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Collagen: The Organic Matrix of Bone [and Discussion]

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Collagen: the organic matrix of bone

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Collagen is the principal organic matrix in bone. The triple helical region of the molecule is 1014 amino acids long. In fibrils these molecules are staggered axially by integers of 234 residues or 68 nm (D). This axial shift occurs by self-assembly and can be understood in terms of a periodicity in the occurrence of apolar and polar residues in the amino acid sequence. Because the molecular length $L = 4.47D$, there are gaps 1.5×36.5 nm regularly arrayed throughout the fibrils. The three-dimensional molecular arrangement is a quasi-hexagonal lattice with three distinct values for the principal interplanar spacings. Analysis of the intensity distribution in the medium-angle X-ray diffraction patterns from tendons has produced the following picture of the molecular arrangement in fibrils (Fraser *et al.* 1983). The molecular helices have a coherent length of 32 nm and are tilted parallel to a specific place within the lattice. A regular azimuthal interaction exists between these helices. This crystalline region could be the overlap region with a non-crystalline gap region. However, the gap is still regular axially and the molecular helices retain their structure; their lateral packing is perturbed although they retain a 'gap'. Neutron and X-ray scattering experiments have shown that calcium hydroxyapatite crystals occur in the gap and are nucleated at a specific though unknown location within the gap. The c-axis of the apatite crystals is parallel to the fibril axis and its length $c = 0.688$ nm is close to the axial periodicity in a protein with an extended β-conformation. If the telopeptides at the end of a collagen molecule do have this conformation they would either have a highly heterogeneous conformation or exist in a folded manner because the overall length of the telopeptides is shorter than a regular collagen repeat of 0.029 nm would allow.

1. Introduction

Collagen is the most abundant protein in metazoan animals. This is largely due to its occurrence as the principal organic constituent of bones. Its mixed etymology from French and Greek means 'glue-producing' and is based on one of its historic uses, but this remains extraordinarily appropriate to the modern understanding of its function in animals. Collagen is the main component of the extracellular matrix, a biomaterial that guarantees the structural integrity of multicellular animals by forming a support or scaffold to which cells or organs may attach. This matrix is also the medium through which cell–cell and cell–substrate communication occurs. The cells and organs of animals are 'glued' together by collagen. Collagen is also the most thoroughly understood biological fibre; the structural molecular biology is known to amino acid resolution.

The known structure of collagen may be used as a basis for a biomaterials science of connective tissue. The strength and flexibility of tendons, the rigidity of bone, the resistance to compression of cartilage, the elasticity of blood vessel walls and ligaments, the transparency of cornea and vitreous humour, the reflectivity of the tapetum and the selective permeability of the kidney glomerulus are all devised by the alloying of collagen with other macromolecules,

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ions or mineral. The first phase of biomaterials science is well underway. It means studying how the structures of collagen and other components are spatially related in the variety of connective tissues. A second phase is only just starting; this involves trying to understand how the structures of these multicomposite materials determine the physical properties such as elastic moduli and refractive indices, which are crucial to the biological function of the tissues. The collagen in these tissues is termed *interstitial*. Another type of collagen occurs in the pericellular region and is involved in cell surface interactions. Here also, the known structure of collagen can be used to examine in molecular detail how it may interact with fibronectin and hence provide a mechanism for some of the events in morphogenesis.

In this paper, the research leading up to the contemporary picture of collagen structure will be summarized. The current state of our knowledge of the three-dimensional molecular arrangement in the fibrils will be described in more detail and related to possible mechanisms of biological mineralization by calcium hydroxyapatite.

In an adult human, approximately one half of the protein is collagen; more than one half of this is in the skeleton and most of the remainder in the skin (Forbes *et al.* 1953, 1956). So while collagen is not the only organic component of bone (Urist *et al.* 1983), it does predominate and its crucial role in bone function is underlined by the dramatic significance of collagen in bone disorder (Smith 1980). During the past decade, studies on the chemistry and biology of collagen have been deeply influenced by the discovery of the precursor molecule pro-collagen (Bellamy & Bornstein 1971), the unravelling of the subsequent sequence of steps that take place in collagen biosynthesis (Grant & Jackson 1976; Minor 1980) and the realization that the collagens in different tissues are genetically heterogeneous (E. Miller 1969; Bornstein & Sage 1980). The framework provided by the knowledge of the biosynthesis has allowed recognition of the biochemical basis of collagen diseases (Bornstein & Byers 1980; Francis & Smith 1983) and resulted in a new classification of these diseases.

At least ten different genes that code for collagen have been recognized. The collagen molecule is a triple helix. In vertebrates, type I collagen, which has two chains identical and one distant [$\alpha1(I)_2$, $\alpha2(I)$] occurs in tendon, skin and bone, type II in cartilage and type III in blood vessels and, as a minor component, in skin. These form the class we have termed interstitial collagens and comprise the extracellular connective tissues. In invertebrates these collagens are even more complex (Tanzer 1978; Adams 1978). A second class of collagens of enormous potential significance is located in the pericellular region and is involved in cell–cell or cell–substrate interactions. Type IV collagen, which occurs in the basement membranes of epithelial cell layers, may be in this class, and type V has been associated with the exoskeletons of fibroblasts, other mesenchymal cells and bone. This second class of collagen is less abundant that the first and much less well understood. It has been detected at the 64-cell stage and probably the 16-cell stage in a mouse embryo. The molecules of the interstitial collagens are highly homologous. Estimates based on comparison of the amino acid sequences suggest that the $\alpha1(I)$ and $\alpha2(I)$ chains diverged some 7×10^8 a ago, i.e. at the Precambrian era around the time of the separation of the vertebrates and invertebrates (Matthews 1980). The $\alpha1(I)$ chain has evolved slowly at about the same rate as cytochrome *c*, while the $\alpha2$ chain has changed more quickly at a rate comparable with that of the haemoglobins. Other collagen genes code for the very different invertebrate collagen structures in annelid cuticle (Tanzer 1978) and drosophila (see Tolstoshev & Solomon 1982) and Clq in the complement system (Reid & Porter 1976). The genetic type of collagen produced by cells is controlled, probably at transcription.

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It is now clear that control of expression of the different collagen genes is a central issue in normal development and differentiation (Hay 1982) in collagen pathology (Smith & Francis 1983) and in neoplasia. The very recent analysis of the intron–exon arrangement in collagen genes (Wozney *et al.* 1981) gives an even more secure foundation for further work on collagen in health and disease.

Bone contains type I collagen, which is therefore the most widespread of the genetic types, and here discussion will be confined entirely to the structure of type I and its relationship to the mineral calcium hydroxy-apatite. However, within the structural diversity that results from the different genetic types there is a substantial similarity, even identity. We may expect, therefore, in the next few years, a fruitful exploitation of the structural picture of collagen described here to the basic problems of evolution, development, differentiation and pathology.

2. The collagen molecule

(*a*) *Molecular conformation*

The size and shape of the collagen molecule was first indicated by the physical biochemical studies of Boedtker & Doty (1956); they suggested that the molecular mass is 3×10^5 Da, the length is 300 nm, and the diameter is 1.25 nm. The type I collagen molecule is a trimer consisting of two identical chains (α1(I)) and a third distinct chain terms α2(I) (Piez *et al.* 1961; Piez 1967). The α1(I) chains contain 1055 amino acids. All three chains contain a central region of 1014 amino acids in which glycine occurs as every third amino acid. The α1 chain has 16 residues at the $-NH_2$ end and 25 at the –COOH end, which lack this regularity in the glycine occurrence. The 1014 region therefore consists of 338 tripeptides of the type -Gly-X-Y-. The amino acid sequences of the entire α1 and α2 chains are known (see, for example, Hofmann & Kuhn 1981).

The conformation of the irregular telopeptides is not known with certainty, but the regular $-(\text{Gly-X-Y})_n-$ regions from two α1 chains and one α2 chain coil around each other to form a three-strand rope. Each α chain is itself a left-handed helix with pitch of about 0.9 nm and they form a right-handed supercoil about a common axis with a supercoil pitch of around 8.7 nm to produce the three-strand rope or triple helix.

