

Classics in Indian Medicine



Professor G. N. Ramachandran

(8 October 1922–7 April 2001)

Gopalamudram Narayana Ramachandran (GNR, as he was known to his colleagues and students) was born in 1922 in Ernakulam, near Cochin, in Kerala. He graduated in BSc (Hons) Physics from St Joseph's College, Trichy in 1942 and subsequently joined the Indian Institute of Science (IISc), Bangalore for his Master's degree in Physics, under the guidance of Sir C. V. Raman. GNR was much influenced by the ideas and motivational personality of C. V. Raman and continued his doctoral research at IISc under him. The research involved studies on photoelasticity and thermo-optic behaviour of different solids such as diamond, fused quartz and zinc blende. The thesis also contained original results on the theoretical calculations of optical rotation of crystals of materials such as quartz and sodium chlorate. In 1947, GNR went to Cambridge to work in the Cavendish Laboratory and obtained a second doctoral degree in 1949. Besides his thesis-related work, Ramachandran enjoyed attending lectures on Quantum Mechanics by Dirac and Heisenberg, as well as western classical music concerts! GNR returned to IISc in 1949 as an Assistant Professor but soon moved to Madras. In 1952, he became the first

Professor and Head of the Department of Physics at the University of Madras at the young age of 30. This was a momentous event and the single most important factor not only in GNR's own career, but also in giving rise to an active and vibrant community of X-ray crystallographers, molecular biophysicists and computational biologists in India. Inspired by Pauling's work on biomolecular structures and at the suggestion of Professor J. D. Bernal (who visited Madras in late 1952), GNR decided to investigate the structure of the fibrous protein collagen. Linus Pauling at Caltech as well as several other groups in the UK and USA were already working on solving the riddle of the collagen structure. In an amazing interplay of serendipity, intellectual prowess and experimental expertise, GNR (along with his colleague Gopinath Kartha) came up with the first correct structure for the collagen molecule, within a short period of 2 years. They stunned the structural biology community with their proposal of a triple helical structure for collagen in 2 landmark papers published in the *Nature* in 1954 and 1955. The Ramachandran and Kartha triple-helical model for collagen has stood the test of time for over half a century, except for some minor details.

One of the objections to the GNR model for collagen was that some of the atoms were in very close proximity to each other, possibly giving rise to 'short contacts'. This led GNR's group to investigate all available crystal structures for arriving at acceptable inter-atomic contact distances. Applying this criteria to the possible conformations of a dipeptide unit, GNR (along with V. Sasisekharan and C. Ramakrishnan) put forth the now well known 'Ramachandran Plot' in 1963, as a way of easily identifying the 'allowed', 'partially allowed' and 'disallowed' conformations for a polypeptide chain. This validation tool is now standard reference material in all research papers and textbooks of Biochemistry and Molecular/Structural Biology.

Apart from conducting cutting edge research, GNR was also the first to realize the importance of personal interaction between scientists. The department at Madras organized two extremely successful International symposia in 1963 and 1967, which were attended by a galaxy of leading crystallographers and structural biologists including several Nobel laureates. The proceedings of these symposia, which were compiled and edited by GNR, contained landmark papers by the various attendees and are referred to even today.

GNR had a long term association with the Department of Biophysics at the University of Chicago, spanning the period between 1967 and 1978. His research at Chicago was facilitated by a grant from the National Institutes of Health (NIH), USA and he did some seminal work on 3-D reconstruction of an object from two-dimensional shadowgraphs of multiple slices, leading to yet another landmark paper in a different area, this time in the *Proceedings of the National Academy of Sciences, USA*. The theory outlined in this paper is the basic principle behind magnetic resonance imaging (MRI) and CAT scan technique, now used routinely in clinical diagnosis.

In 1971, GNR returned to the IISc, Bangalore from where he had started his scientific odyssey. He started the Molecular Biophysics Unit and continued his research on protein structure and conformational variability, while also building up the department at IISc.

