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CHOLESTERIC TWIST OF COLLAGEN *IN VIVO* AND *IN VITRO*

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Abstract In biological structures the spatial distribution of collagen can correspond to the geometry adopted by molecules in cholesteric liquid crystals; in compact bone the twist appears between fibrils, forming helices, and disposed along coaxial cylinders. In thin sections of self assembled collagen gels twisted structures have been observed; the geometry of the fibril aggregates corresponds to a cylindrical twist. In dehydrated drops of collagen solutions the textures observed are related to cholesteric and hexagonal pseudomorphoses. The liquid crystalline behaviour of collagen in these different situations is discussed.

INTRODUCTION

Collagen is a major biological polymer synthesized in cells and secreted in extracellular spaces of animals. The fibrils, visible in electron microscopy, present a typical 67 nm cross striation; they are formed by a quasi-crystalline association of unit molecules (300 nm long), each molecule being a triple helix of polypeptide chains (Review in 1).

In biological tissues collagen fibrils are present in a very concentrated state in the organic matrix of skeletal structures; for example in annelid cuticles (2), in fish scales (3), or in vertebrate bones (4). The three dimensional organization of collagen in these systems is closely related with the spatial geometry of molecules in certain liquid

crystals (5).

The present work describes this analogy in the case of compact bone, *in vivo;* it then considers the behaviour of this polymer in the absence of cells, *in vitro,* both in collagen gels and in dehydrated drops of collagen solutions. The occurence of a cholesteric twist, between collagen molecules, is described and discussed in these diverse situations.

MATERIALS AND METHODS

Human bone fragments, obtained at surgery, are classically fixed for electron microscopy in 2% glutaraldehyde and postfixed in 1% OsO4. The samples decalcified in EDTA for one week, are then dehydrated and embedded in araldite. Thin sections are made with a diamond knife, and contrasted with uranyl acetate and lead citrate. Some air dried bone fragments coated with gold in a vacuum chamber are observed with a scanning electron microscope at 30 Kv (Philips SEM 505).

Type I Collagen is extracted from calf skin in 0.5 M acetic acid, purified and typed by Bioetica.

Collagen Gels are obtained by exposing the acid solutions, at concentrations of 2.5 and 10 mg/ml, to concentrated ammonia vapors in a dessicator for 15 min. Ammonium acetate is removed from the gels by repeated washes in phosphate buffers at pH 7.4. Samples are taken at times ranging from 1 to 6 days from gels kept at 20°C then classically fixed, dehydrated and embedded for the preparation of ultrathin sections (6).

Collagen drops of 50µl are deposited on glass slides and observed, after dehydration, with a polarizing microscope. The textures of dehydrated drops are observed from concentrated solutions (10 mg/ml) either at pH 3 or after dialysis of the acid solution against neutral buffers, that is at pH 7. Samples of the dehydrated drops are prepared, for electron microscopic observations, following the same procedure as considered

above for the gels.

Polarizing microscopy: Preparations are observed with a Nikon, Optiphot X, polarizing microscope, between crossed polarizers.

Transmission electron microscopy: Ultrathin sections are observed with a Philips 201 electron microscope at 80 kV. A Philips 300 electron microscope supplied with a goniometric stage was used to tilt sections by angles of + or - 45°.

RESULTS AND DISCUSSION

CHOLESTERIC ORGANISATION IN COMPACT BONE

Arced patterns in electron microscopy

In a transverse view of long bones, the compact organic matrix appears organized in concentric lamellae around a canal (Fig. 1). Each component is called an osteon, and corresponds, in three dimension, to a cylindrical structure running parallel to the long axis of the bone. Ultrathin sections oblique to the osteon axis are observed in transmission electron microscopy. The decalcified material reveals that the collagen fibrils can be distributed in the form of superimposed series of nested arcs as underlined by the hatched line (Fig. 2). The typical cross striation of collagen is discernable at a higher magnification; the fibrils appear either in longitudinal (L), in oblique (O) or in transverse (T) position within the section plane (Fig. 3).