The triple-helix structure was first proposed by Ramachandran & Kartha (1955) and independently by Rich and Crick (1955) and Cowan *et al.* (1955). These models shared the significant new feature that the three chains were supercoiled about a common axis, but differed in the atomic arrangement within the asymmetric unit, the precise values of supercoil pitch and the number of interchain (i.e. intramolecular) hydrogen bonds per tripeptide. Recent analysis of the medium and high-angle X-ray diffraction patterns from native rat tail tendon (Fraser *et al.* 1983) yield values of h (the axial separation between tripeptides), τ (the azimuthal angle relating tripeptides) and P (the pitch of the supercoil) as 0.289 nm, 8.68 nm or 30 amino acid residues and 108°, respectively. The length of the triple helical part of the molecule is therefore $338 \times 2.89 \times 3 = 293$ nm. The positions of the non-hydrogen atoms in the polypeptide main chain were determined by linked-atom refinement methods (Fraser *et al.* 1979) and found to be close to those of the structure proposed by Rich & Crick (1961) with one interchain hydrogen bond for tripeptide. The conformations of the amino acid side chains are not yet determined.

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(b) *Amino acid sequence*

The amino acid sequences have been determined for the α1(I), α2(I), α(III) and part of the α(II) chains (see Hofmann & Kuhn 1981). These have now been analysed in some detail and they contain interesting features. All the chains have a central triple helical region of some 1014 residues in which glycine occurs as every third residue. At the molecular ends there are short telopeptides, 9 to 25 amino acids in length, which do not show this periodicity and hence are of unknown conformation.

The different α chains are homologous, though to different degrees. The α1(I) chain, for example, is more similar to the α(II) chain than to the α2(I) chain. In the α1(I) chain the occupancies of the X and Y residues of the Gly-X-Y- triplets are uneven. The prolines may be regarded as equally distributed between X and Y but one half of the prolines in Y have been subject to post-translational modification to 4-hydroxyproline. The single hydroxylated proline in position X is 3-hydroxyproline. The apolar residues greatly favour the X position especially Leu and Phe, as does the acidic residue glutamic acid. The basic residues arginine and lysine favour the Y position, while the acidic residue aspartic acid shows an even distribution between X and Y.

The triple helical region also contains long-range periodicities, but since these can be related to the molecular interactions necessary for assembly into fibrils, discussion of these and the collagen gene structure will be reserved until a later section (§§3(iii), (iv)).

The telopeptides contain important lysine residues, one of which is hydroxylated. These form covalent crosslinks with other lysine or hydroxylysine residues in the triple helical region of the molecule. The enzyme lysyl oxidase, which works best on molecules assembled into fibrils (Seigal 1979), converts the lysine residues to aldehydic alysyl groups and crosslinks are formed either between hydroxylysine and an aldehyde group or between two aldehyde groups. The crosslinking is essential for a fibril of sufficient stability. It has been suggested that silicon is involved in this crosslinking (Schwartz 1972, 1973; Carlisle 1974, 1980) but X-ray fluorescence analysis (K. Bowen & A. Miller, unpublished work) makes this most unlikely, at least in rat tail tendon where the silicon level is of the order of $3/10^6$.

3. The fibril structure

(a) *One-dimensional structure*

(i) *Electron microscopy*

The characteristic collagen structure seen in electron micrographs of animal connective tissue are not molecules but fibrils. As we saw in the preceding section, the collagen molecule is about 1.25 nm in diameter and 300 nm long. Collagen fibrils are very long, stretching continuously over several micrometres, possibly several centimetres. They have a tissue dependent diameter varying from 10 nm (in the vitreous humour of the eye) to 500 nm (in mature rat tail tendon) (Parry & Craig 1982). However, a common feature of all fibrils of interstitial collagen seen in electron micrographs of longitudinal views of fibrils stained with heavy metals is a periodically repeating set of bands running across the fibrils perpendicular to the fibril axis. The length of the periodic unit in electron micrographs is 64 nm (Hall *et al.* 1942; Schmitt *et al.* 1942; Wolpers 1943) though it may also be estimated by small-angle X-ray diffraction, which was first reported by Bear (1942) for dry tendon. In conditions close to the native state,

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X-ray diffraction yields a value for this periodicity ($D = 67.8$ nm) (Fraser *et al.* 1983) depending on the degree of stretch.

Molecules 300 nm long can generate 68 nm periodic fibrils if they are staggered axially by 68 nm with respect to each other (Schmitt *et al.* 1954) (see figure 1). Originally it was thought that the molecular length (L) was four times the stagger distance (D) and this was termed the 'quarter-stagger model' in which the molecular ends were in contact. Fortunately collagen molecules can be induced to precipitate in an alternative form termed segment long spacing

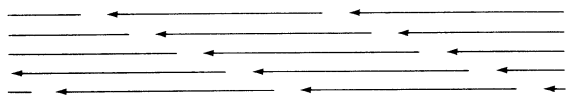

FIGURE 1. The one-dimensional Hodge–Petruska scheme. Collagen molecules, represented here by lines with an arrow at one end, are shifted parallel to the fibril axis by 68 nm with respect to each other. Since the molecules length (L) is 4.47 times the shift ($D = 68$ nm), gaps of $0.53D$ between the molecular ends are spaced regularly along the fibril axis.

(s.l.s.) in which the molecules are parallel and in register with each other. These s.l.s. segments indicate the length of the molecule and by comparing s.l.s. and D-periodic fibrils in the same electron micrograph, Hodge & Petruska (1962) showed that $L = 4.5D$. The importance of the non-integral relation between L and D is that the fibril now contains gaps (of $0.5D$) between the molecular ends, a feature that Hodge & Petuska (1962) instantly recognized as significant for mineralization (Hodge 1967) (see §4).

Electron micrographs of positively stained s.l.s. show a series of fine stained bands traversing the s.l.s. segment perpendicular to the molecular axis. Some 58 bands have been recognized and named as they comprise an asymmetric set (Bruns & Gross 1973). More significantly, as we shall see in the next subsection (3 (*a*) (ii)), these bands represent the location of the amino acids with ionizable side-chains, which take up the heavy metal stain; the unstained regions correspond to the locations of apolar amino acid residues.

An electron micrograph of a positively stained D-periodic collagen fibril also shows a set of fine, perpendicular bands. There are about 12 such bands for D period and they form an asymmetric set within a D-unit. The D-staggered arrangement of molecules in the fibril was confirmed (Hodge & Schmitt 1960) by showing that the peculiar distribution of intensity in the D-periodic fibrils could be synthesized by photographically superimposing several s.l.s. images staggered successively by integers of D.

Electron micrographs of negatively stained fibrils also show D-periodic fibrils, but for those each D period is subdivided into only two broad bands, each approximately $0.5D$ wide, one darkly stained, the other lightly stained. This is readily explained in terms of heavy metal stain collecting preferentially in the 'gap' region between the molecular ends.

(ii) *Electron microscopy and amino acid sequence*

When the amino acid sequence of the α1 chain of the collagen molecule became known, it was clear that the ideas presented in subsection 3 (*a*) (i) could be tested (Doyle *et al.* 1974; Chapman 1974). The collagen molecule was approximated as a linear array of amino acid residues spaced 0.29 nm apart. Acidic (Glu and Asp) and basic (Lys and Arg) residues were represented by black lines to simulate heavy metal stain, while the other residues were left blank.

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The resulting distribution of simulated stain could be compared with the actual distribution of stain in an electron micrograph of positively stained s.l.s. The fit was good. Furthermore the simulated stain patterns of s.l.s. could be used to produce a simulated stain pattern within the *D* unit of a fibril by photographic or computer superposition of staggered s.l.s. patterns. Again the fit was good. This bringing together of electron optical and amino acid sequence studies was first accomplished with collagen. Not only did it clarify the actual effects of heavy metal staining but it meant that instead of giving the bands in a *D* unit arbitrary names, they could be understood in terms of the amino acid residues occurring at that point. This basis proved valuable. Conclusions could be drawn about the chemistry of other collagens available in quantities sufficient for electron microscopy but not for detailed biochemistry.

It was shown that all the bands in a *D* unit are electrically neutral except one. The potential exists (Hulmes & Miller 1975) to compare archaeological specimens and to study the effects of tanning methods on leather artefacts. Other fibrous proteins such as myosin, tropomyosin, fibrinogen and α-keratin will no doubt be similarly studied.

