Brown D. R., Qin K., Hermes J. W., Madlung A., Manson J., Strome R., Fraser P. E., Kruck T., Bohlen A., S.-Schaseffer W., Glease A., Westaway D. and Kretzschmar H. (1997), Nature **390**, 684–687.

Deng H. X., Hentai A., Tainer J. A., Iqbal Z., Cayabyab A., Hung W., Getzoff E. D., Hu P., Herzfeldt B., Roos R. P., Warner C., Deng G., Soriano E., Snyth. C., Parge H. E., Ahmed A., Roses A. D., Hallewell R. A., P.-Vance M. A. and Siddique T. (1993), Amyotrophic lateral scleoresis and structure defects in Cu,Zn superoxide dismutase. Science **261**, 1047–1051.

Floor E. and Wetzel M. G. (1998), Increased protein oxidation in human substantia nigra pars compact in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. J. Neurochemistry **70**, 268–275.

Ito S., Okuno T., Itoh H., Ohba S., Matsushima H., Tokii T. and Nishida Y. (1997), Chemical basis for high activity in oxygenation of cyclohexane catalyzed by dinuclear iron(III) complexes with ethereal oxygen containing ligand and hydrogen peroxide system. Z. Naturforsch. **52B**, 719–727.

Janzen E. G., Nutter Jr. D. E. and Davis E. R. (1978), On spin trapping hydroxyl and hydroxyl radicals. Can.J. Chem. **56**, 2237–2242.

Kobayashi T., Ito S., Hamazaki H., Ohba S. and Nishida Y. (1996), Relaxation of pBR322 Form I DNA by copper(II) complex and hydrogen peroxide. Chem. Lett., 347–348.

Nishida, Y. and Ito, S. (1995), Structures and reactivities of several iron(III) complexes in the presence of hydrogen peroxide: its relevance to induction of tissue damage caused by iron(III) chelates in rats. Polyhedron **14**, 2301–2308.

Okuno T., Ohba S. and Nishida Y. (1997), Oxidation of cyclohexane with hydrogen peroxide catalyzed by copper(II) complexes containing N,N-bis(2-pyridylmethyl)-β-alanineamide ligands. Polyhedron, **16**, 3765–3774.

Peng H.-M., Raner G. M., Vaz A. D. N. and Coon M. J. (1995), Oxidative cleavage of esters and amides to carbonyl products by cytochrome P-450. Arch. Biochem. Biophys. **318**, 333–339.

Pazas M., Goto J. J., Rabizadeh S., Gralla E. B., Roe J. A., Lee M. K., Valentine J. S. and Bredesen D. E. (1996), Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. Science **271**, 515–518.

Prigge S. T., Kolhekar A. S., Eipper B. A., Mains R. E. and Amzel L. M. (1997), Amidation of bioactive peptides: the structure of peptidylglycine a-hydroxylating monooxygenase. Science **278**, 1300–1305.

Prusiner S. B. (1997), Prion diseases and the BSE crisis. Science **278**, 245–251.

Prusiner S. B. (1996), Molecular biology and pathogenesis of prion diseases. Trends Biochem. Sci. **21**, 482–487.

Rabizadeh S., Gralla E. B., Borchelt D. R., Gwinn R., Valentine J. S., Sisodia S., Wongs P., Lee M., Hahn H. and Bredesen D. E. (1995), Mutations associated with amyotrophic lateral sclerosis convert superoxide dismutase from an antiapoptotic gene to a proapoptotic gene: studies in yeast and neural cells. Proc. Natl. Acad. Sci. USA. **92**, 3024–3028.

Rana T. M. and Meares C. F. (1991), Transfer of oxygen from an artificial protease to peptide carbon during proteolysis. Proc. Natl. Acad. Sci. USA. **88**, 10578–10582.

L.-Robichaud P., Shyadehi A. Z., Wright J. N., Akhtar M. E. and Akhtar M. (1995), Mechanistic kinship between hydroxylation and desaturation reactions: Acyl-carbon bond cleavage promoted by pig and human CYP17(P-450_{17a}; 17a-hydroxylase-17,20-lyase). Biochemistry **34**, 14104–14113.

Stadtman E. (1992), Protein oxidation and aging. Science **257**, 1220–1224.

Sohal R. S. and Weindruch R. (1996), Oxidative stress, caloric restriction, and aging. Science **272,** 59–63.

