SECTION - II
2.1 INTRODUCTION

A serious health hazard that threatens the modern world is environmental pollution. It has recently been observed that the input of magnesium, strontium, barium, cobalt, copper, cadmium, mercury, lead and zinc into the environment is increasing during last two decades. The amount of above divalent cations content in the atmosphere shows variations in different localities of industrial cities. The presence of toxic heavy metal ions in industrial waste waters has become a matter of great concern in recent years. Many of the vital industries such as non-ferrous metals, pigments, storage batteries metal processing, finishing and plating discharge heavy metals in waste streams. The heavy metal ions such as cadmium, chromium, cobalt, lead, mercury, nickel, manganese, copper and zinc even in very small concentrations are known to be considerably toxic to human and aquatic life. The increasing use of pesticides in modern land and water management has posed a potential hazard not only to livestock and wild life but also to fish and other animals. These are all responsible either directly or indirectly causing certain diseases in human beings. Foods can get contaminated with divalent cations like manganese, strontium, barium, cobalt, cadmium, mercury, lead, copper and zinc. When the above divalent cations present beyond small concentrations are more or less toxic. Initially, they may combine with proteins and neutralise any poisoning effect. But when
the concentration exceeds the tolerance limit, generally they produce a quick onset of symptoms such as vomiting, nausea, and abdominal pain. Smaller amounts cause gastrointestinal disturbances. Most of these compounds are isomorphous. Copper and zinc from the environment get incorporated into the human skeletal system and cause poisoning. This incorporation occurs due to $\text{Ca}^{2+} \rightarrow \text{Zn}^{2+}$, $\text{Ca}^{2+} \rightarrow \text{Cu}^{2+}$, and $\text{Ca}^{2+} \rightarrow (\text{Cu}^{2+} + \text{Zn}^{2+})$ substitutions in the hydroxylapatite of bones. Such a possibility is based on

(i) the closeness of ionic radii ($\text{Ca}^{2+} = 0.99 \text{ Å}^0$, $\text{Cu}^{2+} = 0.72 \text{ Å}^0$, $\text{Zn}^{2+} = 0.74 \text{ Å}^0$) and
(ii) isomorphism of calcium hydroxylapatite and hydroxylapatite of above divalent cations written as $\text{CuHA, ZnHA}$ and $\text{Ca-CuZnHA}$ for brevity.

$$\text{Ca}^{2+} \rightarrow \text{Cu}^{2+} \text{ and/or } \text{Zn}^{2+}$$ substitution results in the formation of the series of solid solution of $\text{CaHA, CuHA, ZnHA}$ and $\text{Ca-Cu-ZnHA}$ according to the following equation:

$$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + n\text{M}^{2+} \rightarrow \text{Ca}_{10-n}\text{M}_n(\text{PO}_4)_6(\text{OH})_2 + n\text{Ca}^{2+}$$

For the mixed hydroxylapatites

$$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + n\text{M}^{2+} + n\text{N}^{2+} \rightarrow \text{Ca}_{10-(n+m)}\text{M}_n\text{N}_m(\text{PO}_4)_6(\text{OH})_2 + (n+m)\text{Ca}^{2+}$$

where, $n = 1$; $\text{M}^{2+}$ and $\text{N}^{2+}$ are $\text{Cu}^{2+}$ and $\text{Zn}^{2+}$ respectively.
such solid solutions were first prepared as suggested by Muller\textsuperscript{1} by firing mixtures containing various proportions of calcium hydroxylapatite and hydroxylapatites of above mentioned divalent cations at 1300\degree - 1600\degree C. The samples prepared by this solid state reaction were however found to be discontinuous and non-homogeneous. The formation of solid solutions under these conditions could be explained through formal diffusion of ions. This was confirmed by X-ray diffraction studies of the solid phase obtained after thermal treatment. Patel et al.\textsuperscript{2-8} have worked out procedures for the preparation of homogeneous solid solutions over the entire compositional range by the method and co-precipitation in aqueous media. Proof for the formation and homogeneity of the solid solutions is provided by a linear relationship between the lattice constants and the copper and zinc content in the samples. A survey of the work done on CaHA, CuHA, ZnHA and Ca-Cu-ZnHA system indicates that