Such series of nested arcs have been interpreted as the consequence of the twisted plywood architecture of polymers in certain biological structures, this geometry being analog to that of molecules in cholesteric liquid crystals (7). A demonstration of the reality of this model can be given by a goniometric effect observed in electron microcopy (8). In the case of compact bone, by tilting ultrathin sections along an

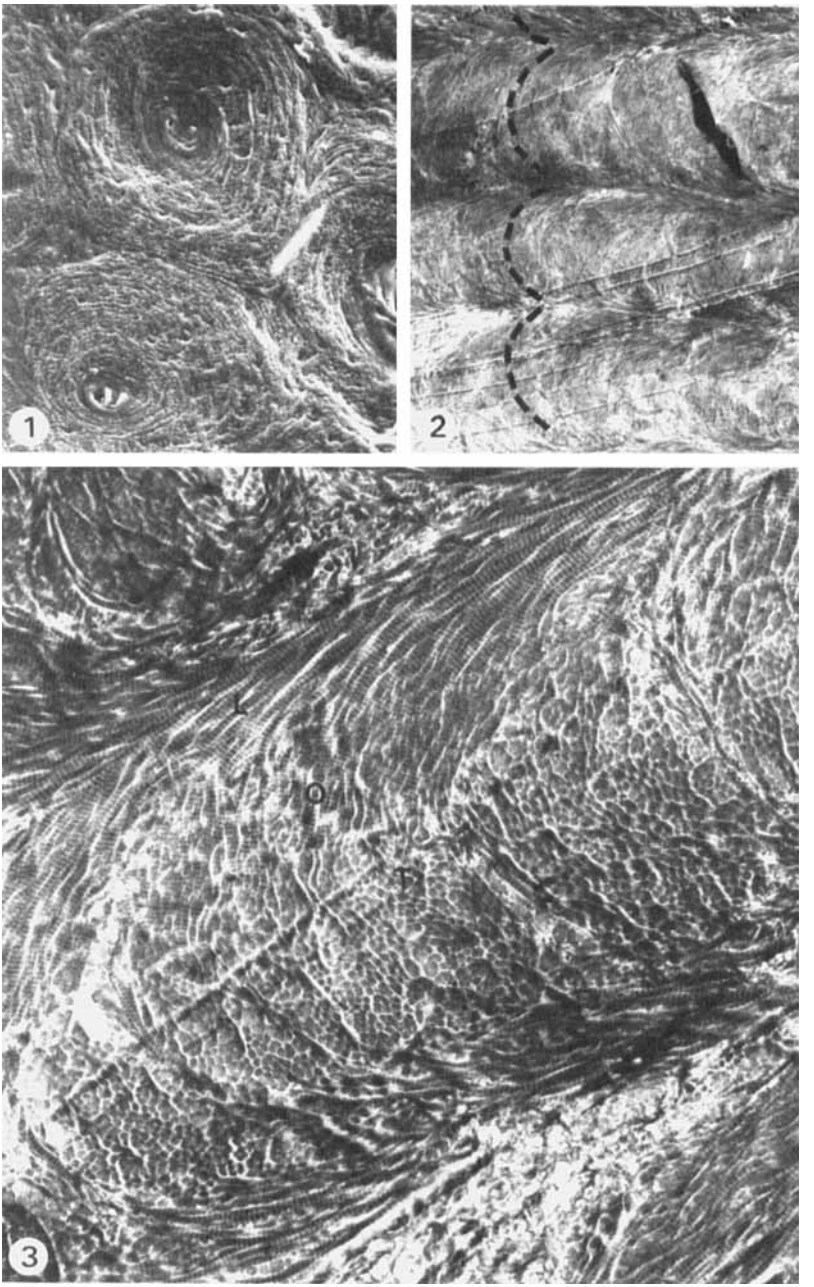


FIGURE 1. Osteons in a transverse view of compact bone; SEM X 200

FIGURE 2. Superposed series of nested arcs in an ultrathin section oblique to the osteon axis; TEM X 3000

FIGURE 3. Collagen fibrils in longitudinal (L), oblique (O), and transverse (T) position within the section plane draw one series of arcs; TEM X 17.500

axis parallel to the laminae, the arced pattern disappears after a tilt angle of 75° (4).