In the late 1970s, GNR became very interested in understanding the molecular basis of diseases, particularly the role of collagen in various connective tissue disorders such as arthritis. He had already proposed a hypothesis on the effect of vitamin C on immune response. Unfortunately, facilities were not available in India to carry out experimental research in these areas. He then started looking at mathematical aspects of biology and published several interesting monographs, particularly on the subject of *Syaad Nyaaya* or 'the doctrine of may be'. This concept also perhaps best sums up the life and scientific career of one of the brightest stars to shine in the Indian scientific firmament—who had many glorious achievements to his name, but with additional support, could have 'may be' accomplished so much more.

GNR was the recipient of several awards, such as the Watumul Memorial Prize, Srinivasa Ramanujan Medal, R.D. Birla Award and Jawaharlal Nehru Fellowship. GNR was also recognized for his contributions to Structural Biology by being elected a Fellow of the Royal Society of UK and the Fogarty Scholar International Medal of NIH, USA.

GNR passed away on 7 April 2001 after suffering for about a decade from Parkinson disease.

Structure of Collagen

A DETAILED X-ray study of collagen fibres obtained from different sources (namely, shark ray, rat tail tendon and kangaroo tail tendon) and a re-examination of the published wide-angle patterns indicate that the unit cell of collagen is hexagonal with $a = 12-16$ A. and $c = 9.5-9$ A., the actual values depending on the moisture content. The essential difference of this indexing from those reported earlier¹⁻³ is that the 2.86-A. meridional arc is here interpreted not as a true meridional reflexion, but as arising from the superposition of two close non-meridional reflexions. A calculation of the angular spread of the arc, using the tilt of the c -axis deduced from the spread of the equatorial reflexions, confirms this interpretation. Table 1 shows the good agreement between the calculated and observed spacings of an air-dried and a wet specimen from kangaroo tail tendon.

A structure has been obtained (Fig. 1) which fits the above unit cell and which appears to be in good agreement with infra-red, X-ray and chemical data for collagen. It consists of nine amino-acid residues per unit cell, which corresponds to the observed density. These are linked together to form cylindrical rods, which occur in a hexagonal array. All the residues have the *trans* configuration, and the latest values of Corey and Pauling⁴ for the dimensions of the amide group were used for the calculations. The residues are arranged in the form of three helical chains, each of pitch 9.5 A. ($= c$) and containing three residues per turn, with the symmetry 3_1 . The three helices are also arranged with a 3_1 symmetry about the c -axis, and they are held together by means of hydrogen bonds to form the cylindrical rods. Two of the three NH groups in each turn of a chain are linked by hydrogen bonds to an oxygen of each of the other two chains, the NH...O distance being 2.80 A. The third NH group points outward from the cylinder, and the nitrogen atom forms part of a proline ring. Of the three α -carbon atoms per turn, a hydrogen attached to one of them (*R*, Fig. 1) could be replaced by a general *R* group to form an amino-acid residue such as arginine or lysine; another (*P*) takes part in forming the proline ring, while the third (*G*) is in such a position that there is no space for either of its hydrogens to be replaced by

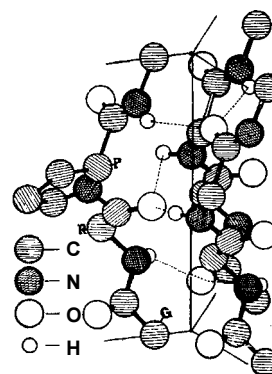


Fig. 1. Diagram showing one of the three-chain cylindrical rods of the structure. The dotted lines indicate the positions of the hydrogen bridges. Only one proline ring is shown, and all hydrogen atoms except those in the NH groups are omitted. The thin lines indicate the directions of the crystal axes.

any other group, so that it could only form part of a glycine residue. These features of the structure could explain the observed range of amino-acid composition for collagen.

