Cartilage Collagen: A Staggered Substructure in Reconstituted Fibrils

Abstract. *In contrast to the typical transverse banding pattern of native and reconstituted skin collagen fibrils, reconstituted fibrils of cartilage collagen have an oblique banding pattern that results from a regular axial shift (89 angstroms) of component "subfibrils." The 89-angstrom shift may be related to the major helix of the collagen molecule.*

The chemically distinct species of collagen molecule isolated originally from developing hyaline cartilage is now partially characterized (*1–7*). The molecule, designated $[\alpha 1(II)]_3$, consists of three $\alpha 1$ type II chains, whereas the common skin-bone type of collagen molecule, designated $[\alpha 1(I)]_2\alpha 2$, is made up of two $\alpha 1$ type I chains and one $\alpha 2$ chain (*1–3*). The two types of $\alpha 1$ chain are similar in molecular weight, although they differ significantly in amino acid composition and sequence (*1–6*). Moreover, ten times more hexose is covalently bound to the cartilage collagen molecule than to the skin-bone molecules (*2, 3, 6*). In molecular weight, intrinsic viscosity, and optical rotatory dispersion the two collagen molecules are similar; but they differ slightly in melting temperature and in the rate of renaturation (*7*). Electron microscopy of cartilage segment-long-spacing (SLS) crystallites reveals an altered staining intensity of several specific bands (*2, 8, 9*).

Stark *et al.* (*8*) found that the banding pattern of reconstituted cartilage collagen fibrils is indistinct or absent, while the bands of reconstituted skin and bone collagen fibrils are well-defined and characteristic of their native form. We report here the occurrence of an unusual staggered banding pattern in reconstituted cartilage fibrils formed under conditions different from those used by Stark *et al.* (*8*).

Cartilage collagen, $[\alpha 1(II)]_3$, was obtained from xyphoid cartilages of lathyritic chicks (*2, 6*). The homogeneity of the protein was established by amino acid analyses of the native collagen molecules and of the isolated α chains, by the chromatographic behavior of the denatured collagen on carboxymethyl-cellulose and diethylaminoethyl-cellulose columns, and by the migratory characteristics of the α chains on polyacrylamide gel disc electrophoresis (*2, 6*). Skin collagen, $[\alpha 1(I)]_2\alpha 2$, was isolated from the same chicks and purified according to the method of Kang *et al.* (*10*). Fibrils of both collagens were formed by dialyzing 1 ml of a collagen solution (0.1 percent collagen in 0.5*M* acetic acid) against two changes of 4 liters of 0.15*M* NaCl for 24 hours at 4°C. The *p*H of the last dialyzate (usually about 5.4) then was increased to 7 by the addition of 1*N* NaOH, and dialysis was continued at room temperature (24°C) for another 48 to 96 hours. The resulting precipitate was applied for 5 minutes to specimen grids covered with carbon-collodion films and blotted dry with filter paper. The fibrils were stained either positively with 0.1 percent phosphotungstic acid followed by 0.1 percent uranyl acetate, or negatively with 4 percent sodium silicotungstate (*11*). Prior to the negative staining procedure, the fibrils were stained with 0.1 percent uranyl acetate.

The electron microscopic findings on cartilage collagen fibrils described below were reproduced with three different collagen preparations.

The precipitate of cartilage collagen contains a large number of tapered, relatively rigid fibrils having wide, dark and light bands inclined at an angle of $61° \pm 7°$ ($N = 147$) (*12*) to the longitudinal axis of the fibril (Fig. 1a). The oblique pattern is either right- or left-"handed," and it occurs frequently in a V-shaped (chevron), or occasionally in an N-shaped, arrangement. In two instances where a single fibril was clearly folded over and the banding pattern was distinct, a reversal of "hand" occurred.

At high magnification, negative staining reveals long, narrow, cross-striated units (subfibrils) (*13*) (Fig. 1d) arranged approximately parallel to one another and to the longitudinal axis of the fibril. The subfibrils have a width of 138 ± 15 Å ($N = 174$) (*12*), and they have been observed to extend uninterrupted in a straight line for 8000 Å. Adjacent subfibrils are shifted with respect to each other by 89 ± 12 Å ($N = 130$), so that they appear to be related by a twofold screw axis. Each subfibril has alternating dark and light transverse bands that are identical in longitudinal dimensions with the wide dark and light bands of negatively stained reconstituted skin collagen fibrils (Fig. 1e and Table 1). Hence, the oblique pattern of the cartilage fibrils arises from a regularly staggered array of parallel subfibrils, each having the transverse banding pattern of reconstituted skin collagen fibrils.

Table 1. Measurements on negatively stained, reconstituted collagen fibrils (*12*).

