CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

All the reagents and chemicals used were of reagent grade and sourced from Sigma Chemicals Co., USA, E.Merck India Ltd., India, Electron Microscopy Sciences, USA, LADD Research Inc., USA and SRL India Ltd., India.

The reagents used were Ammonium persulfate (APS), chloramine-T, collagenase (EC 3.4.24.3) Type I, Coomassie brilliant blue G-250, Dowex 50W-X4, Sephadex SPC-25, N-ethyl-maleimide (NEM), phenylmethylsulfonylfluoride (PMSF), 2-furanacryloyl-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA), poly L-glutamic acid (Sigma Chemicals Co.), bromophenol blue, methyl cellosolve, p-dimethylaminobenzaldehyde (PDAB), methanol, acetic acid, perchloric acid, sulphuric acid and nitric acid (E.Merck India Ltd.), acrylamide, N,N'-methylene bisacrylamide, N,N,N',N' tetramethyl ethylenediamine (TEMED), silver nitrate, tricine, N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulfonicacid) (Hepes) and tris-(hydroxymethyl) amino methane (Tris) (SRL India Ltd.), collodion (Electron Microscopy Sciences) and phosphotungstic acid (PTA) (LADD Research Inc.).
2.2 ISOLATION OF THREE SELECT CHROMIUM(III) SPECIES FROM BASIC CHROMIUM SULFATE

Although Basic Chromium Sulfate (BCS) is known to contain many species, for the present investigation three specific chromium(III) species have been selected. The structure and formulation of the species selected for the investigation has been shown in Figure 1.1. For reasons of convenience, they are referred to as dimer, 1, trimer, 2 and tetramer, 3. The preparation and characterisation of these species have been reported earlier and previously standardised methods have been employed.

2.2.1 Preparation of the Selected Dimer, 1

A stock solution of hexaaquachromium(III) perchlorate \([\text{Cr(H}_2\text{O)}_6\text{(ClO}_4)_3]\) was prepared by using standard methods (Thompson and Connick, 1981). This was done by reducing chromium trioxide (\(\text{CrO}_3\)) in perchloric acid with 30% hydrogen peroxide so that the excess of acid was approximately 0.3 M. Reduction of a deaerated aqueous solution of \(\text{Cr(H}_2\text{O)}_6^{3+}\) with zinc amalgam in a serum capped flask gave a solution of hexaaquachromium(II) perchlorate. The divalent chromium was later oxidised under air. It has been established previously that aerial oxidation of hexaaquachromium(III) perchlorate affords bis(\(\mu\)-hydroxo)octaaquadichromium(III) perchlorate (dimer). The dimer, 1 was separated from other impurities by using ion exchange chromatography on Sephadex SPC-25 column (Ardon and Plane, 1959). The spectrum of the dimer, 1, was recorded using a Shimadzu 160A UV-Vis spectrophotometer. The molar absorption coefficient values of the species isolated in this investigation (\(\varepsilon = 16.8\) and 19.3 M\(^{-1}\) cm\(^{-1}\) at 581 and 416 nm respectively) is in satisfactory agreement with the previously reported values (\(\varepsilon = 17.4\) and 20.4 M\(^{-1}\) cm\(^{-1}\) at 582 and 417 nm respectively) at 25°C.
2.2.2 Preparation of the Selected Trimer, 2

An aqueous solution of trimer, 2 was also prepared from hexaaquachromium(III) (Spiccia et al., 1988). To a solution of hexaaquachromium(III) (0.5 M, 5 mL) in acid (ca 0.66 M perchloric acid), sodium hydroxide (2 M, 10 mL) was added with continuous stirring. The resulting solution was acidified with perchloric acid (2 M, 35 mL) and the solution placed in a water bath at 25°C for a period of 30 h. It was then diluted ten fold to obtain a chromium(III) concentration of 4 x 10^{-3} M and [H\(^+\)] of 0.1 M and adsorbed onto a Sephadex SPC-25 cation exchange column (20 x 1.5 cm). A standard frontal elution technique has been employed to separate the chromium(III) trimer, 2 from other impurities. The cation exchange resin was saturated with trimer by continuous application of a mixture of chromium(III) species until the molar absorption coefficient of the resin is exceeded and the characteristic bands of monomer and dimer were displaced by the trimer (and some higher oligomers). The trimer, 2 was then eluted with acidified 1.0-1.5 M sodium perchlorate (pH 1.7). The observed molar absorption coefficients at the wavelength of maximum absorption were 17.5 M\(^{-1}\)cm\(^{-1}\) and 29.1 M\(^{-1}\)cm\(^{-1}\) at 581.5 and 423.5 nm, respectively. This value compares favourably with the previously reported values of 19.2 M\(^{-1}\)cm\(^{-1}\) and 30.5 M\(^{-1}\)cm\(^{-1}\) at 584 and 425 nm respectively (Spiccia et al., 1988).