(i) no convenient method for preparation of homogeneous solid solutions over the entire compositional range is available
(ii) no systematic procedure for the complicated quantitative separation and determination of calcium, copper and zinc when present together as in solid solutions of Ca-Cu-ZnHA are attempted and
(iii) no physico-chemical investigation, like the detailed X-ray diffraction, electronmicroscopic, infrared etc. have been carried out with the solid solutions with a view to understand the theoretical aspects of the formation of solid solutions and
(iv) no meaningful investigations were carried out with the prepared solid solutions to study the influence of the incorporated copper and zinc on calcification and resorption.

It is thought that the solubility studies of CaHA, CuHA, ZnHA would be helpful to understand the effect of incorporation of copper and zinc on the solubility of bone mineral.

The present investigations are intended to prepare a series of homogeneous solid solutions of CaHA, CuHA, ZnHA and Ca-Cu-ZnHA over the entire compositional range by a new method developed for the purpose and to correlate bone metabolism as observed in the bone of human beings with the physico-chemical properties of synthetic hydroxylapatites. Interesting physico-chemical investigations with the solid solutions of synthetic hydroxylapatites and natural hydroxylapatites of human bone by the application of X-ray diffraction, electron microscopic and infrared spectral studies have been carried out. In addition, the dependence of solubility of hydroxylapatite (synthetic and natural) on (i) the pH of the solvent medium within physiologically important range of 5.0 to 8.0 and (ii) divalent cation content have been investigated.
2.2 EXPERIMENTAL

The experimental work included in this section has been sub-divided as follows:

(i) Preparation of synthetic samples
(ii) Methods of bone deproteination
(iii) Chemical analyses
(iv) Cationic substitution in bone mineral (synthetic & natural)
(v) X-ray diffraction analysis and molar volume determination
(vi) Electron microscopic investigations
(vii) Infrared spectra studies
(viii) Studies on the solubility equilibria.

2.2.1 Preparation of the synthetic hydroxylapatites

Samples of CaHA and solid solution of Ca-Cu-ZnHA, CuHA and ZnHA are prepared by precipitation at 37 ± 0.5°C in carbon-dioxide free atmosphere by mixing stoichiometric quantities of the reactants in the form of their aqueous solutions maintained at a pH of about 12.

All chemicals used for the preparation of these samples were either AR (BDH) or E. MERCK Extra Pure grade. Water used in the preparation and in washing was boiled to remove CO₂ and then used immediately.
Preparation of the sample is based on the following equation:

$$10 \text{Ca(NO}_3\text{)}_2 + 6 \text{NH}_4\text{H}_2\text{PO}_4 + 14 \text{NH}_4\text{OH} = \text{Ca}_{10}\text{(PO}_4\text{)}_6\text{(OH)}_2$$

$$+ 20 \text{NH}_4\text{NO}_3 + 12\text{H}_2\text{O}$$

and for the solid solutions, the proportion of cations being altered as follows:

$$10-(n+m)\text{Ca(NO}_3\text{)}_2 + m\text{M(NO}_3\text{)}_2 + n\text{M(NO}_3\text{)}_2 + 6\text{NH}_4\text{H}_2\text{PO}_4 + 14\text{NH}_4\text{OH}$$

$$= \text{Ca}_{10-(n+m)}\text{M}_n\text{M}_m\text{(PO}_4\text{)}_6\text{(OH)}_2 + 20\text{NH}_4\text{NO}_3 + 12\text{H}_2\text{O}$$

Based on the above equations calculated amounts of nitrates of calcium (and/or) divalent cations as the proportion desired and ammonium dihydrogen phosphate were taken for the yield of about 25 g of the sample. Since the nitrates of above mentioned divalent cations could not be accurately weighed, a stock solution of them was prepared separately and the cation content was determined by complexometric procedure\textsuperscript{9-10}. NH\textsubscript{4}OH or Ethylene diamine was used to maintain the relating solutions at a desired pH.