Item	Cartilage subfibrils (Å)	Skin fibrils (Å)
Major period	657 ± 20 ($N = 238$)	664 ± 23 ($N = 239$)
Dark band	364 ± 25 ($N = 180$)	370 ± 23 ($N = 194$)
Light band	292 ± 21 ($N = 176$)	294 ± 25 ($N = 193$)

The staggered pattern presumably is not the result of asymmetric forces acting on the fibrils during the preparative procedures, since we have observed the oblique and chevron patterns in fibrils fixed in suspension with glutaraldehyde, embedded in Epon, and viewed in thin sections.

With positive staining the fibrils have a complex band pattern that appears to be a composite of the stained bands of individual subfibrils. Regions from the wide part of such fibrils (Fig. 1b), and especially those near the tapered ends, show discontinuous dark bands with a periodicity of 96 ± 13 Å ($N = 252$) (*12*), which appears to reflect the axial shift of adjacent subfibrils (89 Å). The staggered array is still manifested by the superimposed diffuse oblique banding.

We estimate that about 50 percent of the fibrils in the precipitated cartilage collagen show the staggered banding pattern. The remaining fibrils are the narrow, indistinctly banded type (Fig. 1a), already described by Stark *et al.* (*8*) and occasional hybrid forms having the native transverse and the oblique patterns.

We were unable to find oblique or staggered banding patterns in native cartilage fibrils by examining positively or negatively stained homogenates of fresh xyphoid cartilage matrix or thin sections of the fixed and embedded cartilage.

Control fibrils of lathyritic chick skin collagen (Fig. 1, c and e) show the well-known, transverse banding of the common native collagen fibrils (*14*).

A staggered banding pattern apparently has not been recognized before in collagen fibrils although a wide variety of banded patterns and several helical configurations have been described in fibrous structures (*15*).

Our observations suggest that the staggered cartilage fibrils consist of narrow, skin-type collagen fibrils packed together in flat or irregular layers of various widths. In this model the thickness of each layer would be equal to the diameter of the subfibrils, and adjacent subfibrils within one layer would be shifted with respect to each other by 89 Å in an axial direction, while adjacent subfibrils in neighboring layers would be aligned in register. Such a structure could account for the right- and left-handed band patterns observed in our preparations (they would represent views of opposite sides of the fibril) and for the change of "hand" noted in fibrils that are folded over. The structure also would be consistent with prevailing ideas on the staining of collagen fibrils (*16*). Positive and negative stains would be expected to stain, respectively, bands and "holes" in the various layers of subfibrils within the fibril. If bands in the superimposed subfibrils were out of register, in the direction of the electron beam, a diffuse pattern, or perhaps none at all, would appear in the image of the fibril. A sharp native-type band pattern like that seen in individual subfibrils (Fig. 1d) would result only if the stained bands in the superimposed layers were in register.