2.2.3 Preparation of the Selected Tetramer, 3

The preparation of the desired tetrameric complex has been previously reported. The tetramer, 3 was isolated from a solution of basic chromium sulphate (BCS) (Rao et al., 1997). Regenerated Dowex 50W-X4 resin in acid form (100-200 mesh, 3-6 g) was loaded onto a column of 1.5 cm diameter to a
height of 12 cm. The resin was washed free of acid. An aged solution of BCS adjusted to pH 3.0 (to avoid formation of polynuclear complexes on hydrolysis) was loaded onto a Dowex 50W-X4 column. The flow rate was adjusted to 1 mL/min. After sufficient absorption of chromium at the top of the column, the column was washed with dilute hydrochloric acid (0.001 M). Elution was done with solutions of increasing strengths of hydrochloric acid, viz. 1 M to 3 M. The lower charged species were eluted with 1 M and 2 M hydrochloric acid and the fraction collected during elution with 3 M hydrochloric acid was tetramer. The molar absorption coefficient was calculated (16.5 M$^{-1}$cm$^{-1}$ at 582 nm and 25.5 M$^{-1}$cm$^{-1}$ at 425 nm) and compared with previously reported values (17.0 and 27.0 M$^{-1}$cm$^{-1}$ at 578 and 426 nm respectively) (Rao et al., 1997).

2.2.4 Preparation of Basic Chromium Sulfate Solution

A solution of basic chromium sulfate was prepared by dissolving 8g BCS in 100 mL of water (sourced from Golden Chemicals Ltd., India). This was used for various tanning and related studies.

2.3 Desalting of Solutions of Isolated Chromium(III) Species

Since ion exchange techniques had been employed for the separation and isolation of the three select species of chromium(III), desalting of solutions prior to investigations made in this study was essential. For this, the pH of solutions of different chromium(III) complexes was adjusted to a pH of 3.5 using sodium bicarbonate with constant stirring and cooling to prevent hydrolysis of the species. Solutions were desalted by passing through a Biogel-P2 column. The chromium(III) adsorbed on the column was then eluted with water and the first washings were collected. The desalted chromium(III)
species were collected and stored at less than 4°C to prevent hydrolysis. Chromium concentrations of the individual species were then estimated by standard procedures (Haupt, 1952).

2.4 DETERMINATION OF CONCENTRATION OF CHROMIUM IN SOLUTIONS

The concentration of chromium(III) in solutions was determined by using a previously known method without any modifications, after suitable calibration (Haupt, 1952). This method is based on the analysis of chromium as $\text{CrO}_4^{2-}$ after oxidation by alkaline peroxide method. Molar absorption coefficient of $4.82 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ at 372 nm was employed to calculate the concentration of chromium(VI).

2.5 ESTIMATION OF TOTAL IONIC STRENGTH OF SOLUTIONS OF CHROMIUM(III)

Total ionic strength of solutions of chromium(III) complexes was estimated. A known volume of the desalted chromium(III) species was pipetted out and loaded on a Dowex 50W-X4 column free of acid. The amount of hydrogen ions displaced by the chromium(III) species was estimated by titration with standard alkali. The ionic strength of the species was calculated from milliequivalents of alkali consumed for titration.

2.6 TREATMENT OF RAT TAIL TENDON (RTT) WITH SELECTED AND ISOLATED COMPLEXES OF CHROMIUM(III)

Tendons were teased from tails of six month old male albino rats (Wistar strain). The teased tendons were washed extensively in 0.9% saline and
stored in a freezer. The total ionic strength of desalted solutions of chromium(III) species was so adjusted that it was maintained at 0.9 M (3.33 M lithium perchlorate).

2.6.1 Tanning of RTT with Isolated Chromium(III) Species and BCS

Solutions of basic chromium sulfate and selected chromium(III) species of known concentration and ionic strength were prepared. Rat tail tendons were treated independently with BCS, dimer, 1, trimer, 2 and tetramer, 3, for 8 h at 30°C after washing as described above. The solution was then basified with 1% sodium bicarbonate to a pH of 3.8-4.0. The tendons, after basification were washed thoroughly with distilled water and stored in a refrigerator.

