2. MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMAL

*Channa punctatus* (Bloch) is a fresh water teleost belonging to the order - channiformes and family channidae. Its important characteristics include depressed head covered with large scales; fins without spines; dorsal and anal fin long; physoclistic.

*C. punctatus* was chosen as the test animal because of its easy availability and survival capacity in the laboratory conditions. This fish is consumed as food by a large section of people in Sambalpur and elsewhere and hence the effects of metal toxicity to this fish is of particular interest.

Fishes of 10 to 15cms length (irrespective of sex) were collected from local fresh water resources (non source of pollutant input) and brought to the laboratory without mechanical injury. They were washed in 1% solution of potassium permanganate for 5 minutes to avoid dermal infection. The fishes were acclimated to the laboratory conditions for 10 days changing the water at regular intervals under natural photo-period and room temperature of 22±3° C in well aerated large-glass aquaria (35 litre capacity) containing untreated chlorine free deep well water and fed on alternate days with fixed quantity of chopped earthworms.

A.R. grade of arsenic salt was used. Stock solution was prepared by mixing the arsenic salt in carbon dioxide free distilled water and bio-assays were conducted with various dilutions. Unchlorinated well water was used in the test containers. The physico-chemical characteristics of the water used for the experiment were analysed as per the methods of *APHA, AWWA* and *WPCF (1980)*. Again this analysis was checked by microprocessed instrument and the digital pH meter was used to record the pH. The water and arsenic solution were renewed at every 3 days to maintain the desired concentration.
2.2 DETERMINATION OF 96 HOUR LC<sub>50</sub>

During determination of 96 hours LC<sub>50</sub> the test fishes were starved for 2 days before they were finally transferred to the experimental containers. A control was maintained throughout the experiment. Methods of measuring median lethal concentration or LC<sub>50</sub> have been reviewed by Brown (1971). The procedure is to expose groups of fishes to different concentrations of the Na<sub>2</sub>H(ASO<sub>4</sub>).7H<sub>2</sub>O (molecular weight 312.01) and to measure the time taken to kill 50% of fishes of each group. By plotting these median lethal times against concentration of the medium, the 96 hrs LC<sub>50</sub> dose can be determined. After determination of 96 hours LC<sub>50</sub> dose the fishes were kept at various concentrations of Arsenic for determining the toxic effects of arsenic at various concentration and time period. The fishes were fed regularly during the tenure of this experiment. Controls were maintained throughout this experiment. Three replications for each dose were maintained.

STATISTICAL ANALYSIS

(1) Regression Equation

Regression equation worked out by the formula of Snedecor (1961).

Regression Coefficient = \( b = \frac{\sum xy}{\sum x^2} \)

Where

\( X = \bar{X} - X \) and \( Y = \bar{Y} - Y \)

\( X \) = Concentration of arsenic (mg/l)
\( Y \) = Days taken for death of fishes (in days)

\( \bar{X} \) = mean concentration of arsenic
\( \bar{Y} \) = mean time taken for 50% death of fish.

Form the Regression Coefficient thus obtained, 't' value can be calculated as follows:

\( Y - \bar{Y} = b(X - X) \)
(2) The 't' value was calculated using the formula

\[
t = \frac{N_1 \times N_2}{N_1 + N_2} \frac{X_1 - X_2}{(N_1 - 1)(S_1)^2 + (N_2 - 1)(S_2)^2}^{N_1 + N_2 - 2}
\]

Where \(S_2\) and \(S_2\) are standard deviation of sample No. 1 and 2. \(N_1\) and \(N_2\) are number of observations for sample 1 and 2.

The confidence level \(P\) was found out from 't' table (Abramoff and Thomas, 1966). Usually \(P\) at 0.05 level and below were considered as significant and above 0.1 as not significant.

2.3 MORPHOLOGICAL, SKELETAL AND BEHAVIOURAL CHANGES

The fishes were frequently observed during the tenure of this experiment for studying the morphological and behavioural changes due to arsenic toxicity. The fishes developing haemorrhages and lesions were photographed. The fishes showing deformity in the body shape were removed from the experimental aquaria and were preserved in 5% formaldehyde for 15 days. Then X-ray plates were prepared for assessing the skeletal disorder in the fish.

