Stained Native Collagen: Interpretation of the Small Angle X-ray Scattering and Electron Microscopic Pictures in Terms of the Primary Structure

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The average axial electron-density distribution of stained collagen fibers derived from X-ray small angle pattern, is shown to be substantially related to the projection of the polar amino acid residues onto the axis of the microfibril taking into consideration the staggering of neighbouring molecules. In order to arrive ultimately at a quantitative representation of the experimental data, an additional periodic fluctuation of the density in direction of the fibril axis is required. The necessity of this regular microphase structure for an understanding of known physical properties of collagen fibers will be indicated .

Introduction

When native stained collagen specimens are observed under the electronmicroscope, very regularly banded fibrils are seen[1 – 4]. The period of the banding structure is experienced to be slightly dependent on the methods of preparation having fluctuating values in the neighbourhood of $L \sim 65$ nm. But it is essential that the structure within the fibrils of a distinct specimen is always extremely uniform and regular, thus yielding X-ray small-angle patterns the necessary high quality [1, 3]. Moreover, X-ray investigatons combined with light-diffraction experiments resulted in a very accurate representation of the axial electron density distribution of stained native collagen specimens [1, 2]. The quality of this approach can be seen from Fig. 1 where the relative intensity data of the small-angle X-ray interferences (SAXS) are shown compared with the pattern obtained from the light-diffraction experiments using electron microscopic picture as object.

It is almost logical that this regular superstructure within stained collagen fibres is expected to be directly related to the primary structure of the macromolecules. Inspite of the crystallographic accuracy of the superstructure in direction of the chain axes, allowance must be made for a certain internal disorder thus leading to an understanding of typical physical properties of collagen, fibres as, for example, their ability for large elastic bendings.

Nevertheless, on the basis of the knowledge of the electron density distribution wihin the fibrils of native collagen stained with PTA, there was substantial hope for an interpretation of this distribution function in terms of the molecular structure [2], models of which have been developed taking into account the sequence of the amino-acid residues along the polypeptid chains of collagen [4 – 6]. The relevance of each configuration of the chains within

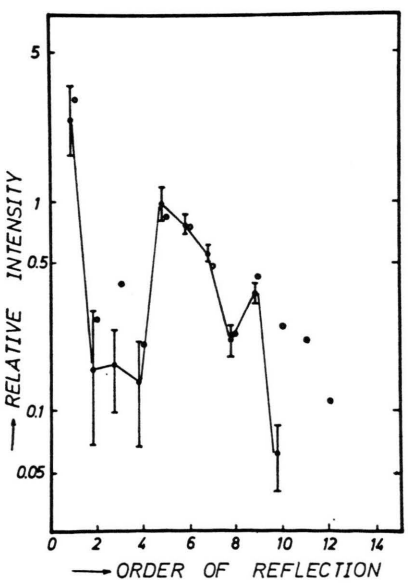

Fig. 1. Relative intensities of the diffraction reflexes of native collagen stained with phosphotungstic acid: (○) small angle X-ray scattering, collagen kept in wet state; (●) light diffraction of electron micrographs.

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Abbreviations: PTA, phosphortungstic acid; SAXS, small angle X-ray scattering; ATP, Adenosintriphosphat; SLS, segment long spacing.

the fibrils according to the model employed [6, 7], is then characterized by the quality of the fit of their calculated intensity distribution to experimental scattering patterns.

The Theoretical Basis

Because of the exact periodicity of the fibril in axial direction, the electron density distribution function with the period of the length L, is properly represented as the following folding product

$$\varrho(z) = \frac{1}{L}\ \varrho_p{}^* \sum_{1=-\infty}^{\infty} \delta(z - n \cdot L) \qquad (1)$$

where the density distribution within the periodic unit of the superstructure is defined by ϱ_p. The periodic electron density function within the microfibrils in direction of their axis is expressed by means of the sum of δ-functions with its characteristic "lattice" parameter L. Hence, we arrive at the following equation for the interference function of a single fibril

$$I(\mathbf{b}) = |F(\varrho_p)|^2 \cdot \sum_{l=-\infty}^{\infty} \delta(b_z - l/L) \qquad (2)$$

with the shape being independent of z.