Vaz A. D. N., Pernecky S. J., Raner G. M. and Coon M. J. (1996), Peroxo-iron and oxenoid-iron species as alternative oxygenating agents in cytochrome P-450-catalyzed reactions: Switching by threonine-302 to alanine mutagenesis of cytochrome P-450 2B4. Proc. Natl. Acad. Sci. USA. **93**, 4644–4648.

Yim M. B., Chock P. B. and Stadtman E. R. (1993), Enzymatic function of copper, zinc dismutase as a free radical generator. J. Biol. Chem. **268**, 4099–4105.

Non-Uniform Triple Helical Structure in Chick Skin Type I Collagen on Thermal Denaturation: Raman Spectroscopic Study*

V. Renugopalakrishnan[a,b], L. A. Carreira[c], T. W. Collette[c], J. C. Dobbs[c], G. Chandraksasan[d] and R. C. Lord[e,†]

[a] Harvard Medical School, Boston, MA 02115, USA
[b] Universidad Nacional Autonoma de Mexico, 0451 Mexico DF, MEXICO
[c] University of Georgia, Athens, GA 30602, USA
[d] Central Leather Research Institute. Council of Scientific and Industrial Research, Madras – 600 020, INDIA
[e] Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Z. Naturforsch. **53c,** 383–388 (1998); received December 18, 1997/February 17, 1998

Collagen, Triple Helical Conformation, Thermal Denaturation

The individual chains in the triple helix of collagen occur in a conformation related to polyproline II because of the presence of large number of imino peptide bonds. However, these residues are not evenly distributed in the collagen molecule which also contains many non-imino residues. These non-imino regions of collagen may be expected to show preference for other than triple helical conformations. The appearance of several Raman bands in solution phase at 65 °C raises the possibility of non-uniform triple helical structure in collagen. Raman spectroscopic studies on collagen in the solid state and in solution at a temperature greater than its denaturation temperature, reported here suggest that denatured collagen may exhibit an ensemble of conformational states with yet unknown implications to the biochemical interactions of this important protein component of connective tissues.

Introduction

Collagen, the most abundant protein in vertebrates, exists in a unique triple helical conformation, in which each of the three intertwined chains can be considered to be a polymer of glycine containing triplets, (X-Y-Gly) (Bhatnagar and Rapaka, 1976 and references cited therein). Collagen contains more imino residues than most other proteins, with proline occurring at position X and hydroxyproline at position Y. The imino residues account for approximately one fourth of all residues and the observed polyproline-like conformation of each chain is ascribed to the restricted, φ rotation about the N-Cα bond of the peptide unit involving each imino residue, as well as to the interactions of the imino residues as derived from preliminary NMR and molecular mechanics studies of (Pro-Pro-Gly) 10 (Bhatnagar *et al.*, 1988). An examination of the primary structure of collagen reveals that the imino residues Pro and Hyp are not evenly distributed along the polypeptide, and extended segments show sparse distribution of these residues. The imino-deficient segments may be expected to have conformational preferences different from the polyproline structure of imino-rich segments. While in the native state of collagen, the predominant stercochemical interactions of the imino residues can be expected to direct the overall conformation, a collapse of the triple helix on denaturation can be expected to relax these constraints, facilitating the local acquisition of other possible conformations. Raman spectroscopy is well suited for monitoring triple helix non-triple helix transition and can be expected to provide insight into structural changes occuring in the triple helical collagen structure.

Previous vibrational spectroscopic studies of imino-deficient and imino-rich model peptides have established characteristic vibrational features of conformationally sensitive amide I and III modes of triple helical structures (Renugopalakrishnan *et al.*, 1984; Diem *et al.*, 1984). More recently FT-

Reprint requests to Dr. Renugopalakrishnan.
Instituto de Quimica, Universidad Nacional Autonoma de Mexico, Circuito Exterior, Ciudad Universitaria, Coyoacan, 0451 Mexico DF. MEXICO.
Fax: 52-5-616-2217 or 2203.
E-mail: renu@servidor.unam.mx.

* Dedicated to Professor G. N. Ramachandran, F. R. S.
† Deceased.

