture of plasma mucoprotein (total concentration 0.2%) and acetic acid solution of ichthyocol (same solution which, when dialyzed without addition of mucoprotein yielded gel shown in figure 7). Stained with PTA. Magnification 13,000 \( \times \).
gen a heavy fibrous precipitate was produced only after dialysis against water. This precipitate consisted almost entirely of typical LS fibrils (figure 8). The intraperiod fine structure (figure 4) and the distribution curve of the axial periods (figure 2) are closely similar to those typical of LS fibrils obtained from citrate extracts of the original tissue. Since our preparations of MP had not been highly purified, the experiment was repeated with an acid glycoprotein from human plasma, described by Schmid,\textsuperscript{14} which was highly purified and proven to be monodisperse.\textsuperscript{15} This material, the composition of which agrees fairly closely with that described by Weimer, \textit{et al.}, produced LS fibrils identical with those produced by our own preparations of MP under similar experimental conditions. It seems safe to conclude, therefore, that it is in fact the MP, or some component of MP, which combines with acid-soluble collagen to produce LS fibrils. It should be noted that this reaction of MP with collagen appears to occur only when the collagen is highly solvated or dissolved in acid. Addition of MP to intact, native collagen fibrils does not produce LS fibrils.

When MP is added to acid filtrates of rat tail tendon or ichthyocol collagen in ratios of the order of 1:1000 or less, few if any LS fibrils are produced after dialysis against water. Rather, large numbers of fibrils having an axial period and intraperiod fine structure similar to collagen are produced. At intermediate ratios, mixtures of LS fibrils and the collagen type occur (figure 6). It is unlikely that this could be a simple reconstitution of collagen fibrils from the acid filtrate for this would require either the presence of salt or neutralization. Dialysis of collagen acid filtrates against water produces a gel consisting primarily of very fine filaments (figure 7). In figure 8 is shown a typical field of LS fibrils produced from the same preparation of ichthyocol but to which a relatively high concentration of MP had been added before dialysis.

These results suggest that MP, or a component of MP,\textsuperscript{16} may be involved in the formation of collagen fibrils. When the MP content exceeds a critical value, LS rather than collagen fibrils are produced \textit{in vitro}. At present, the mechanism of the reaction between MP and acid-soluble collagen is itself unknown. Detailed analytical investigations of this complex and, indeed, of reconstituted and native fibrils themselves, will be required before the above suggestion can be tested experimentally. Investigations along these lines are in progress.

* This investigation was supported in part by grants from the Eli Lilly Company to the Massachusetts Institute of Technology and by the United States Public Health Service to the Massachusetts General Hospital.
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AN HYPOTHESIS OF PROTEIN SYNTHESIS POTENTIATED BY CITRIC ACID*

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Communicated by Henry Gilman, March 27, 1951

The need for understanding protein synthesis is sufficiently great to warrant the presentation of any hypothesis which is frankly recognized as such in the accurate meaning of this term, and which is not intrinsically untenable. The hypothesis presented here concerns the potentiation of peptide bond synthesis by citric acid metabolites. The essential suggestion arises from observations on the effect of increasing concentrations of citrate buffer on the papain-catalyzed synthesis of acylamino acid anilides.¹ ²

The energy barrier in peptide bond synthesis has been evaluated as a figure within the range of 1400–3700 cal.³ and this approximate range of figures has been used in a number of interpretations. Some of these statements are so limiting as to exclude reversal of proteolysis as a mechanism.