(iii) *Origin of fibril structure in amino acid sequence of molecule*

Collagen molecules can reprecipitate from solution to form fibrils, electron micrographs of which have the same *D*-periodic characteristics as fibrils cast directly on to an electron microscope grid. Collagen molecules self-assemble to form fibrils (Schmitt *et al.* 1942). The amino acid sequence of the α1(I) chain of collagen was examined for the chemical basis of this molecular specificity (Hulmes *et al.* 1973). The amino acid sequence was again approximated as a linear, equispaced array and represented in a computer. Two such identical arrays representing two interacting collagen molecules were then moved past each other in steps of one amino acid, and, at each axial displacement (δ), the interaction (I) between the two arrays was calculated. A very simple scoring scheme was used. If two apolar residues were within ± 2 residues of each other, a score of $+1$ was added to I and if two polar residues were within ± 3 residues of oppositely ionizable residues, again a score of $+1$ was added to I. A plot was then made of I against δ. This plot showed that within the amino acid sequence there exists a quasi-periodicity of 234 amino acid residues. The average axial separation between amino acids is determined as 0.29 nm so the quasi-periodicity is $234 \times 0.29 = 67.8$ nm or D. The D periodicity in the amino acid sequence emerges if no distinction is made between the large apolar amino acid residues, which are taken as one group, nor between the acidic and basic residues, which compose a second group. It is therefore termed a quasi-periodicity because it is masked in the real sequence. The consequence of the quasi-periodicity is that when two molecules are staggered by integers of D (234 residues), there is an optimal interaction between them. As we shall see in §3, in the fibril, collagen molecules are staggered by D, $2D$, $3D$, and $4D$ (though not in equal numbers) from their near neighbours. Hence the origin of the molecular specificity (at least in the axial direction) that leads to self assembly of fibrils lies in the amino acid sequence of the collagen molecule.

This sequence specificity was first established for collagen (Hulmes *et al.* 1973) and analogous studies have since been made on tropomysin (Miller 1976; Parry 1975; McLachlan & Steinart 1981), fibrinogen (Dolittle *et al.* 1978), α-keratin (Parry *et al.* 1977; McLachlan 1978; Steinart *et al.* 1983) and myosin (Parry 1981).

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(iv) *Further analysis of amino acid sequence*

In addition to the quasi-D period, Hulmes *et al.* (1973) noted several other interesting features in the sequence. The acidic and basic residues mainly occurred in oppositely ionizable pairs. Furthermore they, and the apolar groups, occurred in clusters so that each D-unit has a subperiod. The apolar residues were markedly grouped around the curious spacing of $\frac{2}{11}D$. Polar groups followed $\frac{1}{6}D$ and proline $\frac{1}{5}D$. However, Doyle *et al.* (1974*b*) did not find convincing evidence that the D units within the α1 chain had arisen by gene duplication.

The sequence was further analysed by McLachlan (1977). He concurred with the findings of the quasi-D period and subperiods by Hulmes *et al.* (1973), but added two important new discoveries. First, the quasi-D period was strongly followed by the X residues in the sequence -(Gly-X-Y)- but scarcely at all by the Y residues. Second, his analysis found marginally in favour of the D period arising by gene duplication.

An even more detailed analysis has been provided by Kuhn and his colleagues (Hofmann & Kuhn 1980). They examined the sequences of the α1(I), α1(III) and α2(I) chains and drew three main conclusions.

(i) All three types of chain showed quasi-D periods of 234 residues in their sequence, though this was more blurred in the α2 chain than in the other two.

(ii) All three chains were highly homologous, especially in the interactive amino acids (i.e. polar charged residues and those of the apolar residues responsible for self-assembly of the molecules). The homology of the residues in the Y position was greater than that of those in the X position and, unlike most globular proteins, the variability of the apolar residues was greater than that of the polar residues. Positive–negative replacements are very rare in collagen compared with globular proteins.

(iii) The D periods within chains of the same type are also homologous (yielding the quasi-D period), but this intra-chain homology is several orders of magnitude weaker than the inter-chain homology mentioned in (ii). Furthermore it is confined almost exclusively to the X residues (McLachlan 1976) and the apolar residues show less variability than the polar residues (in contrast to the intermolecular homology).

(iv) Other intramolecular periodicities were found, for example $\frac{1}{3}D$, $\frac{1}{6}D$, $\frac{1}{5}D$, $\frac{1}{11}D$ (as Hulmes *et al.* 1973; Hulmes *et al.* 1977*a*) and $\frac{1}{13}D$. The $\frac{1}{13}D$ subperiod was most strongly developed in the α2 chain.

The significance of the $\frac{1}{13}D$ periodicity has been underlined by studies on the collagen gene (see reviews by Solomon & Cheah (1981) and Tolstosher & Solomon (1982)). Most information is available about the gene coding for the α2(I) chain, but a similar pattern seems to be emerging for the α1(I) chain. First the collagen gene is broken up into many exons. Five kilobases (kb) of coding sequence (exons) are embedded in about 38 kb of DNA, which makes the ratio of introns to exons one of the highest observed so far. There are some 49 introns of varying length. Secondly, the exons coding for the triple helical region of the molecule are all made up of integers of 9 bases (i.e. corresponding to a Gly-X-Y triplet). Many of the exons are 54 base pairs (b.p.) long (i.e. 18 residues or $\frac{1}{3}D$) or are closely related to 54 b.p. Finally, single exons code for sections of the collagen molecule that start in the triple helical region and continue into the telopeptides.

This is difficult to interpret at present. Did the original collagen gene consist of 9 base pairs, 54 b.p. or 3×234 b.p. corresponding to a D period? Why is the genetic unit of 54 b.p. only

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weakly represented in the amino acid sequence ($\frac{1}{13}D$) compared with some other well developed periodicities such as $\frac{2}{11}D$ and $\frac{1}{6}D$. At present we conclude that, apart from the 9 b.p. quantum, the gene structure is not clearly related to structural features in the protein. Whether this is due to evolutionary change or to our lack of information will no doubt emerge as the gene structure is determined in more detail.

(v) *The axial structure from the X-ray meridional pattern*

By the axial structure I mean the structure of the fibril projected on to the fibril axis. If the one-dimensional array of amino acids along a molecule is known and the axial shift between molecules is known, then the axial structure of the fibrils is fully determined. As we saw in

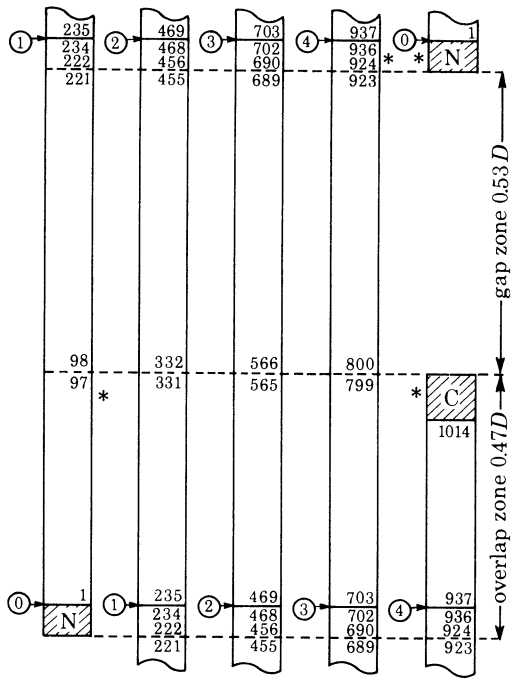

FIGURE 2. A more detailed diagram of the one-dimensional molecular arrangement. The first amino acid residue in the triple helical region of the molecule nearest to the amino terminal end is numbered 1. This makes the residue in the triple helical region nearest to the carboxy-terminal end number 1014. The telopeptides (15 and 21 residues at the amino and carboxy ends respectively), which are compressed axially with respect to the triple helical region, have been adjusted to their experimentally determined lengths in this figure. The positions of residues in known crosslinks are marked with an asterisk. (From Fraser *et al.* 1983.)