The experimental fishes were divided into several batches of 10 each, irrespective of sex, for ascertaining various biochemical changes in the experimental tissues (brain, blood, liver and intestine) due to chronic and acute exposure of the fish to arsenic. Apart from the control group, the other groups were treated with various concentration of arsenic solution, such as 5 mg/l, 8mg/l, 11mg/l, 14 mg/l respectively for a period of 15 days and 17 mg/l (96 hrs \( LC_{50} \)) for 4 days. Another five groups of fishes were treated with a sub-lethal dose of 5 mg/l arsenic for a period of 75 days to assess the long term effect of arsenic.
2.4 CHANGES IN HAEMATOLOGICAL PARAMETERS

Blood was collected directly from the heart using disposable syringes. The blood was kept in 2 vials, one coated with EDTA and other uncoated. The blood kept in EDTA coated vial was used for estimation of Hb%, PCV, ESR, WBC and RBC. Whereas the blood of uncoated vials was used for the estimation serum iron, glucose, protein, triglyceride, cholesterol and VLDL (very low density lipid).

Total RBC and WBC were counted by Neubauer's double haematocytometer using Hayem's and Turk's solution respectively as diluting fluid as described by Dacie and Lewis (1977). Hb% was measured using the photoelectric calorimeter applying cyanomethaemoglobin (haemoglobinocyanide or HICN) method (Drabkin et al., 1932). Packed cell volume (PCV) or haematocrit value was determined by Wintrobe's method centrifuging the blood sample at 3,000 rpm for 30 minutes, (ICSH, 1980). ESR was measured using Westergren's method (1921). The serum iron estimation was done using spectro-photometer applying the modified method of I.C.S.H. (1990) and was based on the development of a coloured complex when ferrous iron was treated with a chromagen solution. Blood glucose was measured with spectrophotometer using GOD/POD method (Henry, 1963; Rabbo, 1969). Total protein, cholesterol, VLDL and triglyceride were analysed using Kodak Ektachem DT System. The data (average of 10 estimations) were analysed statistically by student's 't' test at <0.001 level of significance (Fisher 1980).
ESTIMATION OF Hb% BY HAEMIGLOBIN CYANIDE

(CYANOMETHAEMOGLOBIN) METHOD

For the estimation of Hb%, HiCN method was used. The basis of the method was dilution of blood in a solution containing potassium cyanide and potassium ferrocyanide (Drabkin et al., 1932). Hb, Hi and HbCO (but not SHb) were converted to HiCN. The absorbance of the solution was then measured in a photoelectric calorimeters at a wavelength of 540 nm.

20 µl of blood added to 4ml of HiCN diluent (dilution 1 in 200) and the tube containing the solution was shaked several times for through mixing, then allowed to stand at room temperature for a period of 4 minutes to ensure the completion of the reaction. The solution of HiCN was now ready to be compared with the standard reagent blank in a spectrophotometer at 540 nm. The absorbance of the test sample was measured within 6 hours after dilution as follows:

\[
\text{Hb g/l} = \frac{A_{540} \text{ of test sample}}{A_{540} \text{ of standard}} \times \text{conc. of standard} \times \frac{1000}{\text{dilution factor}}
\]

ERYTHROCYTE OR RED BLOOD CELL COUNT

The apparatus consists of a mixing pipette, a graduated improved Neubaeur's counting chamber and Hayem's diluting fluid. Blood is sucked into a special pipette upto the mark 0.5 when the blood reaches the required mark, the end of pipette is wiped clean and dipped into the Hayem's diluting fluid and
filled up to the mark 101 (dilution 1 in 200). Then the blood and diluting fluid is thoroughly mixed by rotating the pipette while it is held horizontally. About a third of the content of the bulb is discarded by blowing out, holding the pipette at an angle of 45°. Now the contents of the pipette is released in the space between the cover slip and the counting chamber and the fluid is allowed to run under the cover slip by capillary suction. The chamber is left undisturbed for at least 2 minutes for the cells to settle. The counting chamber is adjusted under the objective of the microscope and the counting of Red blood cell is done. The platform is ruled by crossline each enclosing a space of 1/400 of a square millimetre. The depth of each square with the cover glass on is 1/10th of a millimetre. The squares were marked out into sets of 16 by double lines. The red blood cells are counted in 5 such sets, that is in 80 squares, which is equal to 1/50 mm. Then the total RBC number was found out as follows and expressed as million/cmm of blood. Number of cells counted $\times 50 \times 200$ or Cells counted $\times 10,000$.