Osteon model : cholesteric twist along coaxial cylinders

Cholesteric analogs in biological systems usually consist in planar twists; all molecules lie parallel in a given plane and their direction rotates continuously from one plane to the next by a constant angle (6). In the case of compact bone osteons, the parallel and equidistant planes are transformed into a set of coaxial cylinders. Collagen fibrils are parallel helices, the angle of helices relative to the cylinder axis is constant on each cylinder, and changes by a small and constant angle from one cylinder to the next.

A representation of this model is given in Fig.4 a. In the same figure the aspect of the osteon in polarizing microscopy, and the fibrillar directions in electron microscopy (using the nail convention) are represented. In transverse sections light and dark bands are well differentiated between crossed polarizers, and no arcs are visible in ultrastructure (Figs. 4 b and 4 b'). In oblique sections the elliptical maxima of darkness, observed in polarizing microscopy, appear slightly shifted in regard to the osteon axis (Fig. 4 c), and series of nested arcs are visible in ultrastructure (Figs. 3 and 4 c'). This model accounts well for optical properties of osteons observed in polarizing microscopy described as "intermediate type osteons" in the literature (9).

In addition to the cholesteric disposition of collagen in

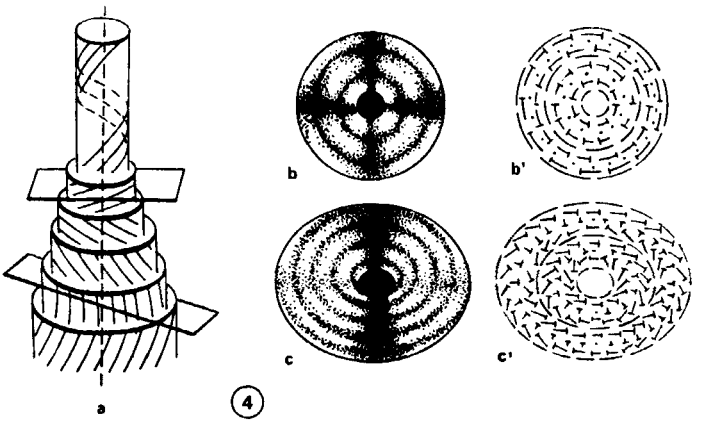


FIGURE 4. Cholesteric osteon model (a) the helices indicate the collagen fibril orientations on coaxial cylinders. Aspect of osteons (transverse and oblique section planes) in polarizing (b,c) and electron microscopy (b', c').

compact bone an orthogonal plywood architecture of the collagen fibrils was also observed, a transition exists between these two types of orders (4).

CYLINDRICAL TWIST IN COLLAGEN GELS

Cholesteric aggregates at the bottom of gels

The formation of fibrils from collagen solutions has been widely studied by biochemistry and electron microscopy (for example:10,11); but only scarce works consider the suprafibrillar order of collagen *in vitro* (12). A recent study has shown that in collagen gels reprecipated fibrils from purified solutions, at neutral pH, assemble into several micron large aggregates (6). Twist is observed at two

FIGURE 5. Regularly twisted aggregate of collagen striated fibrils showing a quasi-continuous passage from fibrils in longitudinal, then oblique and cross view (gel added with fetal calf serum and heated for 2 days at 20°C) TEM x 40.000; courtesy of J.P. Denèfle and J.P. Lechaire.

FIGURE 6. Absence of helicity in abnormally reprecipitated fibrils; in these gels no twist is observed between the fibrils. TEM X 50.000