§3 (*a*) (ii), the positively stained banding pattern in electron micrographs of fibrils in longitudinal view, can be predicted. X-ray diffraction has the advantage that it may be done on tendons in the native state. The high-angle X-ray pattern indicates that the axial shift between amino acid residues along the molecule is 0.29 nm. (Actually this is an average value; more precisely, the axial shift between Gly-X-Y triplets in one α-chain is 3×0.29 nm and the axial shift between triplets in different chains is 0.29 nm). The small-angle X-ray diffraction pattern has a set of meridional (or very nearly meridional) reflections, which index as orders of 67.9 nm (Miller & Parry (1973); the precise value of D depends on the conditions of stretch; see Fraser *et al.* (1983)). The intensities of the meridional reflections are determined by the electron density profile obtained when the fibril structure is projected on to the fibril axis. Hence given the above

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values of 0.29 and 68 nm together with the known amino acid sequence, the one-dimensional axial electron density profile may be calculated and used to predict the intensities of the meridional X-ray reflections. This was done and the predicted intensities compared with the observed intensities of the first 41 orders of the 68 nm period (Hulmes *et al.* 1977*b*).

The fit between observed and calculated intensities was good for orders 1–6, which are dominated by the ratio of the gap and overlap lengths. It was also good above order 18 where it is determined by the clumping of the polar and apolar residues into groups within each D (68 nm) period. In the intermediate range a worse fit was obtained and this was due to the unknown configuration of the telopeptides. The X-ray diffraction data was supplemented by neutron diffraction patterns and the conformations of the telopeptides that best accounted for the observed diffraction intensities were determined. Both telopeptides had a shorter inter-residue axial distance than the triple helical region. The N-terminal telopeptide and C-terminal telopeptides gave average inter-residue axial shifts of 0.25 nm and 0.20 nm respectively (Hulmes *et al.* 1980). A confirmation of this result was provided by applying the isomorphous replacement method to the meridional X-ray reflections (Stinson *et al.* 1979).

In summary, we now understand the one-dimensional axial structure of native collagen to amino acid resolution. This enables us to draw a more detailed Hodge–Petruska structure (figure 2) and insert the positions of known covalent crosslinks, of disaccharides covalently attached to collagen and of the site of attack of animal collagenase (see Gross 1981).

(*b*) *Three-dimensional model of fibril*

(i) *Evolution of the quasi-hexagonal model*

Once the well developed one-dimensional structure of the fibrils is understood, it is natural to proceed to enquire about the three-dimensional structure. It is immediately apparent that the side-by-side or lateral arrangement of the collagen molecules is not nearly so orderly as the arrangement in the direction parallel to the molecular axis. This is common for fibrous proteins. No obvious regularity is seen in electron micrographs of thin transverse sections through fibrils, in fact it was suggested that the lateral interactions in the fibrils was random (Grant *et al.* 1965), the only order being axial shifts between neighbouring molecules of nD where n is randomly 0, 1, 2, 3 or 4. However, X-ray diffraction studies (North *et al.* 1954; Miller & Wray 1971) established that for one collagenous tissue at least, rat tail tendon, the molecules are arranged in a three-dimensionally crystalline manner. The evidence for this crystallinity is sharp Bragg reflections in the equatorial region and on the collagen helix turn layer line (at $Z = (0.95\ \mathrm{nm})^{-1}$) of the X-ray fibre pattern. More recent work shows that this orderly structure occurs in many different tissues (Wray 1971; Jesior *et al.* 1980).

Several points must be made about this crystallinity. It is very precarious. The non-meridional Bragg reflections are only obtained when the tendon is in a solution of ionic strength similar to that *in vivo*. Too dry or too wet tendons show no Bragg reflections and it requires care to produce the conditions that yield these reflections. In other conditions collagen exists with liquid-like packing in the lateral direction (Woodhead-Galloway & Machin 1976). This is undoubtedly important in complex collagens such as elastoidin, but the crystalline phase gives the crucial information on specific molecular interactions. Secondly, even when the Bragg reflections occur on the equator or the helix turn layer line at $Z = (0.95\ \mathrm{nm})^{-1}$ they always occur on a background of more slowly varying diffuse scatter (Miller & Wray 1971). This indicates that there is, superimposed on the undoubted crystallinity, a measure of disorder, the

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nature of which is still not understood. We shall now discuss the lateral order in the fibrils, which, together with the one-dimensional order described in the previous section, results in three-dimensional crystallinity.

Collagen molecules may be regarded as composed of five segments four of which are 68 nm long and the fifth 35 nm. If we consider a transverse section of thickness 68 nm through a fibril, the problem of the lateral packing may be expressed as 'How are the five molecular segments distributed in a 68 nm thick section?' (see discussion of the problem in Miller (1976)). The first problem to settle is the crystallographic unit cell and this requires indexing the Bragg reflections. There is now general agreement that the quasi-hexagonal unit cell (Hulmes & Miller 1979) is the correct solution. The next problem is the positions and inclination of the molecular segments within the unit cell. Again, the X-ray data point to a generally agreed solution; the molecules are tilted by about 5° in a specific plane within the lattice (Hulmes & Miller 1979; Miller & Tochetti 1981; Fraser & MacRae 1981) and the molecular segment position close to, though probably shifted slightly from, the ideal quasi-hexagonal positions (Piez & Trus 1981; Fraser *et al.* 1983). It is not yet clear whether the X-ray data can distinguish between the different 68 nm segments (Fraser *et al.* 1983). It is worth tracing the history of the interpretation of the X-ray pattern because apart from information on chemical crosslinking between molecules, this has been the sole source of information on molecular packing.

The one-dimensional structure of Hodge & Petruska (1963) has equal numbers of the five different molecular segments in each transverse section of thickness 68 nm within the fibrils. If an orderly structure does exist, this is suggestive of a unit cell containing one molecule (i.e. five segments). Long cylindrical structures would be expected to pack hexagonally and it was generally assumed that collagen would do so (Ramachandran 1967; Kuhn 1969; Hodge 1970). Early discussions of molecular packing in collagen revolved around how the Hodge–Petruska one-dimensional scheme could be accommodated in hexagonal packing (Hodge 1970; Ramachandran 1967; Smith 1965; Kuhn 1969). These ideas were interrupted by the elegant model of Smith (1968). He pointed out that the irreducible unit of the Hodge–Petruska model contains five molecular diameters; one from each of the different molecular segments could occur in a transverse section and the neatest arrangement of these would be around the circumference of a cylinder to form a microfibril of five molecular diameters (except in the gap region when there are four) in which the molecules are related by helical symmetry. The molecular diameter of collagen was 1.25 nm and the outer diameter of a Smith microfibril would be 3.8 nm. When John Wray and I (Miller & Wray 1971) obtained improved X-ray patterns from native rat tail tendons, we found, contrary to earlier workers, that the longest equatorial spacing was 3.8 nm and, as a result of studies on tendon stained with heavy metals, that this 3.8 nm was an important lateral vector in the fibril structure. We took this as support of the Smith microfibril (Miller & Wray 1971) and it was shown that many of the equatorial X-ray reflection could be indexed on a square lattice of such microfibrils (Miller & Parry 1973).

Subsequent models were based either on the Smith microfibril or on the square lattice (Fraser *et al.* 1974; Veis & Yuan 1975; Woodhead-Galloway *et al.* 1975); other models relied on a near-square lattice of layers (Nemetschek & Hosemann 1973) and then of so-called octafibrils (Hosemann *et al.* 1974), which were essentially of dimers of the Smith microfibril. The disadvantage of all of these models was that they would not account for the observed density of tendons (Katz & Li 1973; Miller 1976) and they did not explain the general intensity distribution along the equator of the X-ray pattern; only the positions of the Bragg reflections

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along the equator could be explained. Katz & Li (1973, 1974), on the basis of estimates of the intermolecular space in fibrils, emphasized that the density measurements supported hexagonally packed molecules.

In 1971, Macfarlane (1971) proposed a model for the lateral packing of collagen. This was based on an initial threefold symmetric aggregation of molecules, which built up a so-called trill fibril with a threefold axis relating domains in which the collagen molecules were packed hexagonally. In these domains Macfarlane showed a unit cell that would result if Hodge–Petruska packing occurred within two-dimensional sheets and these sheets were then packed parallel to each other with a linear displacement. This gave a monoclinic unit cell with $a = 1.732$ nm, $b = 2.646$ nm and $\gamma = 109°$. Macfarlane said that trill symmetry would explain the off-equatorial splitting (i.e. the Z positions) of the near-equatorial reflections and promised a detailed account of how this model would explain the X-ray data of Miller & Wray (1971). No further publication emerged. It was shown (Miller 1976*a*) that the model gave a poorer fit even with the R-positions of the X-ray reflections than the tetragonal arrangement of Fraser *et al.* (1974) and pointed out (Miller 1976*a*, *b*) that a specific modification of hexagonal packing might solve the problem of fit with the X-ray pattern.