The NH- and CO-bonds are almost exactly perpendicular to the fibre axis, the angle made with the c -axis being about 85° in both cases. This agrees with the observed large infra-red dichroism⁵. Structure-factor calculations show fairly good agreement with observation. The individual helices are unstable, and the stability of the cylindrical rods arises from the hydrogen bonds between the helices. If these are broken, for example, by heating, the structure would crumble down, which would explain the thermal contraction of collagen. The structure could, however, re-form on cooling, as the chain of amino-acid residues in a single helix need not be ruptured in this process.

It may be mentioned that this structure is essentially different from the three-chain structure of Pauling and Corey². The value $c = 9.5$ A. is not critical, and a variation of about 15 per cent is permissible without affecting the main features of the structure.

A detailed paper, containing also a discussion of other features of this structure and a critical comparison with previously proposed structures, will be published elsewhere.

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Table I.

Indices	Dry $a = 13.3, c = 9.55$ A.		Wet $a = 15.0, c = 9.20$ A.	
	obs.	calc.	obs.	calc.
100	11.4	11.5	12.9	13.0
200	5.75	5.75	6.5	6.5
210	4.3	4.34	4.8	4.92
101	7.4	7.35	7.6	7.50
201	4.8	4.92	—	4.46
112	3.9	3.87	3.95	3.93
212	3.3	3.22	3.4	3.36
113	2.86	2.87	2.82	2.84
001	—	9.55	9.2	9.20

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Structure of Collagen

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A STRUCTURE was proposed for collagen by us about a year ago¹. Although it explains the features of the infra-red spectrum and the chemical composition of collagen, it appears to be defective in that it disagrees with the X-ray data for stretched collagen² and in having too large an angle between the NH and N . . . O directions in hydrogen bonds (Pauling, L., personal communication). It is found that a small modification of the earlier structure, which preserves many of its features, fits the above evidence on collagen quite well. In addition, it also explains a number of other properties of collagen, in particular, the important role of hydroxyproline in this protein and the periodicity of 640 Å. along the fibre axis.

The new structure (the projection of which along the fibre axis is shown in Fig. 1) is topologically very similar to the earlier one in that it consists of triple chains of amino-acid residues, each chain being itself a helix. However, the chains now form coiled coils, instead of being arranged with their axes parallel to the fibre axis. Thus, every third α -carbon atom (corresponding to the G-type of carbon atom of the earlier structure) is placed on the surface of a cylinder of radius 1.0 Å., the successive ones being displaced (in unstretched collagen) by 8.58 Å. along the axis of the cylinder and rotated through an angle of 36° about the axis. The single coiled coil repeats itself after thirty residues, and the repeat distance along the fibre axis is 85.8 Å. The two other chains occur in such a manner that the three chains are

symmetrically disposed with respect to one another. Their configuration is most easily described by saying that they are displaced along the fibre axis by + 28.6 Å. and - 28.6 Å. with respect to the first chain. They are also related to the latter by a rotation of $\pm 108^\circ$ about the axis of the cylinder and a translation of ± 2.86 Å. parallel to the axis. For the mathematical discussion of the structure, each chain may be described as consisting of a minor helix (following the nomenclature of Crick³) having ten residues in three turns, with a projection of 2.91 Å. per residue along its axis, wound to form a major helix of radius 2.5 Å., there being thirty residues per turn of the major helix. The major helix is wound in a direction opposite to that of the minor helix, the latter being a left-handed screw if all the residues are of the L-type. Thus, while the thirty residues make nine turns in the co-ordinate system of the minor helix, they make ten turns in the fixed co-ordinate system of the major helix; this ensures that every third residue is similarly situated with respect to the fibre axis.

Because of the above relationships, there are only two types of inter-chain hydrogen bonds, marked A and B in Fig. 1, which are repeated by screw symmetry. In both bonds, the N . . . O distance is close to 2.9 Å. and the angle between NH and N . . . O directions is about 30° . This angle would be less if the NH-bond were not coplanar with the rest of the amino-acid residue.