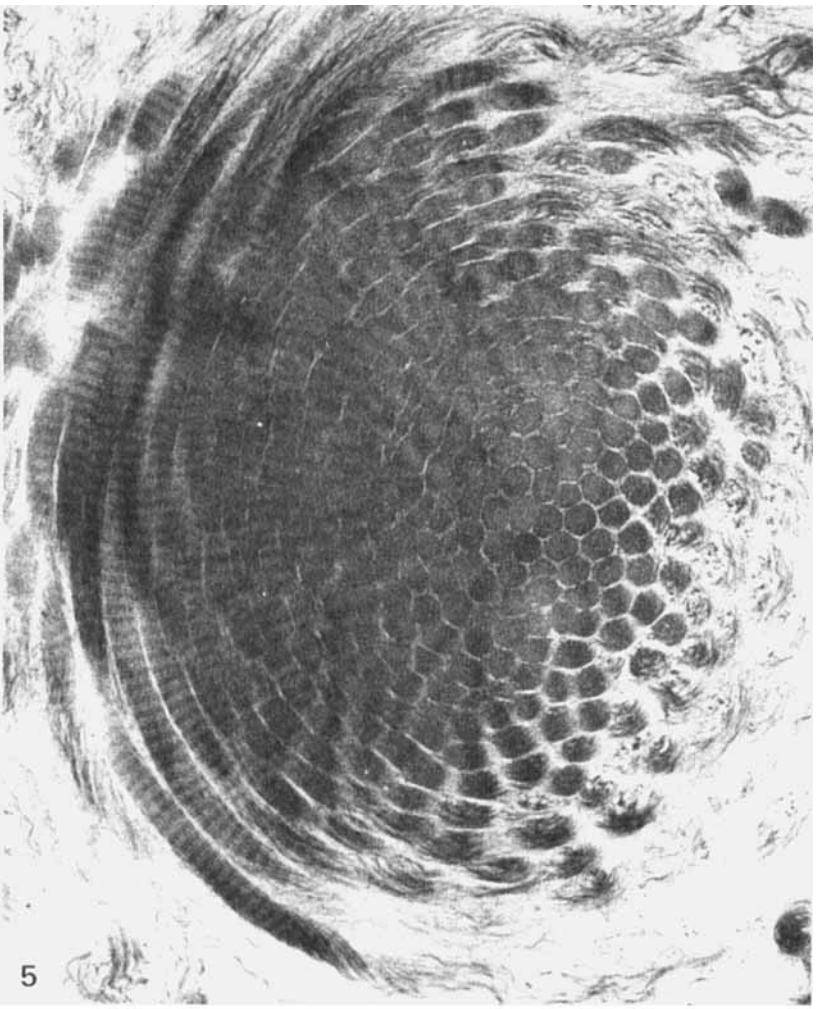
different levels of architecture between microfibrils (as we will call here the subunits of fibrils) and between fibrils (Fig. 5, from Bouligand *et al.* 1985). This ultrathin section shows microfibrils forming
twisted bundles which condense in cross striated fibrils; the fibrils themselves aggregate and show a mutual twist.

At the upper level of the gels thin microfibrils randomly disposed are observed. The collagen aggregates, 10 to 20 µm large, are present only at the bottom of the gels (about 1 cm in height), the density of these twisted structures is much higher than that of the surrounding gel.

Factors influencing the aggregate assembly

The presence of fetal calf serum, which in culture conditions favours the anchorage of cells onto the gel, retards the self-assembly of collagen aggregates (6 days instead of 3); the twist is also less regular in presence of these proteins (6). On the contrary when gels are heated at 37° for several hours, the aggregates reach a higher degree of organization.

The extremities of the cross striated fibrils form twisted bundles (Fig. 5); this character was absent in certain gels where the collagen fibrils self-assembled in an abnormal way for pathological reasons (Fig. 6); the cholesteric aggregates where not observed in these gels. Two reasons can be proposed first the irregular shape of the fibrils in cross section limiting the surfaces of contact between the collagen molecules, and second the rigid extremity of the polymer showing no free helical ends (13).



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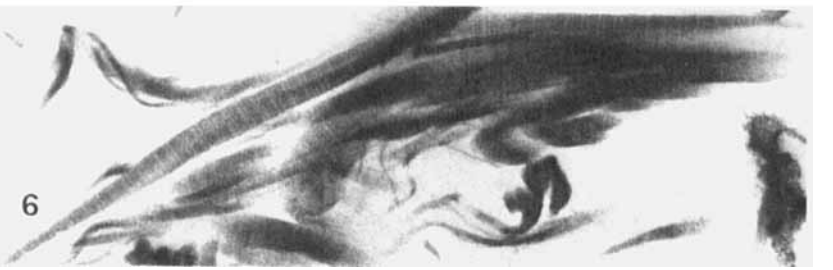