**LEUCOCYTE OR WBC COUNT BY VISUAL METHOD**

This is performed in a manner similar to the RBC count. In which 1 in 20 dilution of blood was taken. The blood collected in EDTA coated vials is sucked to the pipette upto the required mark and then mixed with Turk's solution. Blood is mixed throughly with the fluid and a small portion of it is transferred to the counting chamber after rejection of 1st drop. The WBC cells in as many 1 mm² (0.1 µl in volume) of the ruled area in an improved Neubauer chamber was counted and from it the total WBC/cmm was calculated using the following method.
Then the total WBC number was found out as follows and expressed -

\[ \text{Count} = \frac{\text{No. of cells counted}}{\text{Volume counted (\mu l)}} \times \text{dilution} \times 10^6 \]

Thus, if \( N \) cells are counted in 0.1 \( \mu l \), then the leucocyte count per litre
\[ = N \times 10 \times \text{(dilution)} \times 10^6 \]
\[ = N \times 200 \times 10^6/l \]
\[ (= N \times 200 \text{ per } \mu l). \]

DETERMINATION OF PACKED CELL VOLUME (PCV OR HAEMATOCRIT VALUE)

A macro-method Wintrobe tubes was used.

**Macro-Method (Wintrobe's Method)**: Wintrobe tubes of 2.5 to 3 mm in general diameter and about 110 mm in length, calibrated at 1 mm intervals were employed.

Blood was collected with minimal stress to the animal and rendered incoagulable by EDTA at a concentration of 1.5 mg/ml. Dipotassium EDTA is preferred to the tripotassium salt as the latter produces some shrinkage of the red cells which results in a 2-3% decrease in PCV.

The blood was mixed carefully by repeated shaking and filled into the haematocrit tube at once up to the 100 mm mark by means of glass capillary pipette. Then the tube was centrifuged at 2000-2300 rpm for 30 minutes.

The height of the column of the red cells were taken as the PCV (the volume occupied by the red cells expressed as fraction of the total volume of the blood).
ESTIMATION OF THE ERYTHROCYTE SEDIMENTATION RATE (ESR)

ESR was measured using the method of Westergreen. The recommended tube was a straight glass tube 30 cm in length and 2.55(±0.15) mm in diameter. The bore was uniform 0.05 mm through out. A scale, graduated in mm extended over the lower 20 cm. The tube should be clean and dry and kept free from dust. After use it was thoroughly washed in tap water, then rinsed with acetone and allowed to dry before being reused. Sedimentation rate tests were taken at room temperature (21-25°C) because the sedimentation rate is normally accelerated as the temperature rises (Manley, 1957).

The blood sample was shaked thoroughly and drawn into the Westergreen tube upto the 200 mm mark by means of the teat. The tube was placed exactly vertical and left undisturbed for 60 min, free from vibration and draughts, and not exposed to direct sunlight. Then the height of the clear plasma upto the nearest mm mark above the upper limit of the column of cells was found out. This measurement in mm was the ESR of the sample.

ESTIMATION OF SERUM IRON

This method was recommended by the Committee for Standardisation in Haematology (ICSH, 1978) and is based on the development of a coloured complex when ferrous iron is treated with a chromagen solution.

Protein precipitant: 100 g/l trichloroacetic acid and 30 ml/l triglycollic acid in 1 mol/l HCl. This solution was stored in a dark brown bottle for 2 months.

Chromagen solution: 1.5 mol/l sodium acetate containing 0.025% ferrozine (3-2-pyridil)-5, 6-bis-(4-phenylsulphonic acid)-1,2,4-triazine). Stored in a dark brown bottle wrapped in aluminium foil for 2 weeks.
Iron standard (stock) : Dissolve 100 mg of freshly cleaned pure iron wire in 4 ml of 7 mol/l HCL (overnight) and make up the volume to 1 litre with water.

Iron standard (working) : Dilute 2 ml of the stock iron standard in 100 ml water (2mg/l).

Preparation of the glassware : Contamination by iron was avoided. All glassware, including the reagent bottles were washed in a detergent solution; soaked in 2 mol/l HCl for 24 hours and finally rinsed in iron-free water.