2.6.2 Tanning of RTT with Mixture of Chromium(III) Species

2.6.2.1 Dimer, 1 and trimer, 2

Solutions of chromium(III) dimer, 1 and trimer, 2, of the same concentration (0.01 M) and ionic strength (0.9 M) were mixed in the ratio of 2:1. RTT were treated with the solution for 8 h. The solution was basified with 1% sodium bicarbonate to a pH of 3.8-4.0. The tendons were then washed thoroughly with distilled water after the completion of basification and stored in a refrigerator. The same experiment was repeated with a mixture of dimer, 1 and trimer, 2 in the ratio of 1:1.

2.6.2.2 Dimer, 1 and tetramer, 3

Solutions of chromium(III) dimer, 1 and tetramer, 3 of the same concentration (0.01 M) and ionic strength (0.9 M) were mixed in the ratio of
2:1. RTT after washing as above were treated with the solution for 8 h and the solution was basified with 1% sodium bicarbonate to a pH of 3.8-4.0. The tendons after tanning and basification were washed thoroughly with distilled water and stored in a refrigerator. The same experiment was repeated with mixture of chromium(III) dimer, 1 and tetramer, 3 in the ratio of 1:1.

2.6.2.3 Trimer, 2 and tetramer, 3

Solutions of chromium(III) trimer, 2 and tetramer, 3 of the same concentration and ionic strength were mixed in the ratio of 1:1. Native tendons after washing as above were treated with the solution of chromium(III) for 8 h. The solution was basified with 1% sodium bicarbonate to a pH of 3.8-4.0. The tendons after tanning and basification were washed thoroughly with distilled water and stored in a refrigerator.

2.7 TREATMENT OF TANNED RTT WITH UREA

Samples of RTT treated with various chromium(III) species, described as above in Section 2.6.1 were treated with increasing concentrations of urea, viz., 2 M, 4 M and 6 M for 6h. The fibres were washed with water and subjected to evaluation. A control experiment was also done with native untanned RTT.

2.8 DETERMINATION OF CHROMIUM CONTENT OF TANNED RTT BY INDUCTIVELY COUPLED PLASMA TECHNIQUE

Accurately weighed samples of Cr(III) tanned collagen fibres were digested in acid mixture containing nitric and sulphuric acid in the ratio of 1:1
and made up to a known volume. Perchloric acid usually used for digestion was avoided to prevent damage to the plasma. The mixture was digested on a hot plate till the solution was clear of any suspended organic matter to ensure complete digestion. The samples were then estimated by ARL 3410 ICPAES, which had been previously calibrated for chromium.

2.9 DETERMINATION OF SHRINKAGE TEMPERATURE

The shrinkage temperature of skin or leather was measured using either visual micro-scale determination or differential scanning calorimetric techniques. Both methods are based on the principle that the thermal shrinkage of collagenous matrices and materials can be measured by monitoring the heat induced dimensional changes and phase transitions. The deformation of collagen is apparent as the shrinkage of the fibres, and the temperature interval where shrinkage takes place is a measure of the hydrothermal stability of these fibres.

2.9.1 Microshrinkage

The temperature at which the length of a collagen fibre shrinks to approximately one-third its original length is noted as the shrinkage temperature in this method (Borasky and Nutting, 1949).

Rat tail tendons treated with chromium(III) species (samples from Sections 2.6.1 and 2.6.2), were tested for their hydrothermal stability. The effect of chemical (urea) treatment on the hydrothermal stability was also studied (Section 2.7). About 1 mm of RTT was cut and placed in the groove of a microscopic slide. Few drops of water-glycerol mixture were added to the
groove so that the sample was completely immersed in the medium. The groove was covered with a cover slip. The slide with the sample was placed on an electrically heated metallic disc carrying a thermometer. The disc was in turn mounted on a tripod. A vertically held microscope over the tripod was used to view the sample with the aid of a light source placed beneath the tripod. The changes in dimension of the sample on heating were viewed. The temperature at which shrinkage of the fibre occurred was noted as the shrinkage temperature.

2.9.2 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry is a useful technique for characterisation of parameters like melting point, glass transition temperature and thermal degradation of biopolymers. Principles and procedures involved in the general application of DSC methods for collagen related substances have been presented in Appendix A2 for purpose of documentation.

Generally, RTT (native and chromium tanned, 5 mg at around 60% moisture content) were subjected to DSC investigations by heating at preselected rates of 5°C/min employing standard procedures. A heat flux type TA Instrument model 2910 DSC instrument with 2000 series thermal analyst was employed. Nitrogen gas was purged at a rate of 50 cc/min. The differential heat flow to the sample and reference was monitored using a chromel-constantan area thermocouple. Temperature range employed for the measurements was generally in the range of 25-250°C. Heat flux was measured to a sensitivity of 0.01 mWcm⁻¹.
Uniformly blotted samples of RTT were weighed and used. The fibres were encapsulated in an aluminium hermetic sealed pan. Transition temperatures and enthalpy changes were recorded.