Macfarlane (1971) had obtained one possible class (class I) of hexagonally packing two-dimensional Hodge–Petruska sheets of collagen molecules; Katz & Li (1973) discovered the same solution independently. However, I pointed out (Miller 1976*a*) that there was another class of solutions (class 2) represented by a unit cell suggested by Kuhn (1969), but that the X-ray data were incompatible with this latter cell. In preparing this article, I found that Hodge (1970) had anticipated both of these classes of solution to the packing of two-dimensional sheets and that he illustrates both the general unit cells of class 1 and class 2. Hodge (1970) favoured the class 2 solution but he was the first to discover the class 1, which was later found by Macfarlane (1971) and Katz & Li (1973). Hodge (1970) also made the perceptive comment that in the X-ray diffraction pattern of North *et al.* (1953), the reflection corresponding to the three principal planes of the hexagonal lattice, was split into two reflections (parallel to R) and hence two distinct spacings of the principal planes must exist.

In the improved X-ray patterns Miller & Parry (1973) established that the principal plane reflection was in fact split (in the R direction) into three components. Hulmes & Miller (1979) constructed a quasi-hexagonal unit cell of the type class 1 in which the principal planes had different spacings determined by the observed threefold splitting of the reflection. This immediately produced a unit cell that gave a fit with the R positions of the X-ray equatorial reflections superior to the fit from any other model and, by incorporating straight collagen molecules tilted in a defined plane within the lattice, fitted the (R, Z) positions and the intensities of the near-equatorial reflection (Miller & Tochetti 1981).

Fraser & Macrae (1981) discovered that treating a tendon with phosphotungstic acid produced an X-ray diffraction pattern with improved definition of the row lines. Measurements of the intensity distribution in this diffraction pattern (Fraser *et al.* 1983) have led to the conclusions described below. The original paper (Fraser *et al.* 1983) provides a detailed discussion of the basis of these conclusions (see figure 3).

1. The quasi-hexagonal unit cell of Hulmes & Miller (1979) is correct. It is now possible to define the complete three dimensional unit cell as triclinic with $a = 3.997$ nm, $b = 2.695$ nm, $c = 6.779$ nm, $\alpha = 89.24°$, $\beta = 94.59°$, $\gamma = 105.58°$.

This has a unit cell volume of 7.012×10^2 nm^3 and contains one collagen molecule.

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2. The molecules are inclined parallel to a specific plane in the crystal lattice (approximately the $(\bar{3}, 1, 0)$ planes) and by about 4° to the fibril axis (Hulmes & Miller 1979). However, the precision with which this tilt direction and the unit cell parameters are determined shows them to be geometrically incompatible with the molecules following a completely straight path through the lattice. Furthermore, analysis of the intensity distribution parallel to Z amongst

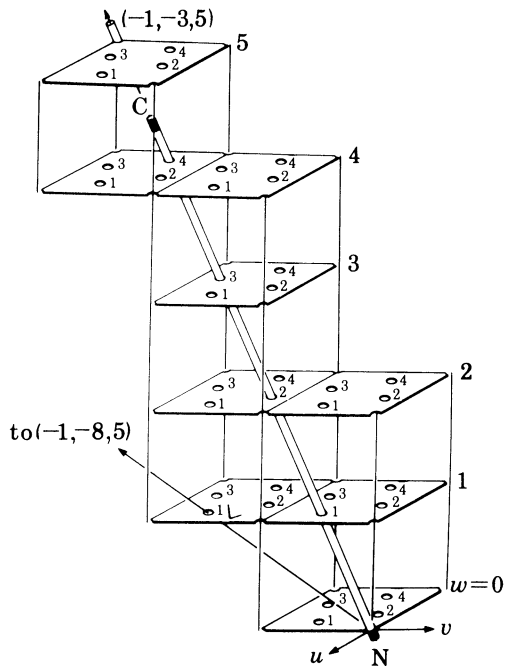

FIGURE 3. Diagram to illustrate the three-dimensional molecular packing of collagen in rat tail tendon as determined by X-ray diffraction. The numbers 0, 1, 2, 3 label the four 68 nm long segments of the collagen molecule and 4 labels the fifth segment size 0.47 nm × 68 nm. The unit cell of dimensions described in the text is necessarily shown distorted. A full molecule is illustrated, which is tilted in the unit cell at such an angle that a completely rectilineal molecule satisfies the lattice symmetry. The X-ray data indicate that in native tendon the molecule is actually inclined at more like the angle illustrated by the single line with an arrow labelled $(-1, -8, 5)$, which are the (u, v, w) coordinates of the intersection of this line with the plane $w = 5$. As explained in the text, the coherent length of the triple helix is only $0.5D$ and the molecule does not follow a rectilineal path through the lattice but is kinked with a D period. From Fraser *et al.* (1983.)

the closely spaced reflections, indicates that the triple helices have a coherent length of $0.46D$. Hence the collagen molecules must be crimped.

3. The intensity distribution in the near-equatorial region indicates that the molecules are packed in a quasi-hexagonal manner (Miller & Tocchetti 1981). However, it is likely that there are slight displacements from the 'ideal' quasi-hexagonal positions, a possibility also suggested on different grounds by Trus & Piez (1980).

4. The intensity distribution along the helix turn layer line has, for the first time, provided information about the relative azimuthal orientation of the triple helices. Several reflections on this layer line are much more intense than the others. These strong reflections correspond to a selection rule that would be imposed if neighbouring helices in each of the principal planes were related by $\frac{2}{5}\pi n$, where n is an integer. The fact that the other reflections do appear, albeit weakly, probably indicates that there are slight deviations from this regularity.

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Note that this analysis of the X-ray pattern leaves undecided which of the five segments of the molecule occupy which of the quasi-hexagonal sites in the unit cell. Several possibilities have been illustrated (Hulmes & Miller 1979; Piez & Trus 1980, 1981; Miller & Tocchetti 1981) and the wide range of other possibilities has been discussed (Fraser *et al.* 1983).

The nature of the molecular crimp is interesting. The coherent length of the crystalline parts is 0.46*D* or 31 nm. Fraser *et al.* (1983) speculate that this crystalline region is the overlap region and that in the gap the molecules, while still triple helical, are less ordered from unit cell to unit cell. The idea of 'bonding zones' of parallel molecules in the overlap and less ordered zones in the gap had been suggested and illustrated by Grant *et al.* (1967) and a similar arrangement proposed on different grounds by Piez & Trus (1981). Hofmann & Kuhn (1980) have suggested that the amino acid residues responsible for intermolecular interactions occur more in the gap than the overlap and the limited coherent length of 0.46*D* would be compatible with an ordered gap and disordered overlap. However, the profile of the equatorial intensity is such that the intermolecular distance in the overlap would be preserved in the gap (Miller & Tocchetti 1981). The molecular crimp indicated by the work of Fraser *et al.* (1983) is *D* periodic and incompatible with the 13.5 nm kinks proposed by Nemestchek & Hosemann (1973).

The three-dimensional structure of collagen is therefore now on the correct lines. However, there is still much to learn, in particular about the arrangement of the different *D* segments within the unit cell, the absolute (as distinct from the relative) azimuthal relation of the molecules, the precise axial registration, the order of the α1 and α2 chains in the molecule and the conformation of the amino acid side chains. Some considerations arise from the present state of our knowledge of the structure.

An important general point is that crystallinity implies a specific interaction between the molecules. A non-specific interaction with axial shifts of random integers of *D* would not generate the observed unit cell. Hence the molecules aggregate in a defined manner during fibrilogenesis. The molecules are covalently crosslinked, yet the geometric regularity of the crystal is precarious and is readily lost by wetting or drying the fibre from the native stage. Such specimens give X-ray diffraction patterns with only the nearest neighbour broad intensity maximum on the equator and no sharp Bragg reflections. Hence the failure to observe Bragg reflections should not be taken as proof of a structure with non-specific lateral interactions. The specific topology of crosslinked molecules, which is of primary importance, can exist in the absence of perfect geometrical crystallinity. The unit cell due to the specific interactions can sometimes be enhanced by heavy metal staining. In this way it was established that turkey leg tendon, which can calcify, has a specific topology (Jesior *et al.* 1980).