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FIGURE 7. Concentric arrangement of microfibrils in a dehydrated drop of collagen solution at acid pH. X 15

FIGURE 8. Varying textures along a concentration gradient in a free drop of collagen solution at acid pH. Polarizing micr. X 150

FIGURE 9. Alternating light and dark bands near the center of the dehydrated drop of collagen. Polarizing micr. X 250

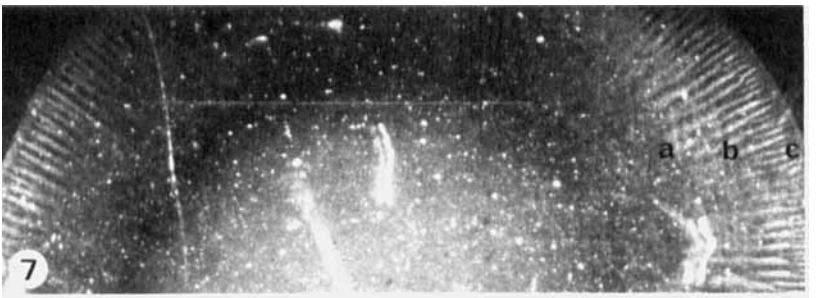
TEXTURES IN DEHYDRATED DROPS OF COLLAGEN

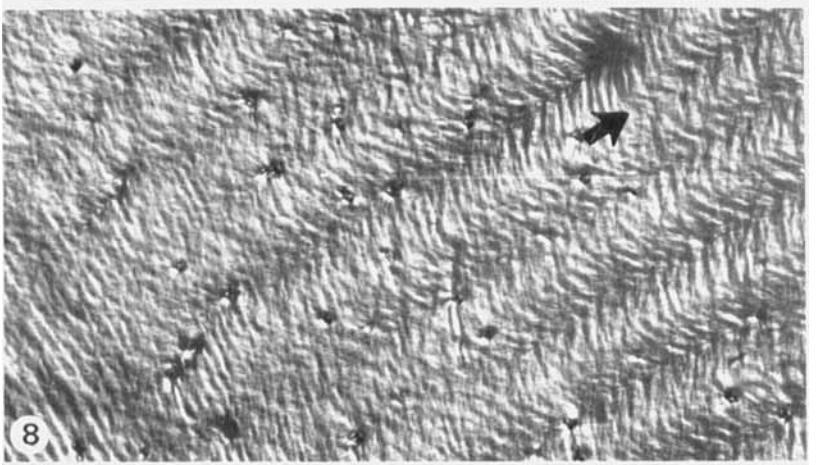
Organization of microfibrils at acid pH

Free drops of concentrated acid solutions of collagen (10 mg/ml) deposited on glass slides are dehydrated at room temperature. At this acid pH the collagen molecules are not organized in cross striated fibrils, but reach an intermediary state between the unit triple helix and the achieved collagen fibril; These molecules arrange concentrically around the center of the drops (Fig. 7). The mean diameter of the drops (50 µl in volume) is 1 cm, and the textures vary along a concentration range increasing from the center of the drop to the edge of the preparation (Fig. 8). The deposition of the molecules, reaches a dehydrated state in a few minutes at the exterior of the drops and progresses inwards; a stable geometry is achieved in about one hour at 20°C. Different textures are revealed in polarizing microscopy (Fig. 8), and we will describe three basic patterns found very reproducibly, with type I collagen.

a : Concentric light and dark bands

This first pattern corresponds to the less concentrated state, it is localized near the center of the drop (Fig. 7, level : a). The texture observed, between crossed polars in polarizing microscopy, consists in alternating light and dark bands disposed concentrically around the center of the drop. Locally, when the direction of the bands lye parallel to the polarizer or to the analyzer, thin extincted bands alternate with larger illuminated bands (Fig. 9 black arrow); when rotating the