Here, the Fourier transform of the electron density distribution within the unit cell of the superlattice is equal to

$$F(\varrho_p) = s(b_x, b_y) \cdot f(b_z) = s(b_x, b_y) \cdot \int_0^L \varrho_p(z) \cdot \exp(-2\pi i\, b_z z)\, dz \qquad (3)$$

with the Fourier transform of the shape factor $S(x, y)$ of the fibril

$$s(b_x, b_y) = \iint S(x, y) \cdot \exp(-2\pi i (b_x x + b_y y))\, dx\, dy\,. \qquad (4)$$

The reciprocal vector $\mathbf{b} = (b_x, b_y, b_z)$ has been employed in the above equations. Using the following notation

$$\mathbf{b} = (\mathbf{e} - \mathbf{e}_0)/\lambda;\ b = 2 \sin\Theta/\lambda \approx 2\Theta/\lambda\,. \qquad (5)$$

(2Θ: scattering angle, λ: wave length of the X-ray radiation, $\mathbf{e}_0$, $\mathbf{e}$: unit vectors in the direction of the incident and scattered X-ray beam).

Due to the assumption of a constant electron density over the cross-section the intensity is equally smeared out into the b_y- and b_x-directions of the reciprocal space for all the (001)-reflections of the fibrils which are considered to be in the direction of the z-axis. Thus, rewriting Eqn (2), we have

$$I(\mathbf{b}) = |s(b_x, b_y)|^2 \cdot |f(b_z)|^2 \cdot \sum_{l=-\infty}^{\infty} \delta(b_z - l/L) \qquad (6)$$

where $|s(b_x, b_y)|^2$ is independent of the z-direction. For a cylindrically shaped fibril we arrive at

$$I(\mathbf{b}) = 2\pi R^2 . J_1(u_0)/u_0 \cdot |f(b_z)|^2 \cdot \Sigma\, \delta(b_z - l/L) \qquad (7)$$
$$u_0 = 2\pi R\, b_R;\ \mathbf{b} = (b_R, b_z)$$

where R is the radius of the circular cross-section of the cylindrically shaped fibrils, $J_1(u_0)$ the Bessel function of the first order. b_R, b_z are the coordinates in the plane of reflection.

The electron density distribution function of stained collagen specimens has been computed from experiments under the assumptions introduced above [1, 2]. Each density distribution derived from a model of the molecular structure makes therefore allowance for a quantitative comparison with the results of the structure analysis only if no relevant contradiction to these assumptions is implicated. Then, it is important to note that the quality of the configuration proposed can be checked twice, first by the direct comparison of the density distributions themselves, secondly by the more sensitive proof whether the intensity functions calculated with the aid of Eqn (7) do fit satisfactorily to the observed intensities obtained from light diffraction or from X-ray small angle scattering experiments.

The Basic Model

According to current models [9], the "molecules" are considered to be composed of three correspondingly twisted polypeptid chains with their amino-acid residues directed nearly perpendicularly to the symmetry axis of the molecule. The fibrils seen in the electron-micrographs are assumed to be formed by nearly parallel micro-fibrils, subunits which are not exactly known and which contain in any case a distinct number of molecules [9 – 11] only. From the lateral width [8] of the crystallographic (001)-reflections observed in SAXS-pattern of rat tail collagen [12] it has been made sure that the X-ray coherence regions identify with the fibrils. Thus, whatever the details of the internal structure of the fibril, all the molecules must be aligned in parallel with crystallographic accuracy in the direction of their axes. Thus, inspite of fluctuations on

account of which the lateral order shows large paracrystalline distortions [13], the total axial electron density distribution should be characterized by the longitudinal structure of a single microfibril only.

For the purposes of our analysis, a distinction of the molecular structure of α_1- or α_2-chains is not necessary at all because of their almost identical chemical structure [4]. According to the so-called "staggering-model" [14], the micro-fibril is considered to be composed of five strands of molecules with a definite longitudinal shift as illustrated in Fig. 2. An equivalent model in the present respect was developed by R. Hosemann [10] (see Fig. 2 b).

To synthezise the axial density distribution of collagen stained with PTA, the spatial distribution of the polar amino-acids, of course, is wanted. For it is suggested at once that the PTA-ions should react quantitatively with amino acids of corresponding polarity. If the linkage between those two reactants is strong enough, all the amino-acids bearing the same charge are approximately equivalent in their reactivity independent of their location in the chain or in the fibrils. From the condition of electrical neutrality an equivalent spatial distribution of the amino acids of different polarity in the collagen fibril is anticipated. Thus, allowance is made for deriving the axial distribution of the PTA from the lateral projection of *all polar residues* to the axis of symmetry. This is best done by taking into account the banded structure which characterizes the essentials of the distribution of the polar residues along the chain axis, as is demonstrated in Fig. 3, b and c [4]. In this case, neighbouring molecules within their microfibril have systematically been shifted for 233 residues. The density of the microfibril is thus easily computed from the number of polar residues related to the length of the corresponding band.