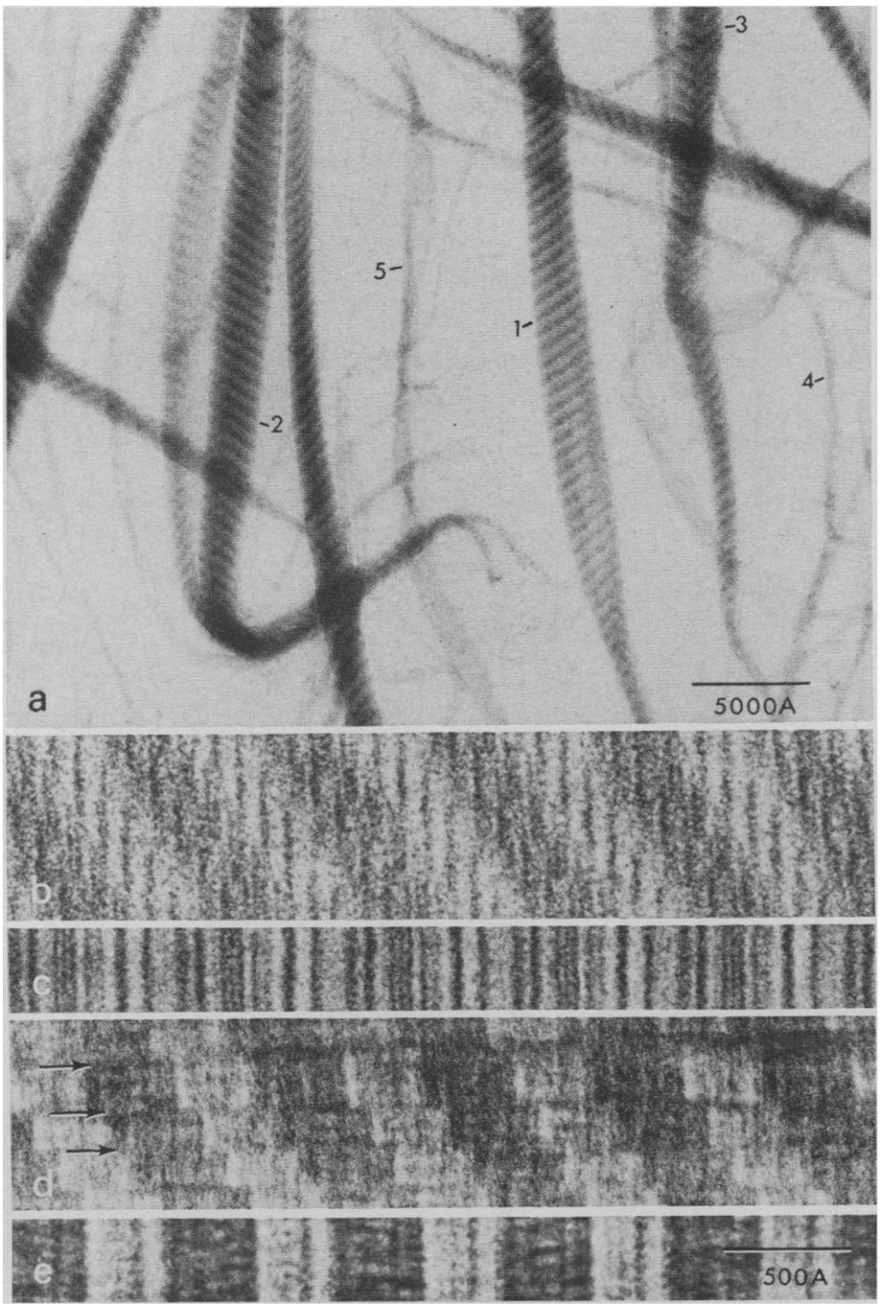

Fig. 1. Electron micrographs of reconstituted collagen fibrils. (a) Fibrils of cartilage collagen showing dark-light bands inclined to the left (1) or right (2) or arranged in a "chevron" pattern (3); narrow (4) and twisted (5) fibrils are shown. Positive stain (× 32,500). (b) Enlargement of an area like that near (1) in (a) showing a complex pattern of interrupted narrow, dark bands superimposed on oblique dark-light bands. Positive stain. (c) Segment of a skin collagen fibril. Positive stain. (d) Segment of a cartilage collagen fibril showing two adjacent subfibrils (between arrows), shifted 89 Å with respect to each other. The subfibrils are better defined when the figure is viewed at a low angle parallel to the length of the subfibrils. A single subfibril and a reconstituted skin collagen fibril (e) have similar dark-light band patterns. Negative stain. (e) Segment of a skin collagen fibril. Negative stain (b to e, × 355,000).

The close correspondence between the axial shift of adjacent subfibrils (89 Å), and the pitch of the major helix of the collagen molecule (85.8 Å) (*17*) suggests that the staggered band pattern may originate from the major helix or from some feature of the molecule directly related to it. This feature may involve, for example, the α chain composition, the covalently bound carbohydrate, or perhaps the interaction of a nonhelical end of one molecule with the helical part of a neighboring molecule. In any case, if the decisive factor resides in the cartilage collagen molecule it also may exist in the skin-bone type of collagen molecule, perhaps to a lesser degree, for a complex oblique banding pattern has been observed by Kuhn *et al.* (*18*) in fibrils reconstituted from highly purified calf skin collagen.

Since a staggered band pattern has not been found in native cartilage collagen fibrils, it seems that the conditions of reconstitution in vitro permit the expression of some characteristic feature of the molecule which is not expressed in vivo. This unidentified feature may be important for understanding the packing arrangement of collagen molecules in the fibril.

The superficial resemblance between the negatively stained banding patterns of the reconstituted cartilage collagen fibrils and paramyosin filaments (*19*) is of interest because the two types of molecular aggregates may have some common structural characteristics.

ROMAINE R. BRUNS
ROBERT L. TRELSTAD
JEROME GROSS
Developmental Biology Laboratory, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

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12. Mean value ± standard deviation and number of measurements.
13. We add to the terminology for the hierarchy of structural elements in collagen [R. S. Bear, *Advan. Prot. Chem.* **7**, 69 (1952)] the term *subfibril* to signify the individual banded elements (mean width, 138 Å) that constitute a staggered cartilage collagen fibril. A subfibril therefore differs from a microfibril [A. Veis, J. Anesey, S. Mussell, *Nature* **215**, 931 (1967)], which is a hypothetical fundamental aggregate of collagen molecules having an overall diameter of ~40 Å.
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Mechanical Resonance Spectra in Human Cancellous Bone