Iron-free water : De-ionized, double-distilled water was used for the preparation of all solutions and for rinsing glassware.

0.5 ml of serum (free of haemolysis), 0.5 ml of working iron standard and 1 ml of iron-free water (as a blank) were taken respectively, in three separate iron-free test tubes. 0.5 ml of protein precipitant was added to each. The contents were mixed vigorously with a vortex mixer, and allowed to stand for 5 min. The tube containing the serum was centrifuged at 1500 rpm for 15 min and an optically clear supernatant was obtained. 0.5 ml of the supernatant, 0.5 ml of each of the other mixtures and 0.5 ml of the chromagen solution was thoroughly mixed. After 10 minutes the iron content was measured in a spectrophotometer against water at 562 nm as follows:

\[
\text{A}^{562\text{ test}} - \text{A}^{562\text{ blank}}
\]

\[
\text{Serum iron (\mu mol/l) = } \frac{\text{A}^{562\text{ standard}} - \text{A}^{562\text{ blank}}}{x 35.8}
\]

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ESTIMATION OF SERUM GLUCOSE

REAGENT USED
1. Enzyme reagent 2 vials
2. Buffer solution 2 x 125 ml
3. Glucose standard 100 mg% 1 x 2 ml
Reagents were stored in refrigerator in dark.

PRECAUTIONS
1. Blood was collected in a clean dry EDTA coated vial. Plasma was separated from the cells at the earliest.
2. To avoid degradation of glycolysis sodium fluoride upto 2 mg/ml of blood was used.

REAGENT PREPARATION
One vial of enzyme reagent was mixed gently with one bottle of buffer solution.

PROCEDURE
It was pipetted into clean dry test tubes labelled Blank (B), standard (S), and Test (T). Mixed well and incubated at 37°C for 10 minutes. Then the absorbance of Test (T) and Standard (S), against Blank (B) was measured in a spectrophotometer at 505 nm as follows.

\[
\text{Glucose in mg\%} = \frac{A_{\text{of}(T)}}{A_{\text{of}(S)}} \times 100
\]

\[
\beta-\text{D Glucose} + O_2 + H_2O \rightarrow \text{D-Gluconic acid} + H_2O_2
\]

\[
2H_2O_2 + 4 - \text{Amino antipyrine} + 1,7 - \text{Dihydroxynapthalene} \rightarrow \text{Red Dye}
\]
ESTIMATION OF PROTEIN, TRIGLYCERIDES, CHOLESTEROL AND VLDL (BLOOD)

Protein, Triglyceride, Cholesterol and VLDL were estimated using the instrument, Kodak Ektachem DT system. Kodak Ektachem DT System uses dry chemistry technology. Kodak clinical chemistry slides incorporated dry layer coated technology. All required reactions take place within the slides themselves. Each test requires only a single, individually warped slide, that is bar coated, so that the instrument can identify the test being performed. Each test employs methodology closely correlated to internationally accepted methods for precise and accurate results. A microprocessor controls all the functions of the analyser including the processing and the sequential operation of transporting the slide from the spotting station to the incubator and then to the reading station, for calculation and reporting of test results by an integral printer.

Each unit in the DT system has a specially designed pipette, which is the key to the module to assure correct spotting of slides and provide quality results.

The blood collected by direct heart puncture of the fish is allowed to clot and then centrifuged. Correct slide is selected and placed on the slide carrier, which is then pushed into the analyser and 10 μl of serum is dispensed onto the slide using a special pipette. The analyser automatically processes the slide and prints out the test result.

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<th>Test</th>
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<td>TRIGLYCERIDE and VLDL</td>
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<tr>
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<tr>
<td>TOTAL PROTEIN</td>
<td>2-11 g/dl</td>
<td>BIURET 555 nm</td>
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Cholesterol measurements are used in classifying the risk of coronary trouble and in the diagnosis of various primary and secondary hyperlipidemias. Cholesterol results are be used together with HDL-cholesterol and triglyceride results to calculate LDL-cholesterol levels.

Kodak Ektachem DT Slide (CHOL) is a dry, multilayered film in a plastic support. It contained all the reagents which determined cholesterol levels in serum or plasma.