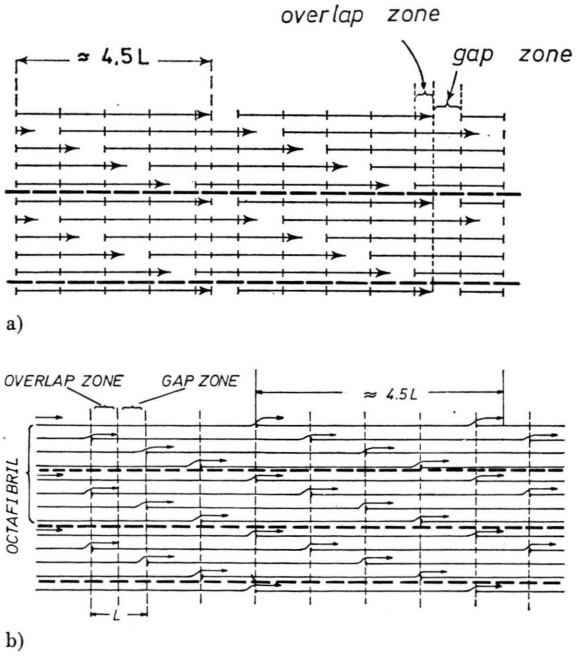

Fig. 2. a) Diagram of the assembly principle of collagen molecules in a five stranded microfibril and in a fibril according to the staggering model for native collagen. The longitudinal displacement, L, of the neighbouring molecules results in a fibril of period, L. Each period embraces an overlap region and a gap region [14]. b) Assembly principle based on the octafibril, the longitudinal displacement, L, results equally well in a fibril with on overlap region and a gap region having the period L. The analogy to the model of Fig. 2 a) is seen by evidence.

Comparison with Experiments

Inspite of some evident simplifications involved in the above procedure, basic correspondence of the banded model density structure within the unit cell

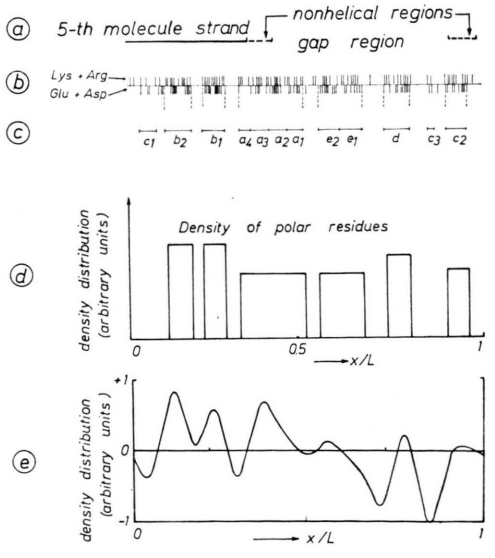

Fig. 3. a) The fifth molecule within the five stranded microfibril according to the assembly principle of Fig. 2 ist marked. b) Projection of the polar residues of a single microfibril (according to ref. [4]). c) Usual notation of the high density bands. d) The distribution of the polar residues (of Fig. 3 b) represented as a density distribution which is locally averaged; the distribution is assumed to be in proportion to the corresponding distribution of the staining material. e) Density distribution of native collagen stained with phosphotungstic acid obtained from X-ray experiments (ref. [1]).

of collagen fibrils to the experimental functions is evident from the comparison of these functions illustrated in the Fig. 3, d and e.

But the satisfactory correspondence of the intensities computed with the aid of Eqn (6) and shown in Fig. 5, could only be achieved by a proper modification of the theoretical banded density distribution derived from the above projection: There was need for increasing the density exactly within that range of z's which are occupied by the fifth strand including the N-and C-terminals (see Fig. 3 a). The better correspondence of the modified theoretical density distribution is illustrated most clearly by the results shown in the Figs 4 and 5.