The following solutions were prepared:

Solution No. [1]:-

500 ml of a solution containing the calculated volume of stock solution of nitrates of divalent cations as the proportion
desired for the solid solutions to be prepared.

Solution No. [2] :-

500 ml of a solution containing the corresponding calculated amount of ammonium dihydrogen phosphate was prepared. Each of these solutions was maintained at a pH of about 12 by addition of liquor ammonia or Ethylene diamine as desired.

Solution [1] and [2] were taken in two separate dropping funnels fitted into the cork of a 2L conical flask with a side tube in which about 200 ml of 25 per cent of ammonium hydroxide was kept. The flask was kept in a water bath maintained at $37 \pm 0.5^\circ C$. The side tube of the flask was connected to the water pump which removed air from the flask when the pump was in working condition. Carbon dioxide free air was admitted into the flask using a series of soda lime and concentrated potassium hydroxide towers. Solutions [1] and [2] were added simultaneously dropwise into the flask while carbon dioxide free air was bubbled through the medium of precipitation to eliminate the formation of carbonate apatite. Such bubbling in addition, kept the medium of precipitation well stirred. The precipitate was aged by boiling it under reflux in contact with the mother liquor to improve the crystallinity and homogeneity. The precipitate was then left over night in contact with the mother liquor and separated by filtration. It was washed till the wash liquid was neutral and
free from calcium or used divalent cations.

A part of the yield was washed with acetone and the rest was heated in about 110°C for 6 h and the product was powdered and sieved to the desired particle size. Care was taken to maintain the experimental conditions scrupulously the same for all the samples. The composition of the reacting solutions being approximately altered depending upon the product desired. The apparatus used for the preparation of the samples is shown in Fig. 10.

Fig. 10 Assembly of the apparatus used in the preparation of the samples.
2.2.2 METHODS OF BONE DEPROTEINATION

INTRODUCTION

It is necessary in the study of bone to remove the organic matrix (which is principally collagen) with minimum alteration of bone apatite crystals. For example, a specific surface determination of bone mineral by the low-temperature nitrogen adsorption method requires a surface with virtually all the organic phase removed in order to obtain meaningful data. In another example, the diffuse background pattern from collagen can sometimes interfere with accurate X-ray diffraction line width and intensity measurements. Similarly, spectroscopic measurements of bone and dentine mineral are complicated by protein absorption. But it is also not possible to undertake some physico-chemical studies of intact bone without removing the organic matrix. Thus it is desirable to obtain hard tissue mineral free of its organic matrix. The best known methods for dissolving away the organic materials are:

(1) dry ashing at about 400°C
(2) autoclaving in steam atmosphere
(3) glycerol ashing i.e. boiling in a KOH of glycerol (Ethylene glycol is often used in place of glycerol and in this case the process is generally known as glycol ashing).
(4) refluxing in ethylene diamine and hydrazine.
Among them the No. 4 method of deprotenation is the best.

X-ray diffraction studies of bone mineral deproteinated with both hydrazine and ethylenediamine show only small alterations in crystal size and/or perfection as indicated by line width comparisons of the bone before and after treatment. The powerful organic bases remove 95 - 98 % of organic matrix of bone. The ethylenediamine ($\text{H}_2\text{NCH}_2\text{CH}_2\text{NH}_2$) extraction\textsuperscript{12-14} requires an extensive water wash to remove all the ethylenediamine and protein. However, X-ray diffraction and scanning electron microscopic measurements of bone deproteinated in this fashion have shown that substantial molecular alterations in mineral phase morphology also result from this treatment. Bone deproteination could be successfully accomplished at 40 - 60° employing 95 % hydrazine itself is a polar, high electric constant material that is a good solvent for organic matrix. The chemical principle operating in this technique is that concentrated (95 % +) hydrazine ruptures peptide bonds within proteins, a reaction which has therefore been used in determining carboxy-terminal amino acid residues in peptide chains\textsuperscript{15}.