10 μl of the sample was deposited on the slide. The sample was spread evenly and diffused into the reagent layers. Cholesterol in the sample undergoes a series of reactions in the slide to produce a coloured compound. The intensity of the colour was proportional to the amount of cholesterol in the sample and it was measured by the Kodak Ektachem DT60 Analyser.

**SLIDE INGREDIENTS**

Reactive ingredients included cholesterol oxidase (source: norcardia cholesteralicum), cholesterol ester hydrolase (source: candida rugosa), peroxidase (source: horse radish), and 2-(3,5-dimethoxy-4-hydroxyphenyl) -4,5-bis (4-dimethylaminophenyl) imidazole. Other ingredients included pigment, binder, buffer, surfactants, preservatives, and a polymer cross linking agents.

**Test Instrument** : Test instrument was the Kodak Ektachem DT60 Analyser.

10 μl serum was collected. EDTA was used as anticoagulants of plasma specimens. Special precaution was taken by promptly removing the serum from the clot and analysing it as soon as possible. Samples were handled in stoppered containers to avoid contamination and evaporation.

**Slide storage and Preparation** : Slides were stored at temperatures below 8°C (46°F). At the time of preparation the slides after removal from the box were warmed to room temperature for 15 minutes.

**Calibration kit** : Kodak Ektachem DT-Plus Calibrator and Diluent Set were used.

**Calibrators** : Kodak Ektachem DT-Plus Calibrators, bottles 1,2 and 3 were used.
PRINCIPLES AND PROCEDURE

The spreaded layer distributes the sample evenly on the slide and caused the cholesterol to be dissociated from lipoprotein carriers. As shown in the reaction sequence, Cholesterol ester were hydrolysed to free cholesterol which then passes through a series of oxidation reactions by the specific enzyme, cholesterol oxidase. In the final reaction a coloured dye was produced. The cholesterol was measured by the light reflected from the dyed layer after a fixed incubation period. The DT60 Analyser then calculated the amount of cholesterol present in the sample.

ANALYSER RANGE

35-425 mg/dl.
(0.91-10.99 mmol/l).

WAVELENGTH

555 nm (green LED).

Surfactant

Lipoproteins $\rightarrow$ Triglycerides + Proteins

Lipase

Triglycerides + H₂O $\rightarrow$ Glycerol + Fatty Acids

Glycerol + ATP $\rightarrow$ L-a-Glycerophosphate + ATP

MgCl₂

L-a-glycerophosphate Oxidase

a-Glycerophosphate + O₂ $\rightarrow$ Dihydroxyacetone Phosphate + H₂O₂

Peroxidase

H₂O₂ + Leuco Dye $\rightarrow$ Dye + H₂O₂

ASSAY TIME

Approximately 5 minutes.

CONVERSION FACTORS

Conventional units = mg/dl
SI units = mmol/L

mmol/l = 0.02586 x mg/dl
ESTIMATION OF TOTAL PROTEIN BY KODAK EKTACHEM DT60 ANALYSER

Protein is essential for growth, the production of new tissue and the repair of the injured tissue. An increase in DT levels may be the result of bone deformities or severe dehydration. A dehydrase of DT levels is seen with kidney damage.

The Kodak Ektachem DT Slide (TP) is a dry, multilayered film in a plastic support. It contains all the reagents necessary to determine total protein levels in 10 μl of serum or plasma. (Fig. 7).

The analysis was based on the Beirut reaction in which the protein was treated with a cupric ion (Cu2+) to produce a violet coloured precipitate which was proportional to the amount of total protein in the sample, and was measured by Kodak Ektachem DT60 Analyser.

SLIDE INGREDIENTS

Reactive ingredients included cupric sulphate, tartaric acid, and lithium hydroxide. Other ingredients were binders, surfactants, and a polymer cross linking agent.

10 μl serum was collected from fish by a disposable syringe. Heparin was used as an anticoagulant for plasma specimen. Samples were handled in stoppered container to avoid contamination and evaporation.

Special Precaution: Always avoid the haemolyzed specimens as protein released from red cells may be measured. Promptly remove serum from the clot.

Analysis of Plasma: Results achieved from the analysis of plasma were higher than serum due to the fibrinogen that remains in the plasma. An increase of 0.2 to 0.4 g/dl (2 to 4 g/L) was observed.