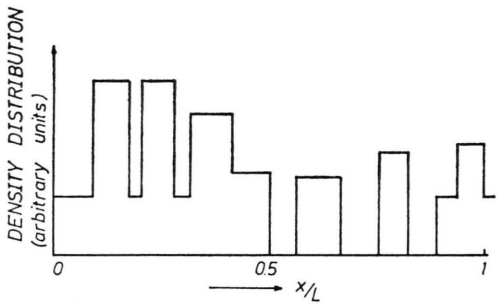

Fig. 4. Model of the axial density distribution developed from that one presented in Fig. 3 d by superimposing a constant contribution in the range of the a, b, c-bands ("overlap-region").

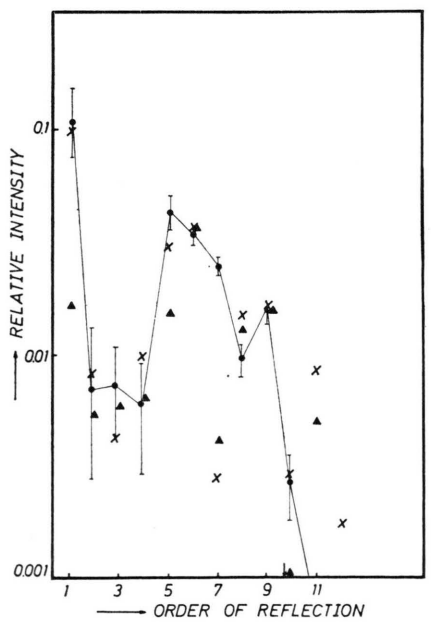

Fig. 5. Diffraction pattern of collagen (see Fig. 1) in comparison with intensities calculated with the aid of the models given in Fig. 3 d, 4 resp. (●) Experiment; (▲) model of Fig. 3 d; (×) improved model of Fig. 4.

The specified correlation between the distribution of the polar residues within the z-projection of the real molecular structure of he stained collagen fibrils and the experimental density distribution points to the importance of the *staggering* of the molecules within the fibrils as well as to the fact that molecules as large as PTA are allowed to be added without disturbing the crystallographical quality of the axial order within the collagen fibrils.

Structure of SLS- and Native Collagen

The formation of fibrils from diluted solutions of collagen can be prevented by proper additives (ATP) [15]. The collagen molecules will then be precipitated in regularly shaped sheets which are composed of nearly parallized single collagen chains in their extended conformation. Thus, the thickness of these sheets identifies with the length of the collagen molecules.

Staining these SLS-patterns, the bands observed under the electronmicroscope are directly related to the localization of the amino-acid residues of proper polarity along the extended collagen chain. According to reference [4 and 16], it should be attractive to synthesize the bands of collagen fibrils by superposing the basic density distribution of the stained extended chains using the staggering model. From the comparison of the synthesized density function with the photometer plots of electron micrographs given by Kühn *et al.* [14] (see Fig. 6, b and c) the fundamental correspondence of both diagrams is evident, thus legitimating a localization of the corresponding polar amino-acids.

Because of the excellent correspondence of the density distribution gained in our investigations [1] (Fig. 6 a) with the constructed densitometer plot of the fibrils published by Kühn *et al.* (Fig. 6 b) [4, 14], it appears interesting to investigate whether the discrepancies in details which are responsible for the more pronounced deviations when comparing the corresponding intensity functions (see Figs 7 and 8), could be diminished by improvements. Thus, a somewhat better correspondence of the synthesized and the experimental pattern is achieved when taking properly into consideration the gap of the staggering model that is demonstrated in Fig. 9. Here the contributions of the fifth chain, taking also care of the telepeptides, have been added to the density function of Kühn [4] which is plotted in Fig. 6 b. As the relevant density differences are determined by

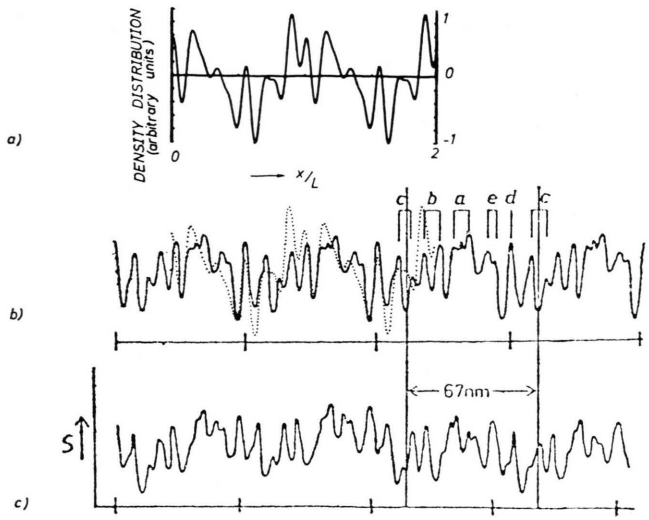

Fig. 6. a) Density distribution obtained from experiments; two periods, L, are depicted. b) Density distribution deduced from the known density of SLS-collagen taking into account 4 molecule strands only (taken from ref. [4]); the dotted line: distribution of a). c) Densitometer trace of an electron micrograph of stained collagen (taken from ref. [4]).