**HYDRAZINE DEPROTEINATION PROCEDURE**

Extraction with hydrazine for 48 h at 60° removed organic matter from the bones. The bone deproteination procedure utilized
in this study was as follows. Sample of bones of human being of suitable size were immersed directly into a suitable volume of hydrazine (95% + Eastman organic chemical) at room temperature in a glass stoppered flask. Following a reagent change at 1 h, the flask was incubated at 55°C. Further reagent changes were made after additional 1, 15 and 24 h periods. Samples were then serially diluted with absolute ethanol (50%, 75%, 87.5% and 100% steps at 30 min.). Samples for chemical and physico-chemical analysis were washed and gently hand ground (1-2 min.) in acetone vacuum-dried and stored in a dessicator. All adhering soft tissue was solubilized through the first two reagent exchanges. Decantation of highly viscous initial supernatant required extra care. Since 95% hydrazine is an extremely caustic and high toxic-flammable chemical, all open work was conducted in a ventilating hood using heavy rubber gloves. Then the dried bones were burnt to ashes at 1000°C in the Muffle's Furnace for about 8 h and powdered and sieved to desired particle sizes.

2.2.3 CHEMICAL ANALYSIS

The chemical analysis of calcium hydroxyapatite is complicated by the mutual interference of calcium and phosphate ions and consequently special analytical techniques are desired for the purpose. These complications became more pronounced when copper and/or zinc are present in addition, as in the case of solid solutions of Ca-Cu-ZnHA. The instrumental methods of analysis
based on fluorescence and flame photometry are frequently used for such determinations. It has been found that the application of the complexometric methods for such analyses offer simple and convenient procedures capable of offering reproducible results of a high degree of accuracy. The following are the procedures used for the analysis of (I) CaHA, (II) CuHA, (III) ZnHA, (IV) Ca-Cu-ZnHA.

(I) COMPLEXOMETRIC ANALYSIS OF CaHA (METHOD-I)

The method is based on the separation of phosphate from a convenient aliquot of a solution of CaHA as ammonium phosphomolybdate first and reprecipitated as magnesium ammonium phosphate. The phosphate content was determined by the method of back titration of the excess of 0.1 M EDTA added using 0.1 M magnesium sulphate at a pH of about 10 using Eriochrome Black-T as indicator.

The calcium content of the filtrate after removal of phosphate as ammonium phosphomolybdate was determined by direct titration of a convenient aliquot with 0.1 M EDTA using Murexide as indicator at a pH of about 10 in the presence of NH₄Cl-NH₄OH buffer.

A convenient weight of the sample (0.4 g) was dissolved in a minimum volume of nitric acid (Sp. gr. 1.42) and later about 10 ml of 8 per cent ammonium nitrate solution were added. Phosphate was precipitated at 50°C as ammonium phosphomolybdate by adding...
10 per cent solution of ammonium molybdate until no further formation of yellow precipitate from the supernatant liquid was perceptible. The precipitate was filtered through \( \text{IG}_4 \) crucible and washed several times with a solution containing equal volume of 1 per cent solution of ammonium nitrate and 20 per cent solution of nitric acid (Sp. gr. 1.42). The filtrate was preserved for the determination of calcium. The precipitate of ammonium phosphomolybdate was dissolved in the minimum quantity of 0.1 M sodium hydroxide and it was reprecipitated as magnesium ammonium phosphate by the addition of slight excess of 1.0 M magnesium sulphate at the appropriate pH indicated by the blue colour of Thymol blue indicator. This condition was obtained by dropwise addition of 9.0 M ammonium hydroxide. The precipitate was kept in contact with the mother liquor over night and was separated by filtration through \( \text{IG}_4 \) crucible, washed with 0.5 M ammonium hydroxide dissolved in 6.0 M hydrochloric acid and a known excess of 0.1 M EDTA was added to it. The solution was neutralised with 210 M sodium hydroxide, adjusted to pH 10 and was finally titrated against 0.1 M magnesium sulphate using Erichrome Black-T as indicator till the blue colour changes to wine red. From the volume of magnesium sulphate consumed in the titration, the amount of phosphate was calculated.