2.10 COLLAGENASE HYDROLYSIS OF CHROMIUM(III) SPECIES TREATED RTT

The enzymatic degradation of native and chromium(III) treated rat tail tendon by collagenase was analysed by 1) estimating the amount of hydroxyproline released in solution after hydrolysis and 2) polyacrylamide gel electrophoresis of the hydrolysates, at different time intervals. The samples for the analysis were prepared as given below.

2.10.1 Collagenase Hydrolysis of Chromium(III) Treated RTT

The Cr(III) species treated RTT from Section 2.6.1 were tested for their resistance to collagenase hydrolysis by the method of Stark and Kuhn (1968).

Equal amounts of RTT treated with the Cr(III) complexes and native untreated collagen fibres were homogenised at 4°C in sample buffer containing 0.1 M Hepes, 0.002 M phenylmethanesulfonylfluoride, 0.01 M N-ethyl maleimide (pH 7.4) and made up to a known volume (0.5 mL). Type I collagenase from *Clostridium histolyticum* was used for enzymatic hydrolysis of collagen. The activity of the enzyme used was 125 collagen units/mg solid. Collagenase (about 10 mg) was dissolved in buffer containing 0.33 M calcium acetate, 0.025 M Tris-HCl (1 mL) at pH 7.4 at 0°C to prepare a stock solution. About 0.1 mL of the enzyme stock solution, was added to the collagen samples.
The ratio of collagen:collagenase was 100:1. After addition of enzyme the reaction was allowed to proceed at 37°C. Aliquots of 40 μL of supernatant were withdrawn after centrifuging at 10,000 rpm for 10 min. Samples were taken at intervals of 2 h, 4 h, 8 h, 10 h, 12 h, 14 h, 16 h, 24 h, 36 h, 48 h, 72 h and 96 h and stored in a freezer.

2.10.1.1 Monitoring collagenase promoted hydrolysis of collagen

The collagenase digests were hydrolysed in sealed hydrolysis tubes with 6N HCl for 16 h. The hydrolysates were evaporated to dryness in a porcelain dish over a water bath to remove excess acid. The residue, free of acid, was made up to a known volume and the hydroxyproline content was estimated according to the method of Woessner (1961) given under Appendix A1.

2.10.1.2 SDS-polyacrylamide gel electrophoresis of collagen hydrolysates

RTT (native and chromium(III) species treated) were subjected to enzymatic hydrolysis of collagenase and the resulting hydrolysates were subjected to SDS-PAGE investigations. Samples for gel electrophoresis were prepared by the method of Berry and Shuttleworth (1988). Sample buffer consisted of 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue, 2 M urea and 5 mM CaCl2. To a weighed amount of fibre, sample buffer (0.5 mL) was added. Activity of collagenase used was 125 collagen units/ mg solid. Collagenase (about 10 mg) dissolved in 1 mL buffer (0.1 M calcium acetate, 0.025 M Tris-HCl, pH 7.4), at 0°C was used for hydrolysis. RTT samples were incubated at 37°C with the enzyme. The ratio of collagen:collagenase was maintained at 100:1. Aliquots of 40 μL were withdrawn at intervals of 2 h, 4 h, 8 h, 24 h and 48 h. In the case of control
(native collagen), hydrolysates were pipetted out at intervals of 10 min, 20 min, 45 min and 60 min. All the samples collected above were then subjected to SDS-PAGE using standard methods (Laemmli, 1970). For purpose of completeness of documentation, the standard procedures of SDS-PAGE are presented in the Appendix A3.