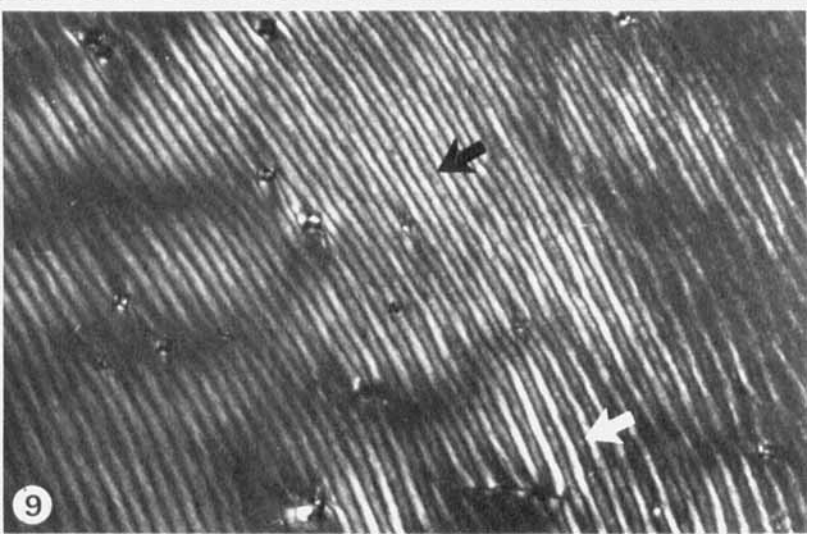


FIGURE 10. Series of arcs radiating from the center of a dehydrated drop of collagen solution. Polarizing micr. X 630

FIGURE 11. Molecular orientations (corresponding to the preparation of Fig. 10) observed in a thin section parallel to the surface of the drop. TEM X 30.000

FIGURE 12. "Undulating" texture at the periphery of the drop Polarizing micr. X 630

FIGURE 13. Thin section parallel to the surface of the drop (corresponding to the preparation of Fig. 12) microfibrils are not resolved. TEM X 30.000

microscope stage by an angle of + 22° one light band out of two, the even series, becomes dark (Fig. 9, white arrow); if the rotation is inversed (by an angle of - 22°) then it is the odd series that become dark. This property indicates that the dehydrated collagen solution is optically active; this rotatory power is due to a disymmetry of the structure. The same observation was made in thin sections of the crab cuticle, analyzed in polarizing microscopy (14), and this material is the best example of cholesteric pseudomorphoses known in biological systems.

b: radiating series of "arcs"

When moving outwards a second pattern is observed (Fig. 7 level b); it consists in arcs disposed along rows radiating from the center of the drop to its periphery; one row of arcs can divide into two when progressing outwards (Fig. 8, black arrow). At a higher magnification (Fig.10) the pattern appears as parallel series of arcs, and is reminiscent of comma textures described in cholesteric phases formed by MBBA (15), but the arcs are further subdivided in dark and light bands, shifting when the microscope stage is rotated. This complex texture was not further elucidated. However information on the molecular orientations in the preparation was obtained in electron microscopy; thin sections, parallel to the surface of the drop, reveal undulating orientations of the collagen microfibrils in this region (Fig.11).