After the separation of phosphate as ammonium phosphomolybdate, the filtrate was made up to a known volume. To a convenient volume of the aliquot about 4 ml of NH₄Cl-NH₄OH buffer to maintain the desired pH was added 3-6 drops of freshly prepared saturated
solution of Murexide as indicator was added. The resulting solution was titrated against 0.1 M EDTA at a pH of about 10 till a brilliant colour change from yellow to purple was obtained.

The accuracy of the method was assessed by analysing the mixtures of calcium carbonate and potassium dihydrogen phosphate of known compositions.

(11) COMPLEXOMETRIC DETERMINATION OF COPPER AND PHOSPHATE IN CuH₂PO₄ (METHOD II)

Since the presence of phosphate interferes in the determination of copper by complexometric method, phosphate was prepared and determined by the method mentioned earlier.

After separation of phosphate, the filtrate was made to a known volume. To a convenient volume of aliquot containing copper, 2.0 M ammonium hydroxide was added dropwise until the precipitate which formed was just redissolved. 3-6 drops of freshly prepared saturated solution of Murexide as indicator was added and titrated against 0.1 M EDTA till a brilliant colour change from yellow to purple was obtained.

The accuracy of the method was assessed by analysing sample solution containing known quantities of CuCO₃ and KH₂PO₄.
(III) COMPLEXOMETRIC DETERMINATION OF ZINC AND PHOSPHATE IN ZnHA (METHOD III)

Since the presence of phosphate interferes in the determination of zinc, phosphate was separated and determined as earlier.

After separation of phosphate, the filtrate was made up to a known volume. To a convenient volume of aliquot containing zinc (the solution should not be too strongly acidic), 3-5 ml of an ammonium chloride - ammonium hydroxide buffer of pH 10 was added and diluted to 150 ml and titrated against 0.1 M EDTA using Eriochrome Black T as indicator (0.2 percent solution of Eriochrome Black T in triethanolamine for every 100 ml of solution). The End point is indicated by sharp colour change from red to blue. The volume of EDTA consumed during titration corresponds to the content of zinc.

The accuracy of the method was assessed by analysing the sample solution containing known quantities of ZnCC₂₃ and KH₂PO₄.

(IV) COMPLEXOMETRIC DETERMINATION OF CALCIUM, COPPER, ZINC AND PHOSPHATE IN (Ca+Cu+Zn)HA (METHOD IV)

Since the presence of phosphate interferes in the determination of calcium, copper and zinc by the complexometric method,
phosphate was first precipitated, separated and determined by the method mentioned earlier.

After separation of phosphate, the filtrate was made to a known volume. To a convenient volume of aliquot containing calcium, copper and zinc (the solution should not be too strongly acidic), 3-5 ml of NH₄Cl-NH₄OH buffer of pH 10 was added. 3 g of NH₄F as maskent for calcium was added and diluted to 150 ml and titrated against 0.1 M EDTA using Erionchrome Black T as indicator (0.2 % solution of Erionchrome Black T in Triethanolamine for 100 ml of solution). The volume of EDTA consumed during titration corresponds to the content of copper and zinc.

In a separate aliquot of calcium, copper and zinc, calcium was masked as earlier, then potassium cyanide was added till the blue colour of copper was discharged. Few ml of 10 % formaldehyde solution was added and titrated immediately with EDTA using Erionchrome Black T indicator till to a colour change from wine-red to blue. The amount of zinc content was calculated from the volume of EDTA consumed in the titration.

The accuracy of the method was assessed by analysing the sample solution containing known quantities of CaCO₃, CuCO₃, ZnCO₃ and KH₂PO₄.
ANALYTICAL PROCEDURE

(1) Disodium EDTA

White crystalline compound, occurring with the molecules of water of hydration. The disodium salt of EDTA (ethylenediaminetetraacetate) has the composition Na$_2$H$_2$C$_{10}$H$_{12}$O$_8$N$_2$.2H$_2$O and represented by the abbreviated formula Na$_2$H$_2$Y.2H$_2$O. Molecular weight is 372.10. The compound is obtained in such purity that standard solutions can be prepared by direct weighing and a standardization procedure is unnecessary. Common names for the salt are: Complexone(III), Trilon B and disodium Versenate. 37.21 g of the dried substance was dissolved in 1 litre to give 0.1 M EDTA solution. Dilute solutions of convenient concentration were prepared from the stock solution.