Abstract. *The dynamic mechanical response of fresh human cancellous bone at low audio frequencies contains two resonance spectra. The spectral frequencies in each series have the ratios 1 : 4 : 9 : 16 . . . n². The frequencies are in quantitative agreement with the concept of momentum wave modes of calcium and phosphorus atoms in the lamellae, with no variable parameters.*

The dynamic mechanical properties of mammalian bone have received relatively little attention, with the exception of fracture and impact-related trauma. However, measurements on intact human long bones (in vivo and in vitro) have shown resonances at low audio frequencies (*1*). Since the elastic compliance of cancellous bone is about an order of magnitude larger than that of cortical bone, it is likely that the dynamic mechanical properties of the former will be more important; for example, in the absorption of shock and in general dynamic response.

We have investigated the complex compressional (uniaxial) elastic modulus of human cancellous bone with a simple viscoelastometer, consisting of an electromagnetic force generator, a piezoelectric accelerometer, and a piezoelectric force transducer. The dynamic mechanical response of disks of human cancellous bone (1 cm in diameter, 5 mm thick) from the distal femur was measured under longitudinal sinusoidal compression. In essence, we measured the mechanical impedance of the disk and converted our data to the complex elastic modulus. Such techniques have been used extensively on engineering polymers for more than a decade (*2*). Ten samples were obtained at autopsy from four cadavers (all male, 43 to 53 years old at death) and machined carefully under water coolant. Measurements on the wet samples were made at room temperature and within 48 hours of death. The signals from the pickups were conditioned by charge amplifiers and fed into an oscilloscope to produce a Lissajous figure. Amplitudes and phase relationships were used to calculate the elastic (E') and viscous (E'') components of the stiffness (*2*). The viscoelastometer was calibrated with standard specimens and with an accurate vibration standard. Typical stresses were of the order of 1 pound per square inch (psi) (1 psi ≈ 52 torr; strains were 0.001 percent). Subsequent microscopic examination attested to the nondestructive nature of these measurements, and indicated the volume fraction of hard tissue to be 0.42 to 0.45. The phase angle, δ, was measurable to 3 degrees.

All ten samples of cancellous bone exhibited sharp resonances of the type shown in Fig. 1. The viscous component E'' was typically equal to E' at resonance, and was relatively insignificant at frequencies away from resonance. By changing the specimen dimensions and using a variety of materials (such as aluminum and various plastics and rubbers) we have determined that the resonances are intrinsic to the cancellous bone itself, and not due to our apparatus.

Audio frequency resonances have been found in a variety of engineering materials (mostly polymeric) (*3*) and also in canine intervertebral disks (*4*); the types of apparatus used all differed markedly from ours. The resonances were characterized by their sharpness compared to other types of resonance based on material properties, and by the fact that the resonant frequencies can be characterized by the ratios 1 : 1, 4, 9 . . . n^2, where n is an integer. However, the resonances previously found were not reproducible, in the sense that they could usually be eliminated by changing the length of the specimen (*5*). Thus, the origin and nature of the resonances have been controversial (*3*). Our data were reproduced in ten separate specimens with thicknesses ranging from 4 to 6 mm, and within the precision of our apparatus (a few percent) the peaks always occurred at the same frequencies.

The equation that best fits our data

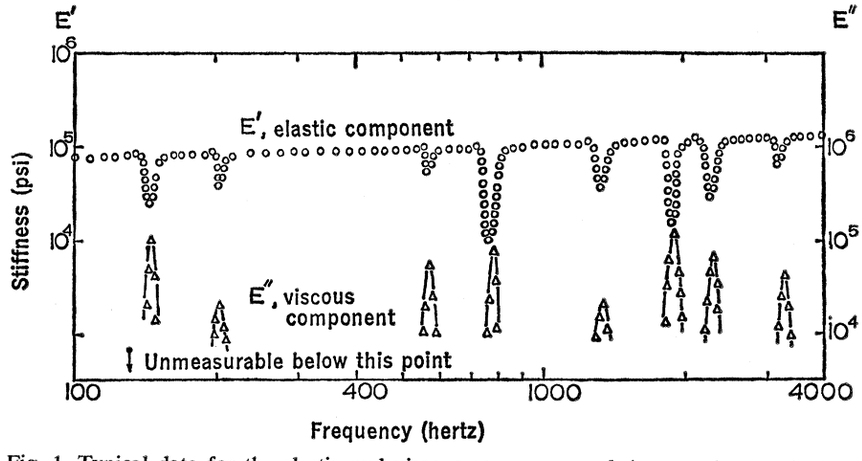

Fig. 1. Typical data for the elastic and viscous components of the complex longitudinal stiffness of fresh human cancellous bone for frequencies between 100 and 4000 hertz.