2.11 PREPARATION AND PURIFICATION OF SOLUBLE COLLAGEN

Collagen was extracted and purified from rat tail tendons as described earlier (Chandrakasan et al., 1976; Miller and Rhodes, 1982). Tendons teased from tails of six month old albino rats of Wistar strain were washed in cold 0.9% saline. All subsequent procedures were performed at 4°C. The tendons were stirred overnight in 0.5 M acetic acid. Protease inhibitors like N-ethyl-maleimide (2 mM) and phenylmethanesulfonylfluoride (PMSF) (1 mM) were added to the mixture. The solution was then filtered and centrifuged at 50,000g for 40 min and the residue discarded. The solubilised collagen in the supernatant was precipitated with slow addition of 5% (w/v) NaCl and left overnight. The precipitated collagen was then recovered by centrifugation at 35,000g for 30 min. The residue was redissolved in 0.5 M acetic acid by stirring overnight. The solution was centrifuged at 50,000g for 40 min and the supernatant collected was dialysed extensively against 0.02 M disodium hydrogen phosphate buffer with 2 to 3 changes of buffer each day. After dialysis, the precipitated collagen was centrifuged at 35,000 g for 30 min and the precipitate redissolved in 0.5 M acetic acid. The dissolved collagen in 0.5 M acetic acid was centrifuged at 50,000g for 40 min and the supernatant was dialysed against 0.05 M acetic acid. After dialysis the solution was centrifuged at 50,000 g for 40 min and the purified collagen was stored at 4°C.
2.12 INVESTIGATION OF CIRCULAR DICHROISM ON COLLAGEN SAMPLES

Circular dichroism spectra of soluble collagen and collagenase samples were investigated at 25°C using J715-Jasco spectropolarimeter. The rate of nitrogen purging was maintained at 5 L/min. A 2-point calibration was done with (+)-10 camphorsulfonic acid. The samples were prepared in double distilled water. Reagents used were of analytical grade. All the solutions were filtered through 0.25 μm filter to remove suspended particles. A scan speed of 20 nm/min was used with an average of 5 scans per sample. A slit width of 1 nm and a time constant of 0.5 msec were used. Both 1 cm and 0.1 cm cells were used for the experiments. Each spectrum was corrected by a baseline measured with the same solvent used in the sample. The intensity of the CD spectra was expressed as Δε per amide band. The mean residue ellipticity was calculated using mean residue weight of the protein samples used.

2.12.1 Binary System

2.12.1.1 Collagen-Cr(III) complexes

Collagen in solution (0.05 M acetic acid) as prepared under Section 2.11 was dialysed against a 12,000 MW cut off dialysis tubing against 5 mM acetic acid overnight at 4°C, with two changes of dialysate, to remove small peptide degradation products and to ensure an equilibrium solvent composition. The solution was then centrifuged at 100,000g for 1 h in an ultracentrifuge to sediment large aggregates. The supernatant from this centrifugation contains soluble collagen. The collagen content was estimated by the procedure of Woessner (1961) given under Appendix A1. The mean residue ellipticity (θ_mew) was calculated using the formula as in equation 2.1.
where \( \theta_{mrw} = \frac{[\theta]MRW}{100} \) (2.1)

Stock solutions of different Cr(III) complexes \( \text{viz.} \) BCS, dimer, 1, trimer, 2, and tetramer, 3, of 5 mM Cr(III) concentration were prepared and filtered through 0.25 \( \mu \)m filter. Final Cr(III) concentration increasing in steps of 100 from 0 \( \mu \)M to 500 \( \mu \)M were used for the study. To minimise the effects of ionic strength on the conformation of the protein, different volumes of 0.33 M lithium perchlorate was added to all the samples. Collagen samples (0.2 mg/mL) were incubated with different concentrations of the various Cr(III) complexes \( \text{viz.} \) BCS, dimer, 1, trimer, 2 and tetramer, 3 and incubated at 25°C for 18 h. After incubation the spectrum was recorded. A reference spectrum containing acetic acid and lithium perchlorate was also recorded. All spectra were then subtracted from the reference. Spectrum of soluble (native) collagen was recorded under similar conditions. A standard subroutine for spectral smoothing and reduction of noise was employed. Spectra are expressed in terms of mean residue ellipticity (\( \theta_{mrw} \)) using mean residue weight of 91.2 (Piez and Sherman, 1970).

2.12.1.2 Studies on polyglutamic acid-chromium(III) interactions

L-PGA (0.3 mg/mL) in 1 mM acetate buffer was incubated with varying concentrations (0 to 250 \( \mu \)M) of BCS, dimer, 1, trimer, 2 and tetramer, 3 and the spectra were recorded. Lithium perchlorate was added to minimise ionic strength variations. Reference spectrum was recorded with acetate buffer and lithium perchlorate and later subtracted from spectra of samples. The
average molecular weight of the peptide was taken as 71,700 Daltons. Spectra were expressed in terms of mean residue ellipticity ($\theta_{\text{mrw}}$).