Slide Storage and Preparation: Slides were stored at temperature below 8°C(46°F).

Calibration:

Calibration Kit: Kodak Ektachem DT-Plus Calibrator and Diluent Set were used and Kodak Ektachem DT-Plus Calibrator’s bottels 1,2 and 3 were used.
PRINCIPLES AND PROCEDURE

10 μl of the sample was deposited on the slide and evenly distributed to the spreading layer. When the fluid penetrated the reagent layer, diffusion occurred and the reaction began. The reaction between the protein and cupric tartrate took place largely in the spreading layer where the protein was contained because of its high molecular weight. The amount of coloured compound formed was proportional to the amount of total protein in the specimen.

After a fixed incubation period, the amount of total protein present in the sample was calculated by the DT60 Analyser and the value was presented as g/dL.

Reaction Sequence:

\[
\text{Protein + Cupric Tartate} \xrightarrow{\text{LIOH}} \text{Coloured Complex.}
\]

Analyser Range:

2.0-11.0 g/dL

(20-110 g/L)

Wavelength:

555 nm (green LED)

Assay Time:

Approximately 5 minutes.

Conversion factors:

Conventional units = g/dL

SI units = g/L

\[g/dL = 10 \text{ g/L}.\]
ESTIMATION OF TRIGLYCERIDE AND VLDL BY KODAK EKTACHEM DT60 ANALYSER:

Triglyceride measurements are used in the diagnosis and treatment of various primary and secondary hyperlipidmias. Triglyceride contents can be used together with the cholesterol and HDL-cholesterol contents to calculate the VLDL-cholesterol level.

The Kodak Ektachem DT slide (TRIG) is a dry, multilayered film in a plastic support. It contains all the reagents necessary to determine triglyceride levels in serum or plasma. (Fig. 7)

10 μl of the Sample was deposited on the slide. The samples were spread evenly and allowed to diffuse into the film layers. Triglycerides in the sample undergoes a series of reactions in the slide to produced a coloured compound. The intensity of the colour was proportional to the amount of triglyceride in the samples and was measured by the Kodak Ektachem DT60 Analyser.

Slide Ingredients:

Reactive ingredients were lipase M, peroxidase, ascorbate oxidase, glycerol kinase, L-α-glycerophosphate oxidase, 2 - (3 , 5 - dimethylaminophenyl) 4,5 - bis (4-dimethylaminophenyl) imidazole, adenosine triphosphate, and magnesium chloride. Other ingredients include pigment - binder, buffer, surfactants, solvent, antioxidants, inhibitors, and a polymer crosslink agent.

10 μl serum or plasma was collected from individual fish. Heparin and EDTA used as anticoagulants for plasma specimens. Analysis was performed very quickly. Samples were handled in stoppered containers to avoid contamination and evaporation.
PRINCIPLE AND PROCEDURE

10 μl of sample was evenly spread on the slide, that caused the triglyceride to dissociate from lipoprotein carriers. Triglyceride molecules were hydrolysed by lipase to glycerol and fatty acids.

The scavenger layer contained an enzyme, ascorbate oxidase, that removed any ascorbic acid present in the sample, so that it will not interfere with the test.

Glycerol molecules were small enough to diffuse through the scavenger layer to the reagent layer where they had gone further reactions as shown by the reaction sequence. Finally a coloured dye was produced. The amount of light reflected from the dye layer was measured in DT60 Analyser from which the amount of triglyceride and VLDL present in the sample was calculated and reported as mg/dl units.

Reaction Sequence:

Analyser Range:

15 - 400 mg/dL

(0.17 - 4.57 mmol/L)

Wavelength:

555 nm (green LED)

Assay Time:

Approximately 5 minutes

Conversion Factors:

Conventional units = mg/dL

Si units = mmol/L

mmol/L = 0.01143 x mg/dL
KODAK EKTACHEM DT SLIDES.
2.5 ESTIMATION OF CHOLESTEROL FROM LIVER, BRAIN AND INTESTINE

The fishes were weighed and sacrificed by severing their heads. The brain, intestine and liver were quickly dissected out and rapidly frozen and processed for the various biochemical analysis.