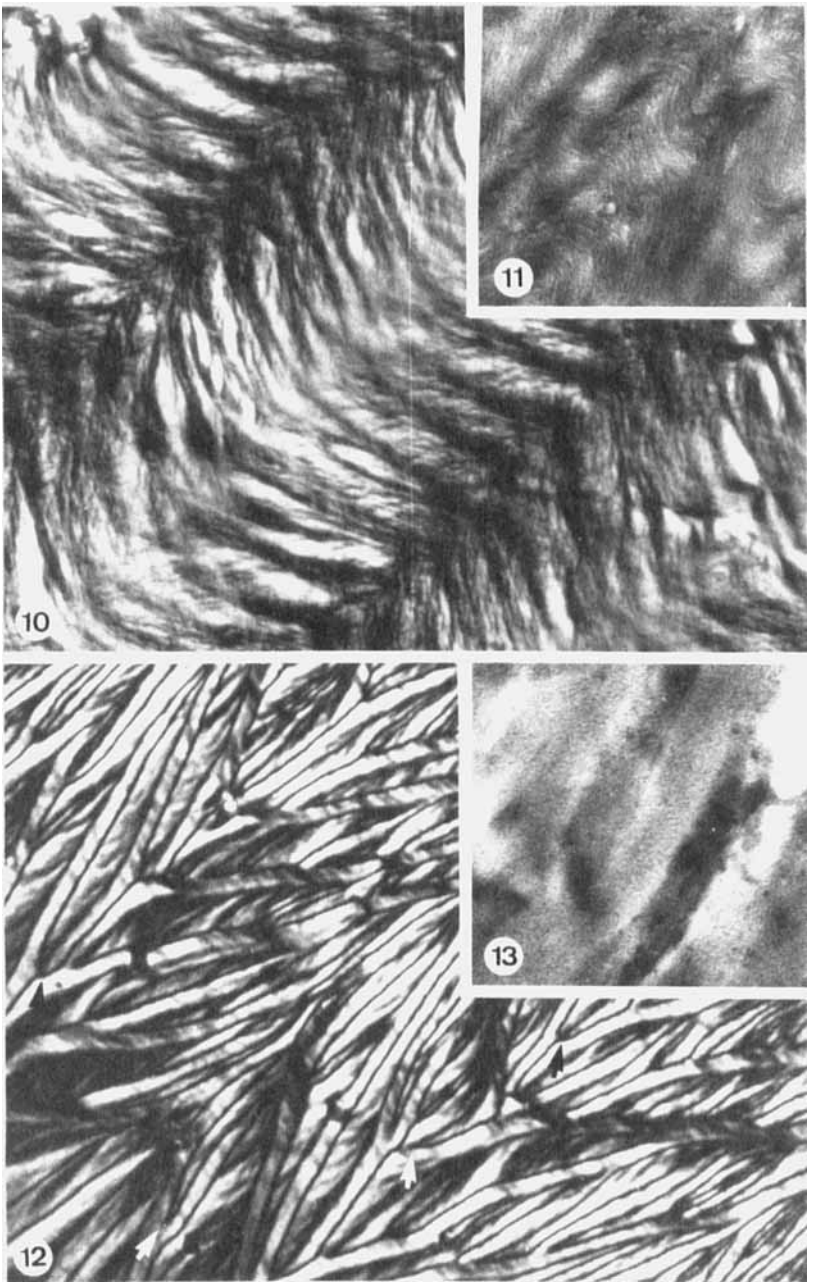


FIGURE 14. Coexisting fibrils and microfibrils at the center of a dehydrated drop of collagen solution at neutral pH. TEM X 30.000

FIGURE 15. Small birefringent domains formed by the collagen molecules in a dehydrated drop at neutral pH. Polarizing micr. X 250

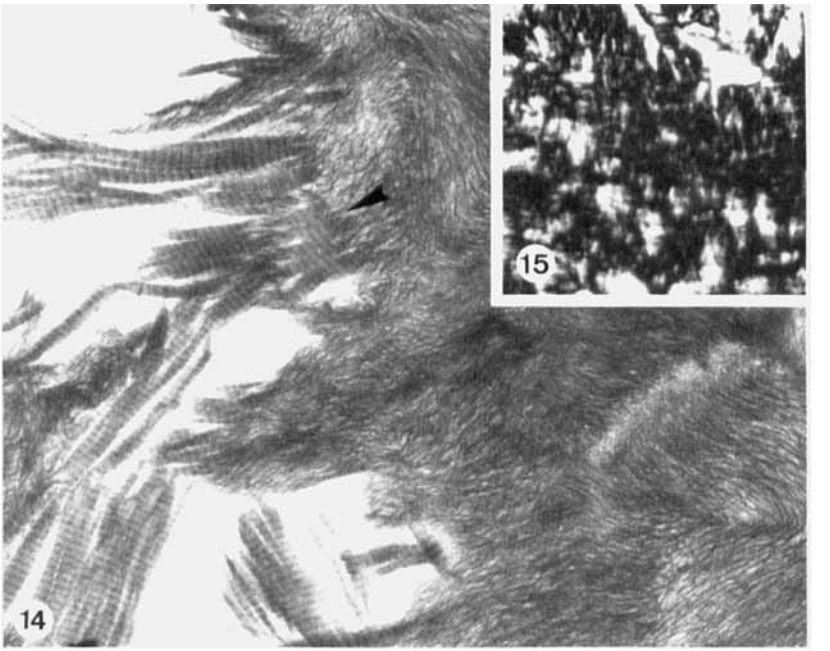
FIGURE 16. Stratified organization of fibrils at the periphery of a dehydrated drop of collagen solution at neutral pH. TEM X X 25.000