The diffuse scatter that appears on the X-ray patterns between the Bragg reflections implies some sort of disorder in the tendons. Various possibilities exist. Perhaps some fibrils are completely crystalline and others completely disordered depending on their diameter or other factors. In view of the arguments presented in §3(*b*) it may be more likely that the molecules in the gap are disordered compared with those in the overlap region. The gap molecules would not contribute then, to the Bragg reflections. As we discussed earlier, there could be variation of this idea with the order in the gap and disorder in the overlap. We do know that the diffuse scatter greatly predominates on the helix layer lines, so the disorder involves collagen molecules.

In the last few years (for a review, see Torchia *et al.* (1983)) nuclear magnetic resonance studies on reconstituted, intact and mineralized fibrils have led to some interesting conclusions. In summary these are first, that the individual collagen molecules are probably performing

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azimuthal oscillations about their axes over an angular range of $\pm 25°$; second, there is evidence that the amino acid side-chains are themselves much more mobile than the side-chain seen in crystals of amino acids (even in highly organized fibrils this implies that the molecules are linked by a fluid domain of residue side-chains); third, in mineralized tendons, this side-chain mobility is absent; the side-chains appear to be locked in position.

What would the effect of these types of mobility be on the X-ray diffraction patterns? The precarious nature of the Bragg reflections must again be noted. The order of the best X-ray patterns is not necessarily comparable with the order in the fibrils examined by n.m.r. It would be of interest to know the n.m.r. spectra from fibrils that are shown by X-ray diffraction to be crystalline.

The azimuthal oscillations of the molecules would cause diffuse scatter along the helix turn layer line but not necessarily along the equator; since diffuse scattering is observed in both of the regions of the X-ray pattern, there is more to the disorder in collagen than the mobility shown up by the n.m.r. The X-ray diffraction patterns from fibrous proteins such as α-keratin, silk and collagen are usually well accounted for on the helix layer lines by the polypeptide backbone alone. The side-chains contribute obviously to the axial structure as we showed in §3 (*a*) (v) and the resolution obtained there would not detect the effects of the mobility picked up by the more sensitive n.m.r.

The X-ray diffraction data may be supplemented by information about the covalent crosslinks between molecules (Bailey *et al.* 1980; Miller & Tocchetti 1981) and hence help to discriminate within the class of structures consistent with the X-ray pattern. We can start by assuming, though this is by no means certain, that crosslinked molecules are nearest neighbours in the fibril. The known solutions are between amino acid residues 9 and 946 and 103 and 1047. So molecules staggered axially by 4*D* are covalently linked; there is no certain knowledge of other crosslinks from tendon collagen. Since these crosslinks would at best crosslink molecules into 4*D* periodic sheets, it seems likely that additional crosslinks between *D* staggered molecules should also exist to stabilize the complete fibril; but this is supposition at present. An important relevant suggestion of Light & Bailey (1980*a*, *b*) is that there are crosslinked polymers of fragments of the molecule from segments 1 and 5. This would perhaps point to an alternating arrangement of segments 1 and 5 at the same level in the fibril and hence favour the arrangement shown in figure 3 (Miller & Tocchetti 1981). It would not be consistent with the random arrangement of compressed microfibrils suggested by Piez & Trus (1981), but this model could be readily modified by substituting the random orientation of compressed microfibrils by a regular arrangement (see Fraser *et al.* 1983).

At the beginning of §3, it was stated that there was no information about the lateral packing of molecules in electron micrographs of thin transverse cross sections of collagen. This was a slight overstatement since claims have been made that either in longitudinal or transverse section, some evidence of molecular packing may be gleaned. Problems with the images in these electron micrographs are their relation to spacings in the native structure and the difficulty of estimating 'edges' with precision in electron micrographs. This has been partially overcome by two pieces of work in which X-ray microscopy was used to monitor specimens prepared for electron microscopy. Hulmes *et al.* (1981) tested tendons prepared for electron microscopy by inspecting the X-ray diffraction patterns from treated tendons for the appearance of the near-equatorial Bragg reflections that correspond to the three-dimensional lattice. It may be recalled that the regularity of this lattice is precarious and is lost even by wetting the tendon

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from the native state. A series of preparative steps involving fixing, staining, dehydrating and embedding a tendon was devised such that the tendon still showed the important 3.8 nm row line in the X-ray diffraction pattern. A tendon thus prepared was then transversely sectioned and viewed in the electron microscope. The fibrils showed arrays of parallel lines estimated by optical diffraction to be spaced apart by around 4.0 nm, and which are thus probably a visualization of one set of lattice planes, the (1, 0, 0), in the crystalline array.

It is also possible by measuring the very low-angle part of the equator of the X-ray patterns, to get information about the diameter and packing of the fibril in the native state. These low-angle maxima were first observed by neutron scattering (see Miller 1976) but have recently been used to good effect on X-ray patterns by Eikenberry *et al.* (1982*a*, *b*). A principal use of this approach is to act as a calibration of the effects of shrinkage and distortion caused to the whole fibril structure by the preparative steps for electron microscopy.

4. Mineralization of connective tissue

The boney skeleton of vertebrates is formed by the mineralization of a matrix, the predominant component of which, is collagen. The process of mineralization is described by Glimcher (this symposium) with emphasis on the mineral component. In this article I will briefly describe the limited knowledge we have of how the mineral may interact with the collagen.

The mineral in bone was identified as calcium hydroxyapatite by Klement & Trömel (1932) by establishing the identity of the X-ray diffraction patterns from both mineral and bone. Hydroxyapatite crystallizes in the space group $P6_3/m$ (C^2_{6h}) with $a = 0.942$ nm and $c = 0.688$ nm. The contents of the unit cell has the formula $Ca_{10}(PO_4)_6(OH)_2$. The structure is one of the fluorapatites, an almost isomorphous set of structures named after its eponymous member fluorapatite $Ca_{10}(PO_4)_6F_2$; this was termed apatite (Gk *apatao*; I deceive) because of its resemblance to some semi-precious minerals such as aquarmarine. The structure of fluorapatite was determined by Naray-Szabo (1930) and Beevers & McIntyre (1946).

In hydroxyapatite, the hydroxyl groups are spaced 0.344 nm apart along the sixfold screw axis. Six of the ten calcium atoms are bonded to the hydroxyls. These form two triangles in planes perpendicular to the hydroxyl axis; the screw-axis symmetry means the two triangles centred on the axis are rotated by 60° about that axis with respect to each other. The calcium atoms not bonded to the hydroxyls form two columns parallel to the sixfold screw axis (the crystallographic *c*-axis) and are coordinated to the oxygen ions in the phosphate tetrahedra.

In bone, the hydroxyapatite crystals are oriented with their crystallographic *c*-axis parallel to the collagen fibril axes. The apatite crystals have been shown by X-ray (Termine *et al.* 1973) and neutron diffraction (Bacon *et al.* 1979) to orient parallel to the stress lines in bone. Well oriented fish bones give a small angle diffraction pattern similar in spacing to that from wet collagen ($D = 68$ nm), but with modified intensities (Engstrom 1972). Normally when a tendon dries, the collagen periodicity shrinks to 64 nm. In dried calcified tissue, which still gives $D = 68$ nm, the mineral appears to stabilize the native periodicity.

When Hodge & Petruska (1963) discovered the non-integral relation between the collagen molecular length and the axial intermolecular shift in the fibrils, they pointed out that this implied 'gaps' regularly arranged throughout the fibril. These gaps are some 35 nm long and 1.5 nm in diameter; Hodge & Petruska (1963) suggested that the gaps might be the site of mineral deposition in bone.

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Electron micrographs of thin sections of mineralizing connective tissue show crystallites occurring at regular intervals coincident with the axial repeat of the collagen fibrils (Robinson 1952; Robinson & Cameron 1957, 1958; Glimcher 1960; Ascenzi *et al.* 1964; Hohling *et al.* 1966). The mineral crystallites are 32–40 nm long and have one lateral dimension of 4 nm (Myers & Engstrom 1965), but uncertainty exists about their shape (needles or plates), which could be tissue dependent. Furthermore it was not clear whether the mineral crystallites were in or around the collagen fibrils, nor was their axial location within a D unit certain (Cameron 1972).