The tissue (liver, brain and intestine) were soaked in whatman filter paper no. 40 and weighed in single pan balance. Then the samples were grinded thoroughly with anhydrous sodium sulphate. A mixture of anhydrous acetone (15 ml) and absolute ethanol (5 ml) was added to the sample and the sample was centrifuged at 3000 rpm. Fifteen ml of anhydrous ether was added to the residue and shaken. Ether layer was separated and then evaporated to dryness in a vacuum protected by nitrogen. Chloroform was added to the residue. Zlatkis and associates (1953) have developed a method for the quantitative determination of cholesterol based upon reaction with a reagent containing ferric chloride, glacial acetic acid and concentrated sulphuric acid. Cholesterol gives a purple colour with this reagent. The intensity of the final colour complex is measured calorimetrically between 560 to 600 nm. Appropriate amount of the sample was taken and 20 ml glacial acetic acid was added to it. Then 5 ml of cholesterol reagent was added. The contents of the tubes were mixed simultaneously for 10 seconds and then placed in the boiling water bath for exactly 90 seconds. It is then immediately cooled in running tap water for 5 minutes. The content of each tube was mixed thoroughly and optical density of the test sample was measured at 560 nm.
2.6 ESTIMATION OF TOTAL PROTEIN CONTENT FROM LIVER, BRAIN AND INTESTINE:

The tissues were quickly soaked in whatman filter paper no. 40 and rapidly weighed in a single pan balance. 8% TCA (ice cold) was added to the tissue and homogenization was done using a homogenizer with toflon pestle at a median speed for about 2 minutes. The homogenized tissue was then centrifuged for 30 minutes at 3000 rpm. Usually this process is repeated twice so that extraction of Ascorbic acid supernatant is complete and the supernatant is used for estimation of Ascorbic acid. Ethyl alcohol, chloroform and ether were added to the residue and centrifuged at 3000 rpm. Then the supernatant was discarded and the residue was dissolved in 4% NaOH solution for protein estimation.

The protein was estimated following the method of Lowry et al., (1951) using folins phenol reagent. 5 ml of protein reagent was added to the test sample after appropriate dilution and after 10 minutes of the addition of propein reagent. Folins phenol reagent was added. The solution was shaked and allowed to stand for ten minutes. A calorimeter was used and the readings were recorded at 660 nm. Bovine albumin was used for preparation of standard curve.

Preparation of Protein Reagent:

Anhydrous Na$_2$CO$_3$ (30 gm) and NaOH (4 gm) were dissolved in a litre of distilled water. To 100 ml of this solution, 1 ml of 4 % sodium tartarate and 1 ml of 2% copper sulphate solution were added. Freshly prepared solutions were used for each set of test.
Preparation of Phenol Reagent:

The reagent is either prepared in the laboratory or purchased from BDH (India). In a 1500 ml round bottom flask, 100 gms of sodium Tungstate, 25 gms of Sodium Molybdate, 700 ml of distilled water, 50 ml of 85% phosphoric acid and 100 ml concentrated hydrochloric acid were taken. The mixture was refluxed gently for 10 hours. Then 150 gm of lithium sulphate, 50 ml of distilled water and a few drops of bromine water was added and the mixture was boiled under hood for 15 minutes, to remove the excess of bromine. The solution was cooled, diluted to 1000 ml and filtered. In case the solution turns greenish on standing, a drop of bromine was added and again boiled as above. This phenol reagent was suitably diluted before use.

2.7 ESTIMATION OF ASCORBIC ACID FROM LIVER, BRAIN AND INTESTINE

After soaking in Whatman filter paper n. 40 the tissue (liver, intestine and brain) were quickly weighed in a single pan balance. A 8% TCA (ice cold) extract of the tissue was prepared after homogenizing it in a homogenizer and centrifuging for 30 minutes at 3,000 rpm. Usually this process was repeated twice and the supernantants were pooled. Samples from this TCA extract were used for the calorimetric estimation of the biochemical constituents.

The ascorbic acid was estimated following 2-4 dinitrophenyl hydrazine method of Roe (1954) with a modification that the samples were incubated at 57° C for 45 minutes as suggested by Tiwari and Pandey (1965). Appropriate amount of the sample was taken in a test tube, 2-3 drops of bromine water was added to it and 0.5 ml of 2-4 DNPH thiourea reagent was added. The test tube was incubated at 57° C for 45 minutes and then transferred to an ice cold beaker containing ice
water for 1 hours. Then 5 ml of 85% sulphuric acid was added to the test tube from the burette and kept for half an hour at room temperature. Calorimetric reading was taken at 530 nm using a green filter.