2.12.2 Deconvolution of CD Spectra

The prediction of secondary structure for proteins from CD data has become an established method in recent years. Characterisation of CD spectral profiles for each of the three secondary structure components viz. $\alpha$-helix, $\beta$-sheet and random coil have been reported (Hennesey and Johnson, 1981; Manavalan and Johnson, 1985; Johnson, 1990). In principle, a protein consisting of these elements would therefore display a composite spectrum from which the three individual contributions to CD spectra can be deconvoluted. The simplest method of extracting secondary structure from CD data is to assume that a spectrum is a linear combination of CD spectra of each contributing secondary structure type viz. pure $\alpha$-helix, pure $\beta$-sheet etc. Several methods have been developed which analyse the experimental CD spectra using a database of reference protein CD spectra containing known amounts of secondary structure (Provencher and Glockner, 1981; Hennesey and Johnson, 1981; Manavalan and Johnson, 1987; Sreerama and Woody, 1993). In this study four programs have been used for deconvolution viz. G&F, K2D, LINCOMB and MLR (Perczel et al., 1992; Andrade et al., 1993). Mean values of secondary structure components obtained from these methods have been reported.

2.13 PREPARATION OF RECONSTITUTED COLLAGEN FIBRILS

For in vitro fibril formation the collagen solution was ultracentrifuged at 100,000g for 60 min. Collagen fibrils were reconstituted from
0.5% collagen (after ultracentrifugation) by mixing with 0.2 M phosphate buffer and 2 M NaCl in an ice bath (Rajaram and Chu, 1990). The pH of the solution was adjusted to 7.4 with 1.25 N sodium hydroxide. The final concentration of the constituents in the mixture is collagen, 3.3 mg/mL, phosphate buffer, 0.02 M and NaCl, 0.13 M. The temperature was slowly raised to 30°C. The collagen gel formed was disrupted and centrifuged. The fibrils, which settled down, were washed extensively with cold distilled water to remove buffer salts.

2.14 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Collagen exhibits a characteristic periodic banding pattern reflective of the quarter staggered arrangement, which is observed under a transmission electron microscope.

2.14.1 Negative Staining for Control

In the negative staining method, collagen fibrils are stained with phosphotungstic acid (PTA) or uranyl acetate. The stain, gives rise to alternate dark and light bands arising out of the axial staggering of molecules. The pattern is explained in terms of charge distribution, bulkiness of the amino acid residue, which is the ratio between the molecular volume and length and hydrophobicity of the amino acids. Other electron rich stains are also known to stain collagen for EM. The use of heavy metals for staining has already been reviewed (Chapman and Hulmes, 1984).

2.14.2 Sample Preparation

Treated and untreated RTT were examined under TEM. After extensive washing with distilled water, RTT was disrupted under cold
conditions in a ground glass disintegrator. After suitable dilution with distilled water, a drop of the sample was placed on carbon coated collodion film (400 mesh grid). The control (untreated) sample was negatively stained with 1% phosphotungstic acid. Chromium (III) species treated fibrils were not stained as the electron dense chromium acts as a self staining agent. The samples on the grid were then examined with JEOL JEM 1200 EX II transmission electron microscope.

2.15 INFRARED SPECTROSCOPY OF COLLAGEN FILMS

2.15.1 Sample Preparation

Collagen, after reconstitution as mentioned under Section 2.13, was poured into a flat container and dried uniformly in air. Two or three layers of reconstituted collagen were poured and dried uniformly. This gives rise to films of uniform thickness. The dried collagen films were cut into squares of 1 cm x 1 cm each and treated with the different chromium(III) complexes for 24 h. The treated films were then washed thoroughly with water, dried and stored in a refrigerator. Prior to analysis, the films were conditioned for 24 h in a dessicator.

2.15.2 Fourier Transform Infrared Spectroscopic (FT-IR) Measurements

The IR spectra were recorded with a Nicolet Impact-400 IR spectrometer using a detector and Omnic data processing software. All spectra were recorded in the range of 2000-400 cm\(^{-1}\). A good signal to noise ratio was achieved over 100 accumulations with a resolution of 4 cm\(^{-1}\). The spectral
calibrations on the instrument had been made using polystyrene standard at regular time intervals. The numerical processing (FFT, smoothing, flattening and normalisation) of the IR data was carried out with a Omnic software package.

2.16 ATOMIC FORCE MICROSCOPY

An atomic force microscope can be used for real-time and high-resolution imaging of hydrated biological specimens ranging from single molecules to whole cells and tissues. High vertical resolution can be achieved. The axial periodicity of mature collagen fibrils has been resolved using AFM (Chernoff and Chernoff, 1992; Baselt et al., 1993; Revenko et al., 1994; Aragno et al., 1995; Raspanti et al., 1996; Hiyama et al., 1998; Lin et al., 1999).