This last problem has been investigated by combined X-ray and neutron diffraction studies on mineralizing turkey leg tendon (White *et al.* 1977; Berthet-Colominas *et al.* 1979). The experiments were done on tendons freshly extracted from turkeys 22 weeks old. The tendons are fully calcified at their distal end but uncalcified at their proximal end. Meridional X-ray diffraction patterns, which originate from the axial structure of the fibril (see §3 (*a*) (v)), were obtained from uncalcified and fully calcified tendon in the native state. The pattern from uncalcified tendon closely resembled that from rat tail tendon with minor differences that may yet yield information about the location of minor components.

The pattern from calcified tendons indexes on the same spacing ($D = 68$ nm) as that from uncalcified tendons. The intensities, however, are greatly modified. The first seven orders have the same strong odd, weak even order feature as those from uncalcified tendon, which indicates a step function of mark–space ratio close to unity. These first few orders are much more intense than the higher orders compared with the pattern from uncalcified tendon, furthermore the calcified tendon gives a pattern with a very intense halo of diffuse scatter, which makes the higher order difficult to observe. In spite of this difficulty the 20th and 21st meridional orders, which are characteristically strong from uncalcified tendon are again recorded as strong from calcified tendon. The reflections of order 10–19 are almost impossible to detect above the diffuse scatter. The experimental problem is that mineral, containing atoms with higher atomic number than collagen, dominates the scattering in the X-ray pattern. It may be concluded, however, that the collagen retains its native periodicity of 68 nm on calcification, that the mineral is arranged D periodically and that the axial extent of the mineral crystallites is about $\frac{1}{2}D$(34 nm). The important question of the spatial relation between the mineral and the collagen remained unresolved.

Neutron diffraction has two advantages over X-rays for this problem. First, the mineral and collagen scattering are comparable; the mineral does not dominate. Secondly, by immersing the tendons in different mixtures of D_2O with H_2O, the contrast between the structure and the background may be varied in a known way. This approach led (White *et al.* 1977) to the following conclusions.

The first order intensity from calcified collagen in 0% D_2O is actually less than that from uncalcified collagen in 0% D_2O. This suggests that the amplitudes of the collagen and mineral scattering are adding with a phase relation, which implies a specific interaction between the collagen and mineral.

The variation of the intensities of the neutron reflections at different contrasts can be analysed and indicate that the mineral block is scattering some 134° out of phase with the collagen 'overlap' step function. This starting result was then used to obtain a more precise value for the axial location of the mineral on the collagen from the intensities of the first nine meridional reflections in the X-ray pattern. Advantage could be taken of the detailed understanding we

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already have of the axial structure of the uncalcified tendon (see §3 (*a*) (v)). The result showed clearly that the mineral block occurs at the axial level of the 'gap' in the collagen fibrils, possibly displaced a little towards the N-terminus of the collagen molecule.

The knowledge of the uncalcified collagen structure means that we have this one-dimensional structure; if it is on an absolute electron density scale, and assuming the electron density of apatite to be $900 e\,\mathrm{nm}^{-3}$ the volume of the apatite block may be calculated as 30 nm^3 (Berthet-Colominas *et al.* 1979). This is about the volume of a 35 nm long section of a triple helical collagen molecule, but it is much smaller than 560 nm^3, the volume of a $4 \times 4 \times 35$ nm^3 block. This strongly suggests that there is also apatite, possibly amorphous, between the collagen molecules in the overlap region as well as the 'gap'. The diffraction method gives an estimate of the excess mineral at the axial level of the gap. The volume determined from the experiments is too large to derive only from single blocks forming external girdles round the periphery of the fibril. Hence the most likely inference is that the apatite occurs in the gaps throughout the fibrils.

By placing a narrow slit before the specimen, the region of a mineralizing tendon at the edge of the mineralized zone could be studied by X-ray diffraction. This gave a remarkable low-angle diffraction pattern, which was different from either that from fully calcified or uncalcified tendon. It represented the region of mineralization and indicated an intermediate structure. The absorption of the tendons were measured with an ionization chamber and the first six orders of diffraction recorded. The new feature was a strong second order, never obtained from either fully calcified or uncalcified tendons. Analysis of the intensities of the reflections by model building indicates that mineralization must start at a sharply defined locus within the gap rather than at loci evenly distributed throughout the gap. A less certain conclusion was that the locus was at the end of the gap containing the N-terminus of the collagen molecule (Berthet-Colominas *et al.* 1979).

The role of collagen in mineralization is still uncertain. It is the principal component of the calcifying matrix but there are other macromolecules present, glycosaminoglycans, proteoglycans and glycoproteins, which have been implicated in the mechanism of mineralization either as putative nucleators or inhibitors. Here we have not discussed the question of calcium transport and the part played by phospholipids or matrix vesicles. The aim here has been to procede slowly by establishing the structure of the major component in the calcifying matrix and to establish the stereochemical relation between that and mineral in both calcified and calcifying tendons.

The mechanism by which the mineralization occurs could be either directly on to collagen (see, for example, Glimcher 1976) or by way of special intermediary macromolecules (glycoproteins, phosphoproteins (Veis *et al.* 1977)), γ-carboxyglutamate containing proteins (Hauska & Reddi 1980), or osteonectin (Termine *et al.* 1981). In either case, a detailed knowledge of the underlying collagen fibrils structure is essential. An epitaxial relation between collagen and mineral would require a geometrical fit between the two structures. The distribution of polar amino acids such as aspartic and glutamic acids are distributed along the collagen molecule in groups separated by about 11.3 nm. The pitch of the collagen triple helix is 8.68 nm and inter-residue axial repeat 0.389 nm. None of these is an integral multiple of 0.688 nm, the *c*-axis of the calcium hydroxyapatite, nor even of 0.344 nm, the axial period of the calcium ions or the phosphate ions. The ratio of the different structural periodicities can only be expressed as the ratio of pairs of large integers. Unless, of course, some leeway is allowed.

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The mobility of the protein side-chains discussed in §3 indicates that the leeway is to be sought in the collagen rather than the apatite and the extent of the leeway can only be settled by model building. Even if side-chain flexibility permitted say, glutamic or aspartic acid side-chains to reach calcium ions in apatite, the interaction would not be regular.

Epitaxy depends on numerology and the N-terminal telopeptide is interesting in this respect. It was shown (Hulmes *et al.* 1980) that the N-terminal telopeptide is compressed to 0.25 nm as the average axial inter-residue distance compared with 0.289 nm in the triple helical region of the molecule. Then Helseth *et al.* (1979) pointed out that the actual amino acid residues occurring in the N-terminal telopeptide strongly indicated that it would adopt a β-conformation. A β-conformation is extended compared with the collagen triple helix so Helseth *et al.* (1979) postulated a hairpin structure with two hydrogen bonded β-chains and a plausible β-bend. Now the axial repeat of a β-structure is 0.69 nm with half this distance as the inter-amino acid residue repeat. Since the N-terminus was the favoured site for nucleation (Berthet-Colominas *et al.* 1979), this near identity of apatite *c*-axis and β-chain repeat is worth note. However, a hairpin structure will imply lateral compression with less intermolecular space at the level of the β-structure, so it is not obvious that there could be space for crystal nucleation. The observed mobility of the side chains implies a flexibility that may relax the requirement for precise registration between the protein backbone repeat and the crystal repeat. However, it is based on the several assumptions listed above and remains speculative, especially in view of the probable involvement of intermediary macromolecules between collagen and mineral.

The locus of interaction of proteoglycans with collagen in intervertebral disc (Berthet-Colominas *et al.* 1978; Berthet-Colominas *et al.* 1982) and cornea (Meek *et al.* 1981) has also been established by methods similar to those used to locate the apatite in mineralizing tendons.