As in the earlier structure, two of every three NH groups are hydrogen bonded to an O of a CO group, but unlike it, the two oxygens to which they are linked are different. Thus, of every three carbonyl oxygens, only one does not take part in internal hydrogen bonding in the triple-chain cylindrical rods. This

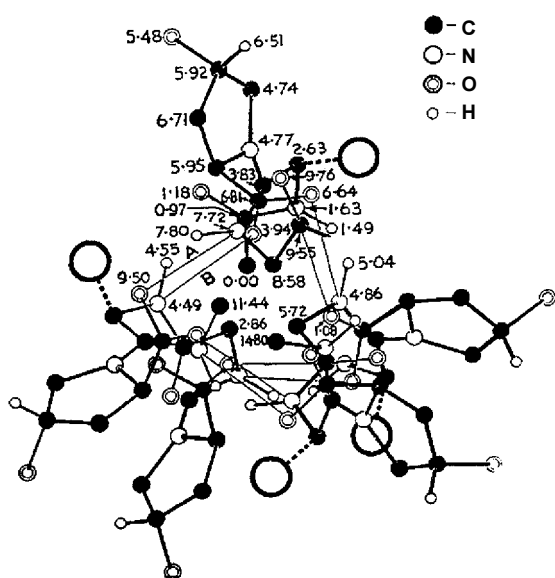


Fig. 1. Projection of the proposed structure along the fibre axis. The numbers denote the heights of the atoms above the plane of projection. Only a part of each of the three individual chains is shown. The thin lines indicate the N . . . O directions in the hydrogen bonds

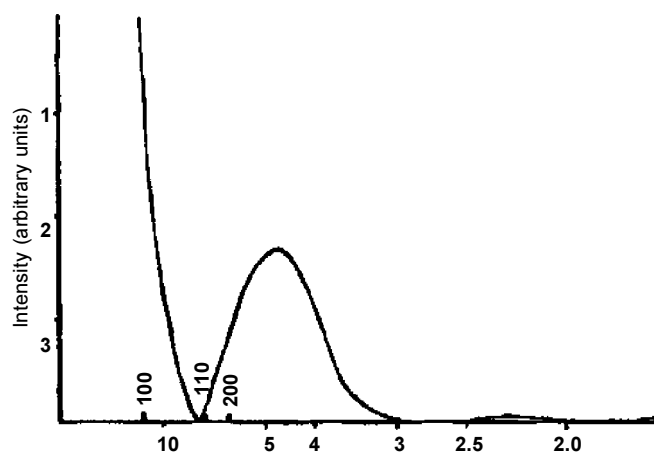


Fig. 2. Radial intensity distribution along the equator calculated from the structure. The abscissae are proportional to the reciprocal of the spacing. The positions corresponding to the 100, 110 and 200 reflexions of the hexagonal unit cell with $a = 12$ Å. are also marked

oxygen points outward from the rod and plays an important part in the cross-linking of the rods by side-chains, as shown below. As before, every third residue must be a glycine residue, and similarly a maximum of one-third of the residues may be proline or hydroxyproline.

The essential difference introduced in the structure, so far as its X-ray pattern is concerned, is that the 2.86 Å reflexion is meridional, and is the only meridional reflexion apart from its higher orders. Further, the exact repetitive unit along the fibres axis is ten residues (28.6 Å.), which agrees well with the observed layer-line spacings⁴. The intensity distribution among the spots on the layer lines has been found to agree with the structure qualitatively. The distribution of intensity along the equator has been worked out in detail and is shown in Fig. 2. This is in good agreement with observations (Ramachandran, G. N., and Ambady, G. K., unpublished results), particularly with regard to the large peak at 4.7 Å. and the minimum at 7.4 Å. The absence of the expected spot at 7 Å. is thus explained. In wet collagen, the water would be expected to occur between the cylindrical rods and, under these conditions, the minimum shifts towards smaller angles and continues to occur in the region of the unobserved reflexion of 7–8.5 Å., even though the lattice expands. Further, the three-dimensional cylindrical Patterson diagram for collagen published recently by Yakel and Schatz⁵ is in excellent agreement with the structure. The peak at about 1 Å. in the level $z = 0$ is due to the projected component of bonds joining neighbouring atoms perpendicular to the fibre axis; that at 2.7 Å. is due to the component of the almost horizontal hydrogen bond interactions; the strong one at 4.5 Å. is due to the distances between the three approximately parallel amino-acid residues at the same level.