2.16.1 Principle

The atomic force microscope provides topographic images of conducting and non-conducting surfaces by measuring the interaction forces between a sharp tip and the sample, thereby revealing the details of the area examined at a molecular or even atomic resolution.

2.16.2 Instrumentation

The AFM records contours of force, i.e. the repulsion generated by the overlap of the electron cloud at the tip with those of the surface atoms of the sample (Lieber et al., 1996). The essential components of the AFM include an integral tip-cantilever assembly, the tip acting as a local probe, and an optical
detection system that measures cantilever deflection. In effect the tip, like the stylus of a phonograph, 'reads' the surface. When the cantilevered tip is brought extremely close to the surface of the sample, an atomic force is generated between the tip atoms and the sample atoms. The atomic force depends on the distance between the tip and the sample. The sample is placed on a xyz piezo-translator and scanned by using the sharp tip mounted on a microfabricated cantilever, in a x, y-raster. The atomic force changes according to the contour. The cantilever deflection is linearly related to the force and the experimental signal is a measure of the force. There are different systems to measure the deflection of the cantilever due to tip-sample interactions. The most commonly used deflection sensor detects the angular displacement of a laser beam reflected off the back of the cantilever. In operation, variations in cantilever position, which result from sample topography and/or differing sample-tip interaction, lead to a deflection in the laser beam on a quadrant photodiode detector. By measuring the vertical and horizontal displacements on the photodiode it is possible to extract both normal and lateral forces (Meyer and Heinzelmann, 1992). The force between the tip and the sample usually varies from $10^{-7}$ to $10^{-9}$ N. AFM can operate in gaseous as well as in liquid environments and generally requires an easy and non-destructive sample preparation.

2.16.3 Sample Preparation

2.16.3.1 Treatment of rat tail tendon with the different Cr(III) species

RTT teased from six month old rats were washed extensively in 0.9% saline and stored at less than 4°C. The tendons were then washed in 1:100 Triton X-100 for 10 min to remove extraneous matter. The tendons were then
rinsed with 5 mL of 1:100 Triton X-100 followed by running distilled water. The samples were mounted on a stainless steel disk.

2.16.3.2 Reconstitution of collagen in the presence and absence of the different Cr(III) species

Substrate consisted of freshly cleft mica fixed to 15 mm steel disks with an adhesive. For fibrillogenesis collagen solution (0.5 mg/mL) was mixed gently with BCS, dimer, trimer, and tetramer, to give a final concentration of 0.01 M as Cr(III). After incubation for 30 min, the substrate was placed over the sample, removed after 30 min, washed with water, drained and dried.

2.16.4 AFM Imaging

Images were captured using a Nanoscope II AFM (Digital instruments, Santa Barbara, CA) with a ‘J’ scale scanner (horizontal ranges of 1 μm). The instrument works in a constant height mode with scan rate varying from 0.1 Hz to 150 Hz. The range of bias voltage is +10 volts with 16 bit resolutions. The X and Y voltage are in software selectable range of +220V or +12V with the Z voltage range of +220V or +55V. The tunneling current range is +50 nA. Rat tail tendons were fixed to the stainless steel disk using an adhesive. The mica substrates were also glued to the discs. The discs with the sample (either rat tail tendon or collagen adsorbed on mica) were then mounted on the sample holder. Examinations were made on a 1 μm piezoelectric scanner in air in the contact mode. The tip of the cantilever was adjusted to touch the sample, monitored using the laser spot. The scanned images were stored and analysed.
2.17 STUDIES ON THE BINARY SYSTEM CHROMIUM(III)-COLLAGENASE

2.17.1 Steady State Kinetic Studies on Collagenase Hydrolysis

2.17.1.1 Collagenase assay

Collagenase assays using FALGPA as substrate were performed according to the method reported earlier (Van Wart and Steinbrink, 1981). Assays were carried out spectrophotometrically by continuously monitoring the decrease in absorbance of substrate after addition of the enzyme. The wavelength used varied from 324 to 345 nm, depending on the FALGPA concentration. Typical mixtures for incubation contained FALGPA concentrations of 0.02 –1.5 mM, 10 µL of 15 mg/mL Type I collagenase in Tricine buffer (0.05 M Tricine, 0.4 M NaCl and 10 mM CaCl₂, pH 7.5) in a final volume of 1 mL. The course of hydrolysis was monitored on a Shimadzu UV-Visible spectrophotometer by measuring the decrease in absorbance at 324 nm when [FALGPA] = 0.02-0.19 mM. When higher concentrations of FALGPA were used viz., 0.19-0.25 mM and 0.25-1.5 mM, the decrease in absorbance was measured at 338 nm and 345 nm respectively. The hydrolysis was monitored till no further change in absorbance was observed. The initial velocity corresponding to the first 10% of hydrolysis was determined from the slope of the reaction curve using standard methods (Espenson, 1981). Initial velocities were expressed as millimoles/sec.