0939–5075/98/0500–0383 $ 06.00

IR and FT-IR photoacoustic studies have been extended to chick skin type I collagen (Renugopalakrishnan *et al.*, 1989). A low frequency Raman vibrational mode in the region 300–400 cm^{-1} was found to be characteristic of triple helical conformation (Renugopalakrishnan *et al.*, 1985). An inelastic neutron-scattering study of type I calf skin collagen (Berney *et al.*,1987) revealed a number of low frequency vibrational modes at 296 °K and at 110 °K and were interpeted as a consequence of significant structural changes in collagen. The unique triple helical structure in collagen assumes a polyproline II type of conformation and should be expected to impose stereochemical constraints by the occurrence of Pro or Hyp occuring at least once at position X and Y in the repeating triplet sequence, Gly-X-Y, on the amide vibrations of collagen. Raman studies of collagen have been previously reported by Frushour and Koenig (1975) and Goheen *et al.* (1978). The appearance of two amide I vibrational modes was interpreted as arising from two non-equivalent species of C=O groups, "associated with the polar and non-polar regions of the collagen polypeptide chains". A distinction between polar and non-polar regions based on amide I stretching frequency observed in collagen Raman spectrum is hard to justify. In the past, there has been considerable difflculty in identifying amide I vibrations from collagen Raman spectra due to extensive hydration of samples and the virtual impossibility of complete subtraction of water background, strong fluorescence background, especially in the previously reported studies in the literature utilizing earlier versions of Raman spectrometers.

Proteins can be regarded as delicately free energy balanced systems and hence the equilibrium that determines the unique conformation of a protein is the one that exists between the native low energy conformation and the lack of obvious long range order in conformational states present in the denatured state (Anfinsen, 1973). It is this equilibrium that is influenced by thermal denaturation favoring the "random" correlate which is essentially devoid of structural regularity (Bhatnagar and Rapaka, 1976) characteristic of the native low energy conformation. Thermal stability of proteins is a subject of contemporary research in our laboratories and progress has been slow in understanding the free energy change (ΔF) that occur at the characteristic melting temperature, T_m, of proteins. Thermal stabilization of proteins by increasing their T_m is one of our long term goals in order to increase their utility in technological applications of proteins (V. Renugopalakrishnan, U. S. Patent to be submitted) and hence the present study is a part of ongoing research on the thermal denaturation and free energy (ΔF) changes of proteins (Oobtake and Renugopalakrishnan, to be submitted).

Information concerning various conformations that may be generated by segments of collagen after their release from the triple helix is necessary for understanding the many biologically important interactions of collagen. Several of the reactive sites of collagen have been identified as lying entirely on individual chains (Hay, 1984). The ability of certain regions of the molecule to assume distinct conformations is likely to be an important mechanism in the regulation of the specifIcity of the interactions of collagen with cell surface receptors and with other macromolecules such as fibronectin.

Material and Methods

Chick skin type I collagen was generously provided by Dr.Jerome Gross, Harvard Medical School and Massachusetts General Hospital, Boston, MA. The experimental protocol for its isolation and characterization have been described elsewhere (Heighberger *et al.*, 1978).

Solid phase Raman spectra: To obtain the Raman spectrum of the solid collagen, a small amount (~1 mg) of the material was packed into the indented end of a small metal rod. The exposed surface of collagen was smoothed with a knife blade. The metal rod was then positioned in an assemblage which held the exposed solid in the path of the laser beam in such a way that the specular reflection would not be collected. The scattered light was then frequency-analyzed in order to observe the Raman bands. This configuration was somewhat troublesome in that, even at large Raman shift values, >600cm^{-1}, a large background signal was present due to scattered light from the excitation source. However, this method was found to be superior to that of placing the solid in a capillary tube since true glass-free spectra could be recorded.

Collagen in aqueous solution is denatured at 41 °C; however, collagen fibers when in alignment exhibit a rather broad phase transition characterized by shrinkage at elevated temperatures. The center of this transition is at approximately 55 °C (Ramachandran, 1967). In order to maintain uniformity in these studies, we have selected a temperature of 65 °C for the examination of the denatured state of collagen both in solution and in the solid phase. The elevated temperature was maintained by a hot air blower directed at the sample holder and was regulated by a variable resistance device. All of the collagen spectra presented in the study reported here, solid and solution, were obtained with a Spectra-Physics Argon ion laser (Model 164) operating at 547.9 nm with output power typically about 200mW. A Spex Ramalog Model 1401 spectrophotometer was used. The spectral band width(slit width) was generally about 8 cm^{-1}. Points in the spectra were taken every 3 cm^{-1} with counts averaged at each point for ten seconds. Typically 100 scans were performed and the results presented in this paper represent the average of 100 scans. The spectrophotometer was automated by a Digital PDP computer which was used to store, manipulate, and display the data (Town *et al.,* 1981). All of the displayed spectra have been subjected to a standard three-point smoothing procedure. It was also necessary to digitally mutiply certain spctra in order to compensate for any artificial intensity variations between sample runs.