(2) Magnesium sulphate solution

About 22.60 g of MgSO$_4$.7H$_2$O was dissolved and made up to 1 litre to give an approximate 0.1 M solution. This was subsequently standardized by titrating complexometrically against 0.1 M EDTA solution. Solutions of desired concentration were prepared by dilution of the stock solution.

(3) Buffer solution of a pH of about 10

Many of the EDTA titrations are carried out in solutions.
buffered at pH 10. For the most part, ammonium chloride-ammonium hydroxide buffers were used. The one most widely used was prepared as follows:

54 g of ammonium chloride in 200 ml of water was mixed with 350 ml of 25 per cent ammonium hydroxide and diluted to 1 litre with distilled water.

(4) Indicator Solutions

(a) Murexide Indicator

This compound is the ammonium salt of purpuric acid \( \text{C}_8\text{H}_3\text{N}_3\text{O}_6 \). Saturated solution of the indicator was prepared in doubly distilled water. A fresh solution was prepared for each set of titration. Because such solutions are stable for only a day or two.

(b) Eriochrome Black T

This compound is one of the most widely used metal indicators in EDTA titrations and is sold under various trade names such as Eriochrome Black T, Eriochrome-schwartz T, Pentachrome Black TA, Potting Black C, Diamond Blue Black EBS, Omega Chrome Black S and Chromogen Black special ETOO (Russian). It is Nc. 203
in the "Colour Index". Chemically it is sodium 1-(1-hydroxy-2-naphthylazo)-6-nitro-2-napthol-4-sulphonate. 0.2 g of Eriochrome Black T was dissolved in about 20 ml of absolute alcohol. A fresh solution of it was prepared for each set of titration. A stable (at least for 7 months) and convenient form of Eriochrome Black T indicator was prepared by dissolving 2 g of the indicator in 100 ml of triethanol amine.

(c) Phthalein complexone Indicator

Other names applied to this indicator are metal-phthalein, o-cresol phthalein complexone, phthalein purple and phthalein purpur. Phthaleincomplexone is almost colourless and is insoluble in pure water. A stable form of indicator was prepared by dissolving in triethanolamine.

(5) Use of masking reagents in EDTA Titrations

(a) Masking with potassium cyanide

The cyanide ion forms very stable complexes with zinc and copper(II). All these complexes are stronger than the corresponding complexes with EDTA. It is therefore, possible to determine calcium in presence of above mentioned metals by masking its potassium cyanide.
(b) Masking with Ammonium Fluoride

The fluoride ion forms strong complexes with some cations such as alkaline earth metals etc. It has, therefore, been used as a masking reagent for the determination of zinc and copper etc. in the presence of alkaline earth metals.

2.2.4 CATIONIC SUBSTITUTION IN THE BONE MINERAL (SYNTHETIC AND NATURAL)

During the past decade, the chemistry of hydroxylapatite has excited a great deal of interest both amongst biologists and chemists in view of its importance in understanding the mechanism of calcification and dental caries production. Hydroxylapatite is assigned the formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, CaHA. It is now recognised as the most important inorganic mineral constituent of animal bones. The term mineral, in the biological sense refers to the elements or compounds which are non-organic in nature. It undergoes a series of cationic substitution reaction, which are of biological and physico-chemical significance. Ca$^{2+}$ (0.99 Å) can isomorphously be substituted by Cu$^{2+}$ (0.72 Å) and Zn$^{2+}$ (0.74 Å) of the solution (cations whose ionic radii are close to that of Ca$^{2+}$), which explains the mechanism of their incorporation into human skeletal system, has not been thoroughly investigated as is evident from the literature and hence the present work has been undertaken in order to determine the manner in which divalent cations
are incorporated into the inorganic crystal of bone. Therefore, an attempt has been made to correlate the physico-chemical properties of bone mineral and synthetic hydroxylapatite which has been prepared from solution resembling body fluids.