2.17.1.2 Kinetics of collagenase inhibition

Enzyme reactions were performed according to the conditions mentioned above in the presence of increasing concentrations of different Cr(III) species viz. dimer, 1, trimer, 2, tetramer, 3 and BCS. To assay inhibition
by Cr(III) complexes, appropriate volumes of the complexes to give final concentrations of 5, 10, 20 and 40 μM of Cr(III) were mixed with 10 μL of collagenase and pre-incubated for 18 h at 20°C. These incubated samples were then added to the substrate in Tricine buffer (pH 7.5) and the hydrolysis monitored as mentioned earlier. The substrate concentrations used were in the range of 0.02-1.5 mM. The initial rates were calculated and all data followed Michaelis-Menten behaviour.

Kinetic data were analysed by double reciprocal plots and computer fitted to appropriate rate equation by means of non-linear regression analysis program (EZ-FIT) (Perrella, 1988). The nomenclature used in this study is that of Cleland (Dixon and Webb, 1979). From the Lineweaver-Burk plots of $v^{-1}$ against $[S]^{-1}$, the kinetic parameters such as maximum velocity, $V_{\text{max}}$ and the Michaelis-Menten constant, $K_m$ of the enzyme were calculated. Dixon plots of $v^{-1}$ against $[I]$, were used to determine the inhibition constant ($K_i$) values for competitive and non-competitive inhibition. Secondary replots of slopes and intercepts of Lineweaver-Burk plots against $[I]$ were also used for $K_i$ determination. $K_i$ for mixed inhibition was determined using secondary plots, by replot of intercepts and slopes of the double reciprocal plots against inhibitor concentration $[I]$. Cornish-Bowden plots of $[S]/v$ against $[I]$ were used to calculate $K_i$ for uncompetitive inhibition. In all the cases, the X-intercept on the $[I]$ axis gives $-K_i$.

2.17.2 Circular Dichroism Spectral Studies

CD spectra of collagenase were acquired with a Jasco J-715 dichrograph. Collagenase at a concentration of 0.00034 M in 1mM acetate buffer (pH 4.0) was used. A reference spectra was recorded with buffer and
lithium perchlorate (LiClO$_4$). LiClO$_4$ was added to all the samples to minimise the effect of ionic strength variations on the conformation. Addition of LiClO$_4$ to collagenase did not alter the spectra of collagenase significantly. CD spectra were recorded in the far UV region (190-250 nm), under nitrogen, using 1 mm cells at 25°C. A slit width of 1 nm was used. The scan speed was 20 nm/min with 5 accumulations per sample. The effect of Cr(III) complexes viz. BCS, dimer, 1, trimer, 2 and tetramer, 3 on collagenase was investigated by titrating the enzyme with Cr(III) complexes and recording the spectral changes. Stock solutions of the Cr(III) complexes (500μM) were prepared and metal ion titrations were performed by adding volumes of stock solution to collagenase so as to give final concentrations of 25, 50, 75, 100, 125, 150, 175, 200 and 220 μM of Cr(III). The mean residue ellipticity [θ]$_{mrw}$, was calculated using mean residue weight of 110 for collagenase. The spectra obtained were deconvoluted using G&F, K2D, LINCOMB and MLR programs and the mean values of the secondary structure components were tabulated (Perczel et al., 1992; Andrade et al., 1993).

2.17.3 Gel Electrophoresis

The SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) as given under Appendix A3. To 10 μL of 15 mg/mL collagenase was added to different Cr(III) complexes viz. BCS, dimer, 1, trimer, 2 and tetramer, 3 to obtain final concentrations of 8, 16, 24, 30, 60, 90 and 120 μM of Cr(III) and the resulting mixture was incubated for 18 h. The samples were then centrifuged and protein concentrations estimated according to the method of Lowry et al. (1961). Samples with equal amounts of protein in 20 μL was mixed with 5 μL of 5X sample buffer and heated at 100°C
for 2 min. The samples were subjected to electrophoresis under denaturing conditions on a 10% polyacrylamide gel using bromophenol blue as the tracking dye. Gels were then fixed in 50% methanol, 10% acetic acid and 0.05% (v/v) formaldehyde and subsequently stained with silver (Nesterenko et al., 1994).