Solution phase spectra: The solutions used for the Raman study were prepared by dissolving the solid collagen in 0.2 M acetic acid at a concentration level of 14.3 mg/ml. A small amount of this solution was then sealed in a standard melting point capillary which was supported in a Harney-Miller cell whose temperature could be controlled. To obtain the elevated temperature spectra, nitrogen gas was passed first over a heating element onto the capillary tube, then over a chromel-alumul thermocouple junction. Voltage applied to the heating element was automatically controlled by a proportional heating source which monitored the thermocouple output. Even though not essential to this study, the temperature of the sample could be accurately maintained to within a fraction of a degree with this configuration. In order to digitally subtract the solvent contribution from the solution spectra, spectra of acetic acid were recorded at both 25 °C and 65 °C within the Harney-Miller cell. The solution spectra displayed are those that resulted from the subtraction. All of the other experimental parameters described for the solid phase spectra apply for solution phase spectra.

Results and Discussion

Raman spectra of collagen in the solid state at 25 °C and 65 °C are shown in Fig. 1 and the frequencies of bands with their assignments are listed in Table I. Solid type I collagen at 25 °C exhibits two bands with roughly equivalent intensities in the amide I region, at 1670 cm^{-1} and 1690 cm^{-1}, the 1670 cm^{-1} band occurs in a fequency range normally ascribed to β-sheet structures (Chirgadze and Nevskaya, 1976; Bandekar and Krimm, 1979 and references cited therein). The previous vibrational spectroscopic studies focused on (Pro-Pro-Gly) (Diem *et al.,* 1984) and on a tripeptide, Ala-Gly-Gly (Renugopalakrishnan *et al.,* 1984), known to pack into a triple helical conformation in the solid state from x-ray cystallographic studies (Subramanian and Lalitha, 1983). The vibrational spectral data from the above synthetic collagen-like polypeptide and the two Raman spectroscopic studies of collagen reported in the literature (Frushour and Koenig, 1975; Goheen *et al.,* 1978) have shed some light on the characteristie amide I vi-

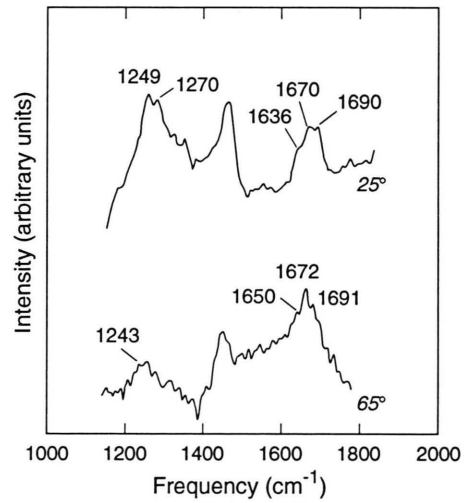

Fig. 1. Raman spectra of type I chicken skín collagen in the solid phase at 25 °C (top) and 65 °C bottom., see text for experimental conditions.

Table I. Major Raman Spectral frequencies (cm^{-1}) a of chick skin type I collagen at 25 °C and 65 °C, respectively, in solid and solution phases.

Solid		Solution[b]		Assignment
25 °C	65 °C	25 °C	65 °C	
1249(s)	1243	1257	multiplet	**amide III**
1270(m)	1261	1284(sh)	structure	
1318(sh)				CH 3
1346(sh)				symmetric deformation and CH2 Wagging
1420(sh)	1460	1434(sh)	1455(sh)	CH 3 assym.
1457(s)	1474	1461(s)	1473(a)	deformation
		1473(sh)		and CH2 deformation
		1527(sh)	1536	**amide II**
		1542	1559	
		1563(sh)		
1636(sh)	1650(m)	1641(s)	1641(sh)	**amide I**
1670(s)	1672(s)	1662(m)	1664(s)	
1690(s)	1691(m)	1677(s)		
1695(m)				

[a] Frequencies are accurate to ± 3 cm^{-1}
[b] In acetic acid solution.
s, strong, m, medium, sh, shoulder.