Since CaHA, CuHA and ZnHA are isomorphous, Ca$^{2+}$ in CaHA can be replaced by Cu$^{2+}$ and Zn$^{2+}$ of the solution. This substitution reaction first takes place at the surface of the crystal of CaHA and subsequently occurs in the interior of the lattice. This is favoured by the factors like

(i) pH of the medium of equilibration
(ii) concentration of divalent cations in solution
(iii) mean radius of the particle size of the solid phase
(iv) period of equilibration.

The dependence of uptake of divalent cations on the factors were investigated.

The dependence of uptake of divalent cations by solid CaHA (synthetic and natural) of human being was studied by equilibrating about 0.5 g of powdered CaHA at 37 ± 0.5°C with 100 ml of solution of Cu(NO$_3$)$_2$ and Zn(NO$_3$)$_2$ by constant shaking, changing the factors effecting the uptake under investigation each time while other factors were kept constant. At the end of the desired period of equilibration each time, the contents were filtered through IG$_4$ crucible, the residue
was washed till it is free from absorbed divalent cations. The Ca\(^{2+}\) and Cu\(^{2+}\) or Zn\(^{2+}\) contents in the solid were determined complexometrically\(^{18-20}\). Thus the dependence of uptake of Cu\(^{2+}\) and Zn\(^{2+}\) by CaHA was investigated to correlate the relation between environmental pollution and uptake of divalent cations by skeletal system. The concentration of the solutions of Cu(NO\(_3\))\(_2\) and Zn(NO\(_3\))\(_2\) varied from 0.01 M to 0.1 M. The pH range of investigation was between 5.0 to 8.0 which is physiologically most important. The particle size chosen for equilibration was between 300 \(\mu\)m to 50 \(\mu\)m. The buffer combinations used for the purpose are mentioned later.

2.2.5 X-RAY DIFFRACTION ANALYSIS AND MOLAR VOLUME DETERMINATIONS

The formation and homogeneity of the solid solutions of CaHA, CuHA and ZnHA (synthetic and natural) can be confirmed by X-ray diffraction studies of the samples. Based on the consideration of the ionic radii of Ca\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\), it is evident that lattice parameters showed unit cell contraction consequent upon the introduction of smaller ions in the apatite lattice. This can be investigated through the determination of lattice parameters of the samples. The X-ray diffraction patterns of the samples which was previously heated to 110°C for 6 h were taken using CuK\(_\alpha\) (Nickel filtered) radiation with a graphite monochromator using a Siemens Powder Diffractometer. Sharp peaks separated by less than 0.1 degree (2\(\Theta\)) can be resolved
with a 0.006 inch entrance slit at a scanning speed of 1 degree (2θ) per minute using tube voltage and current of 30 kV and 24 mA respectively.

The d-spacing and the relative intensities of the investigated materials were theoretically calculated using available programmes by the well known Azaroff method. The lines were indexed and the lattice parameters were calculated knowing the lattice parameters, the unit cell volume was obtained using the formula $\sqrt{3} \over 2 a^2 c$ for the hexagonal apatite lattice. The unit cell dimensions are very sensitive to atomic substitutions in the structure and are useful for exact phase identification.

**Molar volume determination**

One of the criteria for a given pair of solids to be capable of forming solid solutions is that their molar volumes should not differ appreciably from one another. In order to supplement the evidence provided by the X-ray diffraction patterns, the molar volumes of the samples were determined to scrutinise the homogeneity of solid solutions prepared. The densities of the samples were determined using a specific gravity bottle and toluene as solvent. Knowing the molecular weight of the sample calculated on the basis of their molecular formulae, the molar volumes were determined.
2.2.6 ELECTRON MICROGRAPHIC INVESTIGATION

Electronmicrographic investigations are premierly intended for identification of the samples of hydroxylapatites and to confirm the homogeneity of the samples prepared. The investigations are also intended to correlate the alteration of dimensions of the crystals with inclusion of divalent cations (Ca\(^{2+}\) and Zn\(^{2+}\)) into the apatite lattice.