The recent infra-red data⁶ of Sutherland *et al.* are also in agreement with the present structure. The expected dichroism of the non-coiled 3–10 helix agrees with the observed data⁷, and the further coiling of this helix produces only small changes and the results are not materially different.

The distribution of the proline and hydroxyproline rings can be calculated exactly, and they are found to be such that the oxygen of hydroxyproline is nearly as far away from the axis of the cylinder as is possible. Thus internal hydrogen bonding of the OH in hydroxyproline is ruled out. Rather, these groups take part in linking the cylindrical rods with one another; the hydroxyproline OH group in one rod is nearly at the same level as the exposed non-hydrogen-bonded carbonyl oxygen of the neighbouring rod, so that they could readily be linked by a hydrogen bond. This is in agreement with the results of Gustavson⁸ on the nature of the hydroxyproline OH...O bond. Assuming these bonds are 2.7 Å. long, the distance between the centres of neighbouring rods works out to be 11.6 Å., which is close to 12 Å. (10.4/0.866) found for well-dried collagen⁹. The theoretical value is not precise, for it would change if the bond angles in the amino-acid

residue and the α -carbon atoms are altered by even a small amount.

If the coiled coils described above are simply put together in a hexagonal array, then only some of the hydroxyproline OH groups are bonded to carbonyl oxygens of neighbouring rods. However, it is found that a very slight change in the pitch of the major helix is sufficient to make the number of hydroxyproline-carbonyl hydrogen bonds a maximum. Instead of a rotation angle (say ϕ) of 36° per three residues about the axis of the major helix, the angle must be changed to 35°. This immediately introduces a 6₁ symmetry for the major helix, which now repeats after 216 residues (618 Å.). The spacing 618 Å. is highly suggestive of the 640 Å. spacing found in the small-angle X-ray pattern and in electron micrographs of collagen. The condition for stabilization of the structure by forming the maximum number of hydroxyproline cross-links thus automatically leads to the long spacing of collagen. Further, on working out the number of hydroxyproline residues which occur in such a position that the hydrogen bonds are fairly short, the number is found to be twelve for every thirty-six of the *P* type residues. The percentage of hydroxyproline residues occurring in collagen is variable with the source of the material⁸; but if we assume that the hydroxyproline residues which actually occur are all hydrogen-bonded, then the maximum proportion of hydroxyproline residues should be 11 per cent. It is noteworthy that the maximum observed (namely, in bovine collagen) is about 10 per cent. Further, the correlation of the shrinkage temperature with hydroxyproline content reported by Gustavson⁸ is also to be expected if hydroxyproline is mainly responsible for the cross-linking of the cylindrical rods. When collagen shrinks, the individual chains in the triple chain are not likely to be separated, but the triple chain itself would take up a highly folded configuration. The X-ray pattern of gelatin, in fact, supports this. The detailed evidence will be discussed elsewhere.

Having fixed the configuration of the rods with reference to hydroxyproline cross-linkages, a consideration of the length of the *R* side-chain of the third residue needed to form hydrogen-bonded linkages between one rod and another shows that it is variable from 5.5 to 7.5 Å. In view of this, there must be a periodic distribution of the *R*-type residues. The details are not easy to work out, but an interesting result emerges, namely, that the distribution of *R*-groups along the fibre axis will not have a period of 618 Å., but one-sixth of this. It is indeed noteworthy that electron micrographs of collagen stained with phosphotungstic acid exhibit¹⁰ such a six-fold subdivision of the gross period of 640 Å.

The agreement of 618 Å. with 640 Å. observed for dry collagen is not close enough, nor is the six-fold subdivision exact¹¹. However, it must be mentioned that it is not at all certain that the unit cell of collagen is hexagonal. It is probably monoclinic even in the dry state with slightly differing *a* and *b* (taking *c* as monoclinic axis), as is found to be the case with wet collagen¹². The subdivision of 618 Å. would then not be

exactly into six equal parts, and further the angle ϕ may be different from 35° and the macro-period may also differ from 618 Å. The variation of the long spacing with moisture⁹ is to be attributed to the changes in the angle of the monoclinic cell.