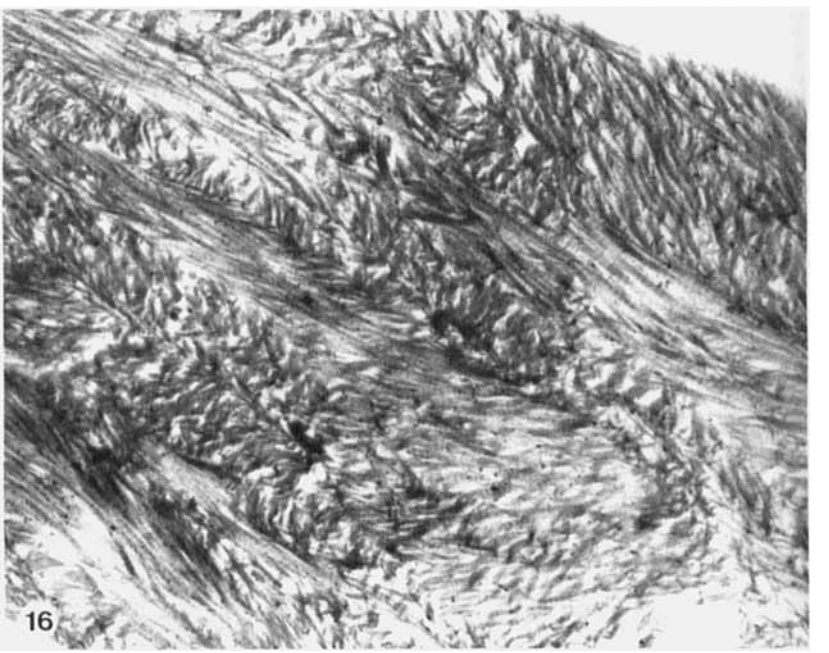
C: "Undulating" patterns

At the periphery of the dehydrated drop a third typical pattern corresponds to the most concentrated state of collagen molecules (Fig. 12). This texture recalls undulating patterns given by other helical biological polymers, in particular PBLG forming hexagonal phases in very concentrated solutions (16); similar defects are observed giving either branched patterns (black arrows) or flame patterns (white arrow). In this region, observed at high magnification in electron microscopy, microfibrils are not anymore resolved, but parallel domains appear of different electron opacity (Fig. 13).

Fibril order at neutral pH

Free drops of concentrated neutral solutions of collagen (10 mg/ml) deposited on glass slides are dehydrated at room temperature. Like in the acid solutions the molecules arrange concentrically around the center of the drop; however the textures observed in polarizing microscopy do not present the same degree of order as just described. Only small birefringent domains are observed (Fig. 15). Interesting observations were made in thin sections parallel to the surface of the drop. Towards the center of the drop (the less concentrated state) the microfibrils and cross-striated collagen fibrils coexist, with passages between these two state of aggregation of the polymer (Fig. 14, black arrow). Towards the periphery of the drop (The most concentrated





state) only fibrils are present, their compaction leads to a stratified organization (Fig. 16), very close to what is observed in compact bone.

CONCLUSION

This work emphasizes the presence of a cholesteric twist between collagen molecules at different stages of their organization (microfibril and fibril level). This liquid crystalline behaviour is related to a very concentrated state of the polymer which is present in biological structures and that we have obtained *in vitro*. More extended studies are needed to interpret the molecular orientations in the dehydrated drops of collagen. It is however possible to say that ordered distributions of collagen are obtained in the absence of cells; these self-assemblies lead to textures close to cholesteric and hexagonal pseudomorphoses which are also observed in many skeletal structures in biology.

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