Electronmicroscopic scanning were obtained in order to determine the degree of cry-stallinity of the samples. An Etec Autoscan Scanning Electron Microscope was employed with a 20 kV secondary electron mode using samples which were prepared from an alcohol dispersion on a polished sample stub and sputtered with 100 A\(^{0}\) coating of a 60-40 Gold-paladium alloy.

2.2.7 INFRARED SPECTRAL STUDIES

The degree of splitting of certain IR absorption bands and their breadth and shape give useful information about the internal structure and atomic order of a solid material. During the formation of the solid solutions, in addition to the static field, the dynamic interaction between the ions also has strong influence on the internal vibrations of PO\(_4^{3-}\). Ths results in the alteration in the position and shape of infrared spectra corresponding to the phosphate ion. The shift in the vibration of frequencies of phosphate ion depend
upon the binding energy of the atomic mass of the substituent in the lattice structure according to the equation of Barnes et al.\textsuperscript{23} which

$$\nu = \frac{1}{2\pi c} \sqrt{\frac{K}{M}}$$

gives a relationship between frequency, atomic mass and force constant. The vibration frequency is dependent on the reduced mass $\mu$ of the participating atoms and the restoring force $K$ between atoms, all other terms remaining constant. When the equilibrium distance between the positive and negative atoms of the molecule is decreased, $K$ generally increases in the above equation. This equilibrium distance depends on the ionic radii of the participating atoms in the molecule.

The infrared spectra of the samples were recorded as KBr Pellets on PERKIN-ELMER Spectrophotometer.

\section*{2.2.8 STUDIES OF SOLUBILITY EQUILIBRIA}

The studies of solubility equilibria of the samples are concerned with micro analytical determination of calcium and divalent cations in presence of phosphate. It is evident that the glass containers are unsuitable for setting of the systems for longer duration of equilibration, the calcium and silicate of glass under these conditions dissolve and interfere with the analysis of the solution. In addition, carbon dioxide should be eliminated from the systems lest the pH of the dissolving
medium may be changed and also carbonate apatite be formed. Based on these considerations perfectly airtight polyethylene bottles of about 250 ml capacity were used as containers for setting up of the systems. About 0.5 g of the samples powdered and sieved to 200 mesh (BSS) in each case was equilibrated with 100 ml of the solvent (potassium acid phthalate-sodium hydroxide for a pH of 6.0) by constant shaking at a regulated speed using a mechanical flask shaker at 37 ± 0.5 °C. The time required for attainment of saturation was determined in preliminary experiments by analysis of phosphorus content in the filtrate obtained by filtration through IG- crucible at the end of each convenient time of equilibration.

The solubility of the sample was determined by similar analysis of phosphorus in the filtrate after attainment of saturation.

**SELECTION OF BUFFERS**

The investigations were principally concerned with the dependence of the solubility of the samples on

(i) the pH of the dissolving medium and
(ii) the cation content in the solid solutions.

Determination of solubility of the solid solutions as a function of pH of the dissolving medium are based on selection of appropriate buffer combinations for the desired pH range. The suitability of
the buffer is based on the subsequent determination of calcium, copper, zinc and phosphate for the desired pH range of 5.0 to 8.0. Such a condition was fulfilled by the following combinations:

Potassium acid phthalate - Sodium hydroxide for the pH range 6.0 to 6.5 and sodium diethylbarbiturate-hydrochloric acid for the pH range 7.0 to 8.0. The microdetermination of phosphate by complexometric procedure was found unaffected by the buffer over the wide range of their concentrations in the dissolving medium. The determination of the solubility of the samples was done at regular intervals. The pH of the dissolving medium was measured with a line operated in Beckman pH meter and constancy was confirmed by repeating the measurement after the period of equilibration. The fluctuations were found to be less than 0.1.

**Attainment of equilibrium:**

The system were equilibrated in usual way at convenient pH values for 3.0 h and the phosphorous content in the known volume of the solvent was determined each time at the end of the desired period of equilibration after separation of the colloidal component through IG₄ crucible. It was found that the saturation was attained within about 1 h of equilibration in all the pH values.