5. Biomechanics of connective tissue

In the *Timaeus*, one of the main sources on the nature of Greek science, Plato devotes a whole section (40) to fibrous proteins. He says, 'The sinews he [the Demiurge] made from an unfermented mixture of bone and flesh, producing a substance intermediate between the two and adding a yellow colour. The sinews are thus tenser and tougher than flesh, but softer and more elastic than bone'. Earlier, Plato had attributed contractility to tendons, a progenitor in the honourable history of wrong theories of muscle contraction. In our own century, d'Arcy Thompson (1941) provided a framework for the analysis of biological form and that more inscrutable biological characteristic, growth. d'Arcy Thompson's approach was geometrical and physical. More recently McNeil Alexander (1978, 1979) has given an account of the whole of the vertebrate and invertebrate subphyla in terms of animal mechanics. An important feature of both of these treatments, that of d'Arcy Thompson in particular, is that they are non-molecular. Now, with the emergence of structural molecular biology, it is possible to start from a basis of molecular structure and arrangement and reinterpret the mechanical properties of organisms.

It is the aim of structural molecular biology to relate the structure of biological macromolecules or macromolecular assemblies to their biological function. The function of biological fibres is usually structural. They support the organism in the face of gravity, determine its external shape, maintain the relative positions of and afford protection to the major organs and, in animals, permit locomotion. Examples are cellulose in plants, collagen in metazoan animals,

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chitin in the arthropod phylum, silks in the insect class, keratin in the mammal, bird and reptile classes and muscle in most animal phyla. Because of this mechanical function of biological fibres, our aim is to relate their structure to their mechanical properties, which is the basis of their biological function. We wish to show why various structures, usually molecular helices packed into fibrils, have the mechanical properties they exhibit in organisms. We wish to take steps towards a science of biomaterials analogous to the materials science of alloys or ceramics.

This general project has quite broad significance. The question of form and function in biology was a preoccupation of nineteenth and early twentieth century morphologists and elegantly epitomized in d'Arcy Thompson's (1941) classic *On growth and form*. We can ask purely functional questions about biological structures and seek an account in terms of mechanics of the design in the organism's framework and even of optimal design. Or we can go on to ask an evolutionary question, how did these optimized structures come about? Here we wish to know about the role of mechanical considerations in natural selection. A similar question, dealt with by d'Arcy Thompson, can be put about the development of form during the lifetime of an individual organism. Recently there has been a resurgence of interest in biomechanics (Wainwright *et al.* 1976; Society for Experimental Biology 1980). We can investigate at a molecular level and relate molecular structure and arrangement to microscopic mechanical properties by probing these by Brillouin light scattering. This will yield separable elastic moduli of the constituents of polycomposite materials and hence reveal how these properties are combined to yield the macroscopic mechanical properties of the material. Bone is a composite of two components of widely different elastic moduli and we can try to determine how the structural arrangement of the two components affects the mechanical properties of the composite. At present physical theories of bone lack much of the basic experimental data on microscopic properties necessary to test and develop the theories. Precise parameters would allow further theoretical development and guide the fabrication of replacement tissues.

Brillouin (1922) first pointed out that at thermal temperatures condensed matter should contain waves of compression and rarefaction propagating through it. These are now termed phonons. In liquids these are simply sound waves of specific velocity, but in crystals the lattice symmetry imposes systematic restrictions on the directions of the sound waves and on the relations between the velocities in different directions. Radiation of appropriate energy (light in the visible region or thermal neutrons) can interact with the phonons and the energy exchange may be obtained by measuring the change in wavelength between the incident and scattered radiation. For visible light these phenomena are termed Brillouin and Raman scattering, which are due to scattering from, respectively, the so-called acoustic and optical branches of the phonon dispersion curve. Phonon dispersion curves are obtained by plotting the phonon frequency against wavevector; at low wavevector the frequency of acoustic phonons in linearly dependent on wavevector while that of optical phonons is independent. We are interested in acoustic phonons and hence Brillouin scattering. The frequency shift of the scattered light may be measured with a Fabry–Perot etalon and a plot of I (scattered) against ΔE will show a maximum at the phonon energy shift. From the frequency of the phonon its velocity may be determined and this is related via the density of the material to the elastic moduli. For a crystal of known symmetry, variation of the angle between the incident radiation and the scattered vector can yield the c_{ij} tensors in the matrix relating stress to strain throughout the crystal lattice. These c_{ij} tensors in turn may be used to calculate the familiar Young modulus, bulk modulus and compressibility ratio of the crystal (see, for example, Fedorov 1968).

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This approach has been applied to biological fibres (Harley *et al.* 1977; Cusack & Miller 1979; Randall & Vaughan 1979; Vaughan & Randall 1980; Maret *et al.* 1979). These experiments have established the feasibility of measuring c_{ij} tensors from appropriate materials and of obtaining results of biological significance.

In the first experiments (Harley *et al.* 1977) tendon fibres were inclined at 45° to the incident beam and the scattered beam analysed at 45° to the fibre axis. In this configuration (scattering angle 90°) the scattering is independent of the refractive index of the material. Phonons are observed and are confirmed by dependence on wavelength of irradiant light and polarization analysis to be of the longitudinal acoustic branch. Similar simple measurements were made on tendons at different humidities, and on clam muscle, which contains predominantly α-helical protein. These experiments demonstrated the dependence of the phonon energy on humidity and molecular conformation. A more detailed study was made on tendon and comparative measurements made on muscle, silk (containing protein in the β- or extended conformation), α-keratin and feather keratin (Cusack & Miller 1979). These experiments established that for the quasi-hexagonal lattice symmetry (Hulmes & Miller 1979) in collagen, it is possible to map the c_{ij} tensors and hence to determine the complete set of elastic moduli of the fibrils. The microscopic moduli were an order of magnitude greater than those determined before, thus establishing the value of the method for obtaining precise values for these moduli. The comparative study showed that the longitudinal elastic moduli were inversely related to the degree of extension of the polypeptide chain conformation and that it was modified when regular helices were embedded in amorphous matrix, as in the keratins.

Randall & Vaughan (1979) have repeated some of these studies and gone further by measuring the breadths of the Brillouin peaks and have also made a comparative study of eye lenses and related the relative stiffness of the lenses to the mode of visual accommodation in different animals.

Experiments on wet tendons close to the *in vivo* state gave a value for the Youngs modulus of 5.1 GN m^{-2} (Cusack & Miller 1979). For dry collagen, it was possible to measure all the c_{ij} values and the Young modulus was measured as 11.9 GN m^{-2}. The measurements may be compared with estimates of 1.0 GN m^{-2} for the Young modulus of native tendons obtained by Torp *et al.* (1975) with a strain gauge.

Fish bone was selected because of its transparency, as an example of mineralized tissue. The value deduced for the velocity of longitudinal elastic waves along the bone axis is 3.7 km s^{-1}. By using the measured value of the density of bone, 1.9 g cm^{-3}, this yields a value for c_{33} of 25.6 GN m^{-2}. This compares with a value of 32.5 GN m^{-2} obtained by Yoon & Katz (1976) by ultrasonic measurements at 5 MHz on dried human cortical bone.

These types of investigation are now at an interesting stage and in the next few years we should see a bridge from the molecular structure to the macroscopic mechanical properties of mineralizing tissues by way of testing the applicability of various models (Currey 1964; Currey 1969).

6. Conclusion

Collagen makes up 88% of the organic matrix of bone (Urist *et al.* 1983). There are several minor components that affect bone differentiation and growth but for the fundamental mechanism of mineralization we must look for an interaction of mineral with collagen, possibly

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by way of an intermediary molecule. The three-dimensional structure of collagen will be the framework for interpreting mineralization and for explaining, in interaction with mineral, the mechanical stability with controlled plasticity, essential for the life of vertebrates.

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Discussion

S. B. Parker (*Inorganic Chemistry Laboratory, Oxford, U.K.*). (1) Has Dr Miller found any evidence that the telopeptides play any role in the mineralization process? If not, what is their fate?

(2) Osteonectin has been suggested as the nucleator of bone, by binding to both hydroxyapatite and collagen. Are there any sites on the collagen fibril that could act as a binding site for osteonectin?

A. Miller. Dr Parker has confirmed that since he asks about the *fate* of the telopeptides, he means the peptides cleaved from procollagen. There is no evidence to my knowledge that they are involved in mineralization and their fate is presumably degradation.

We have not yet looked in detail for possible osteonectin binding sites since not quite enough is known about the osteonectin molecule. Obviously it would be difficult to fit large (molecular mass *ca.* 10^5 Da) molecules into the 'gap' of the collagen fibrils unless these molecules were fibrous.

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