brational modes of collagen. The 1680 cm^{-1} band also occurs in solid bovine achilles tendon collagen (Frushour and Koenig, 1975). The barely discernible shoulder at 1636 cm^{-1} observed in the present study is low frequency shifted compared to the shoulder at 1646 cm^{-1} in solid bovine achiles tendon collagen (Frushour and Koenig, 1975). In contrast, Poly-L-proline II exhibits a strong Raman band at 1650 cm^{-1} (Smith, 1969) which has no counterpart in the Raman spectrum of collagen. The band at 1241 cm^{-1} with a shoulder at 1261 cm^{-1} in the Raman spectrum of poly-L-proline II bears resemblance to the Raman doublet at 1249 cm^{-1} (strong) and 1270 cm^{-1} (medium) of collagen. The amide III frequencies observed in collagen are indicative of a Ramachandran angle, $\psi > 90°$ (Lord, 1977). Ramachandran angle, $\psi > 90°$, are characteristic of collagen-like structures (Ramachandran and Sasisekharan, 1968). The amide III region of Raman spectra of type I collagen manifests a doublet at 1249 cm^{-1} (strong) and 1270 cm^{-1} (medium), which are remarkably close to the amide III frequencies at 1248 cm^{-1} and 1271cm^{-1} (shoulder) observed in calf skin collagen (Frushour and Koenig, 1975). Raman spectrum of collagen in solid phase at 65 °C, above its T_m of 41 °C (Privalov *et al.*, 1979; Privalov, 1982), is shown in Fig. 1. On thermal denaturation, the amide I region exhibits a marked difference with the 1672 cm^{-1} band gaining intensity which is slightly different from the intense 1668 cm^{-1} band and its shoulder at 1636 cm^{-1} observed earlier in calf skin collagen (Frushour and Koenig, 1975). The most striking difference on thermal denaturation of collagen can be seen in the amide III region, a conformationally sensitive region of Raman spectra of polypeptides and proteins. The Raman doublet at 1249 cm^{-1} and 1270 cm^{-1} observed at 25 °C collapse to produce a strong band at 1243 cm^{-1} at 65 °C. Raman spectra of collagen in acetic acid solution at 25 °C and 65 °C are shown in Fig. 2 and their frequencies are listed in Table I. In contrast to the observation in the solid phase, collagen solution shows a strong doublet amide I pattern with bands at 1641 cm^{-1} and 1677 cm^{-1}, respectively. A medium intensity band also occurs at 1662 cm^{-1}. The amide III region in contrast has an intense band at 1257 cm^{-1} with a shoulder at 1284 cm^{-1}. Frushour and Koenig, 1975 have reported only amide III bands for calf skin collagen at a concentration level of 2% in acetic acid solution at 25 °C.

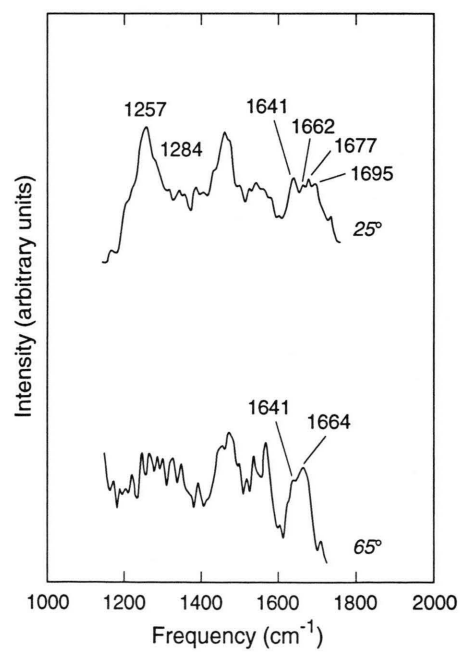

Fig. 2. Raman spectra of type I chicken skin collagen (14.30 mg) in 1 ml. of 0.2 M acetic acid at 25 °C (top) and 65 °C (bottom), see text for experimental conditions.