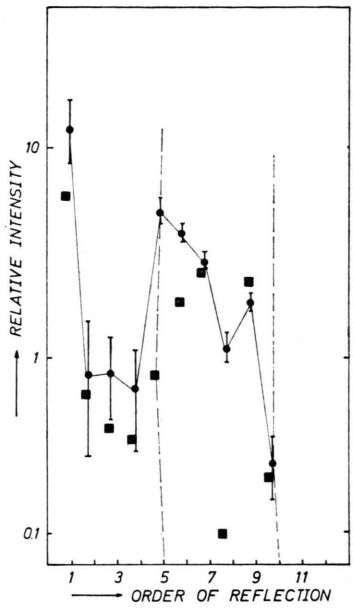

Fig. 8. The experimental diffraction pattern compared with the pattern computed from the density distribution given in Fig. 6 b: (●) experiment, (■) calculated.

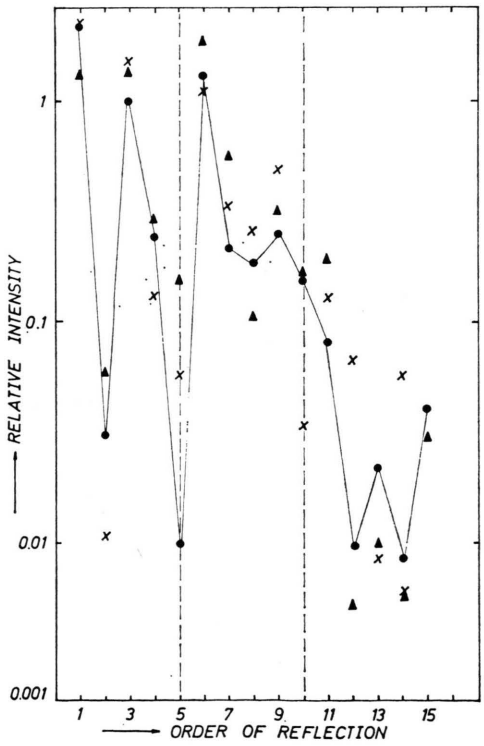

Fig. 7. Diffraction pattern computed with units (L) where the density function within the units has been taken to be identical with one of the units given in Fig. 6 c, thus yielding four different calculations.

the staining additives only which are concentrated within the bands, allowance is made for adjusting the density within the gaps between the bands to zero. Further, the density along the axis of the 5th molecule is assumed to identify with that average value gained for the other molecules. The diffraction pattern of the improved distribution function shows equally well a satisfactory approach to the experimental data of SAXS-measurements (Fig. 10).

We obtained surprisingly good correspondence between different experiments inspite of the fact that the staining of the SLS-specimens has been performed with uranyl acetate, the ion of which bears a charge with opposite sign compared with the ion of PTA. This agrees logically with the idea that electrical neutrality seems widely be established within the bands as can be estimated from Fig. 3 b. However, the correspondence is limited to the accuracy of the description of the super-structure which is at least determined by the resolution obtained in the electronmicrographs, thus restricting the use of the number of reflections in our analysis of the SAXS-pattern to 9 or 10 [1].

Discussion

Although the essentials of the axial-electron density distribution in stained samples of collagen are

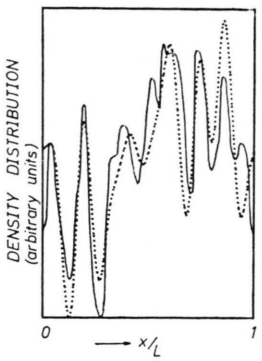

Fig. 9. The improved density profile derived from SLS-collagen compared with the density determined by experimental means: (——) SLS-collagen model, (· · ·) determined from experiment.

obtained by calculating the axial density of the polar residues within a micro-fibril employing the staggering model, the necessary correction of this projection is of interest.