Further details of this work are reserved for a separate communication.

June 3

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Collagens are the most abundant proteins in the human body, constituting about 30% of its protein mass. It is now well established that collagens and proteins with collagen-like domains form large superfamilies in many species and are associated with several connective tissue-related disorders at the molecular level. Hence, elucidation of the molecular structure of this protein was an important event. In two landmark papers, published in *Nature* in 1954 and 1955, G. N. Ramachandran's group from Madras (now Chennai) outlined, for the first time, the correct molecular structure for collagen. In the first paper, a triple helical model was proposed consisting of an assembly of 3 parallel helical chains, each with 3 residues per turn. One critical feature of this structure was the requirement that one-third the total number of amino acid residues should be glycine.¹ An additional feature was its ability to accommodate a large proportion of amino acid residues (viz. proline and 4-hydroxyproline). This unique triple helical structure was stabilized by inter-chain hydrogen bonds. Every third position in the structure, which lies towards the centre of the triple helix cannot have any side chain attached to it, since presence of even a β -carbon atom (as in alanine) leads to unacceptable inter-chain atomic contacts. Hence, this position must necessarily have only glycine residues, thus providing a rational explanation for the unique amino acid composition and triplet repeat sequence (-Gly-X-Y-) of collagen.

The slightly modified structure proposed by Ramachandran and Kartha, in the second paper, published in 1955, introduced the concept of a rope-like coiled-coil triple-helix, with 10 residues in 3 turns of each chain.² In this structure the requirement for glycine at every third position is even more stringent. In addition to inter-chain hydrogen bonds between the peptide N-H and C=O groups, tightly bound water molecules as well as the hydroxyl groups of hydroxyproline residues, present at the Y position of the repeating triplet sequence can stabilize the structure,³ thus providing an explanation for the observed correlation between the stability and hydroxyproline content of various collagens. Recent experimental studies have confirmed that the Ramachandran-Kartha triple-helical structure is essentially correct, except for some minor differences in the inter-chain hydrogen bond geometries.

The individual triple helices or tropocollagen molecules, as they are sometimes called, are arranged to form fibrils which are of high tensile strength and flexibility and can be further assembled and cross-linked so as to support stress

efficiently. Imperfections or abnormalities in the collagen molecular structure or its organization into mature fibres lead to different diseases associated with connective tissues. For example, perturbation in the proper distribution of charged amino acids in the chains causes loose packing of fibres, affecting their tensile strength and leading to diseases such as Ehlers-Danlos syndrome, osteogenesis imperfecta and some types of osteoporosis and dentinogenesis imperfecta. Missing Gly/X/Y in the standard repeating sequence can cause kinks or bends in the structure. Most common mutations in the collagen gene are single base substitutions that convert the codon of the critical glycine residue to that of a bulkier residue, which causes considerable distortion of the triple helix or even prevent its formation beyond this point. Amino acid changes in the other two positions of the triplet have milder effects. The sites of two mutations in type II collagen, one leading to achondrogenesis-hypochondrogenesis and the other to spondyloepiphyseal dysplasia congenital are both shown to lead to local destabilization of the triple helix.

Interestingly, mutations that produce some structural alterations in the polypeptide chain but still allow the chains to assemble into a triple helix, generally manifest as more severe phenotypes than those that prevent triplex formation altogether. This is because the triple helices containing the mutated chain will have an abnormal structure, which will affect the formation of higher order structure or alter their assembly and function. Thus, while the exact relationship between an alteration in the amino acid sequence and the structure and lethality of a mutation in the collagen molecule is still not clear, a knowledge of the collagen molecular structure, first proposed by G. N. Ramachandran, has helped considerably in comprehending collagen behaviour *in vivo* and in attempts to find cures for collagen-related diseases.

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