The amide II region in Raman spectrum of collagen contains a band at 1542 cm^{-1} with two shoulders at 1527 cm^{-1} and 1563 cm^{-1}, respectively. The amide III band on thermal denaturation splits into a multiplet structure exhibiting a complex pattern. The amide II band gains intensity with a doublet at 1536 cm^{-1} and 1559 cm^{-1}, respectively. Therefore, it is interesting to observe that Raman studies suggest that the denatured solid collagen manifests an amide I band at 1672 cm^{-1} and an amide III band at 1243 cm^{-1} which are archtypical of β-sheet structures (Chirgadze and Nevskaya, 1976 a,b). The complex amide III pattern observed on thermal denaturation of collagen is indicative of several conformational states accessible in the denatured state. The accessibility of conformational states could be due to the loss of ordered structure by the rupture of inter-chain hydrogen bonds, extensive dehydration which will remove water molecules usually associated with collagen structure (see Lim and Griko, 1981; Renugopalakrishnan *et al.,* 1989), and possibly induction of cis-trans isomerization of peptide bonds. Although several conformational states are indicated on the basis of Raman studies, the denatured collagen may lack long range order. The appearance of several Raman bands in solution phase at 65 °C also raises the possibility of non-uniform triple helical structure unlike α-helical structures which give rise to a much narrower range of Raman amide I bands, 1650–5 cm^{-1} (Renugopalakrishnan and Bhatnagar, 1984). The conclusions derived here may have important implications to the biochemical interactions of this vital protein component of connective tissues.

Acknowledgements

This research was partially supported by funds from Harvard University. V. R. would like to express his sincere thanks to Dr. Francisco Lara-Ochoa, Director, Institute of Chemistry, UNAM for his generous support in the completion of this manuscript. Mr. Vivas Cortes Jose Trinidad, Institute of Chemistry, UNAM and Mr. Masahiro Tanikawa, Mexico City, Mexico rendered valuble help in the preparation of the art work which is sincerely acknowledged.

Anfinsen C. B. (1973), Principles that govern the folding of protein chains. Science **181**, 222–230.

Bandekar J. and Krimm S. (1979), Vibrational analysis of peptides, polypeptides, and proteins. 4. Charactertic amide bands of β-turns. Proc. Natl. Acad. Sci. USA **76**, 774–777.

Berney C. V., Renugopalakrishnan V. and Bhatnagar R. S. (1987), Collagen. An inelastic neutron-scattering study of low-frequency vibrational modes. Biophysical J. **52**, 343–345.

Bhatnagar R. S. and Rapaka R. S. (1976), Synthetic polypeptide models of collagen: Synthesis and applications. In: Biochemistry of Collagen (Ramachandran, G. N. and Reddi, Eds.). Plenum Press, New York, NY, USA. pp. 479–523.

Bhatnagar R. S., Pattabiraman N., Sorensen K. R., Langridge R., MacElroy R. and Renugopalakrishnan V. (1988), Inter-chain proline:proline contacts contribute to the stability of the triple helical conformation. J. Biomol. Struct. Dynamics **6**, 223–233.

Chirgadze Yu.N. and Nevskaya N. A. (1976a), IR spectra and resonance interaction of amide I vibration of antiparallel-chain pleated sheet. Biopolymers **15**, 607–625.

Chirgadze Yu. N. and Nevskaya N. A. (1976b), IR spectra and resonance interaction of amide I vibration of parallel-chain pleated sheet **15**, 627–636.

Diem M., Bhatnagar R. S., Druyan M. E. and Renugopalakrishnan V. (1984), Solution phase Raman spectroscopic studies on synthetic collagen analogs: Prolyl-prolyl-glycine and (prolyl-prolyl-glycine) 10. Biopolymers **23**, 2955–2961.

Frushour B. G. and Koenig J. L. (1975), Raman scattering of collagen, gelatin, and elastin. Biopolymers **14**, 379–391.

Goheen S. C., Lis L. J. and Kaufman J. W. (1978), Raman spectra of intact feline corneal collagen. Biochim. Biophys. Acta **536**, 197–204.

Hay E. D. (1984), The Role of Extracellular Matrix in Development. Alan R.Liss, New York, NY.

Heighberger J. H., Corbett C., Kang A. H. and Gross J. (1978), The amino acid sequence of chick skin Collagen α1-CB7: The presence of a previously unrecognized triplet. Biochem. Biophys. Res. Commun. **83**, 43–49.

Lim V. I. and Griko N. B. (1981), Model of collagen with ice-like packing of water molecules between polypeptide chains in the triple helix. Dokl. Akad. Nauk SSR **259**, 743–746.

Lord R. C. (1977), Strategy and tactics in the Raman spectroscopy of biomolecules. Appl. Spectroscopy **31**, 187–194.

Privalov P. L. (1982), Stability of proteins which do not present a single Cooperative System. Adv. Protein Chem. **35**, 1–104.

Ramachandran G. N. (1967), Treatise on Collagen, Vol. **I**, Academic Press, London, U. K. and New York, NY, USA, pp. 103–183.