Assuming that the staining of the amino-acids is different as a result of their correspondingly different reactivities, the $\varrho(z)$-correction can not be explained at all by the use of the sequence of the amino-acids even when very synthetical conditions are considered (Fig. 11) as, for example, selective

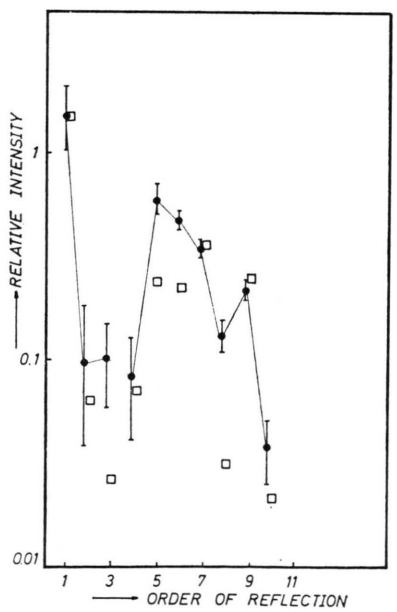

Fig. 10. Experimental diffraction pattern of stained native collagen (●) in comparison with the pattern calculated with the aid of the density model of Fig. 9 deduced from SLS-collagen (■).

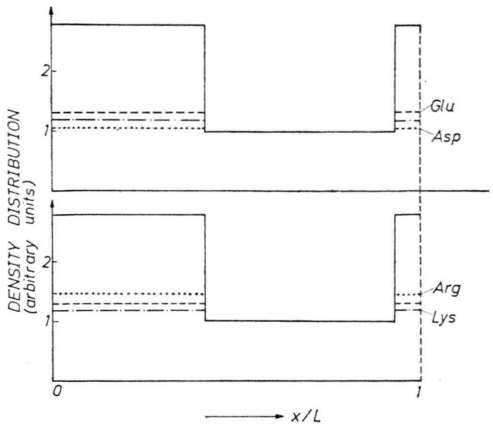

Fig. 11. Density distributions; the density is averaged in the overlap region and in the gap region resp.; the density of the amino acids was calculated in terms of the number per length unit according to the staggering model (Fig. 2). (——) Improved model of Fig. 4; (— —) Glu+Asp and Arg+Lys resp. (according to ref. [4]).

reactions of parts of the amino-acids with staining molecules.

The correction term is not needed in the improved calculation employing the SLS-patterns. Hence, we arrive at the interesting conclusion that the lateral interactions between a single stranded extended collagen chain within an SLS-structure unit should topologically not be different in what is considered to be established between staggered triple helices within the collagen fibrils.

The architectonic concept of the collagen fibril is evident: In order to form a "macroscopic" fibril from chains of uniform length, a regular longitudinal shift gives an excellent opportunity for a lateral crosslinking (see Fig. 2) which seems to be energetically profitable. But it might be that an additional cooperative interaction between the molecules is necessary to form a stable fibril.

Adopting these considerations, a "two micro phase" model with phase 1 and phase 2 comprising the overlap- and the gap-region resp. seems to be imperative. By assuming an adequate periodical fluctuation of the electro-chemical potential in axial direction, which characterizes the thermodynamic situation within the fibril, the correction function of the synthesized $\varrho(z)$-function can be related to corresponding properties of the staining process within the two regions involved. Though this is an explanation of a very hypothetical origin, such a model is in good conformity with a "two-phase" model proposed in earlier times for an understanding of the physical

properties of the collagen fibrils [17]. According to our idea, phase 1 should propably appear as the microphase with a stiffness as that of solids. Allowance is only made for a marked bending of the fibre in macroscopic dimensions because of the properties of the phase 2 the stiffness of which should be reduced accordingly.

Hence, the results presented in this paper clearly suggest that the existence of a flexible collagen fibril seems to be based on the existence of longitudinal microphase structure the correct description of which must uniquely be related to the chemical structure of the collagen chains and the triple helix resp. The collagen fibrils appear to be characterized by an alternating regular serie of micro-phases which differ in their elastic properties, the solid part comparable with crystallites, the more flexible part comparable with the soft "amorphous" parts in "partially crystallized" fibres formed from synthetic polymers. The segments in the "amorphous regions" are considered to be highly oriented thus yielding a sufficiently regular longitudinal arrangement of the chemical units even within the regions having no crystallographical lateral order.

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