2.8 PREPARATION OF SYNTHETIC HYDROXYLAPATITE

Samples of AsHA is prepared by co-precipitation at 37° to 50°C in a CO₂ free atmosphere by mixing stochiometric quantities of the reactants in the form of their solutions maintained at pH of about 12 (Patel, 1983).

All chemicals used were of either A.R. (B.D.H.) or E. Merck quality. All the interacting solutions were prepared in CO₂ free distilled water. The preparation of the samples of calcium arsenate hydroxylapatites was based on the following equation:

\[
10 \text{Ca(NO}_3\text{)}_2 + 6 (\text{NH}_4\text{)}_2\text{H}_2\text{PO}_4 + 14 \text{NH}_4\text{OH} \rightarrow \text{Ca}_{10}(\text{PO}_4\text{)}_6(\text{OH})_2 + 20\text{NH}_4\text{NO}_3 + 12 \text{H}_2\text{O}
\]

The proportion of phosphate to Arsenate (ASO₄⁻³) was altered in the interacting solutions according to the product desired. Based on the above equations calculated amounts of calcium nitrate, diammonium hydrogen phosphate and diammonium hydrogen arsenate were taken for an yield of about 25.0 g of the sample in each case. Since calcium nitrate, Ca(NO₃)₂·4H₂O could not be accurately weighed, stock solutions of it was prepared and its calcium content was determined by complexometric procedure (Nakamoto, 1971). The following solutions were prepared. (Fig. 8).
ASSEMBLY OF APPARATUS USED FOR THE PREPARATION OF THE
SAMPLES OF SOLID SOLUTIONS OF HA AND AsHA.
Solution No. 1: 500 ml of a solution containing the calculated volume of the stock solution of calcium nitrate.

Solution No. 2: 500 ml of a solution containing calculated quantities of diammonium hydrogen phosphate and diammonium hydrogen arsenate, so that phosphate to arsenate ratio corresponded to the desired solid solution of calcium arsenate hydroxyapatite to be prepared.

Solution No. 3: 500 ml of 25% liquor ammonia, solution 1 and solution 2 were taken in two separate dropping funnels, each of 150 ml capacity fitted into the cork of a suitable sized 3-necked flask of 2 litre capacity in which 200 ml of calcium nitrate solution was taken. A 25% solution of liquor ammonia was taken in another separating funnel of the flask the flask was kept in a water bath maintained at 37±0.5°C. A side tube was inserted into the central neck of the flask which was connected to a pump which removed air from the flask when the pump was in the working condition. This operation kept a continuous bubbling of CO₂ free air into the flask using a series of sodalime and conc. KOH towers. Solution 1 and 2 were maintained at a pH of about 12 by addition of liquor ammonia solution. The solutions 1, 2 and 3 were added simultaneously dropwise into the flask while CO₂ free air was bubbled through the medium of precipitation to eliminate the formation of carbonate apatite. Such a bubbling, in addition, kept the medium well stirred. The precipitate was aged by boiling under reflux in contact with the mother liquor for about 45 min. It was then left overnight in contact with the mother liquor and connected to soda lime towers. The precipitate was separated by filtration, washed till the washed liquid was neutral and free from calcium ions. A part of the yield was washed with acetone.
and the rest was heated to about 100°C for 8 h. It was then powdered and sieved to the desired particle size. For preparation of the end members, the solution No. 2 contained only diammonium hydrogen phosphate of diammonium hydrogen arsenate. Care was taken to see that all the experimental conditions were scrupulously maintained the same during preparation of all the samples to check the likely alteration in the nature of the precipitated samples. The yield was found to be 80 to 90% of the theoretical value in each case. AsHA, however was found to be of higher yield.

2.9 PREPARATION OF NATURAL SAMPLES FROM FISH, CHANNA PUCTATUS

The study of AsO₄³⁻ uptake was extended to natural hydroxylapatite obtained from fish bone. The fishes were killed with one blow on the head and boiled for one hour. The muscles were teased out and the bones were removed and dried then deproteinated as follows:

**Step - I**: The bone was treated with 25% hydrazinehydrate at room temperature (37°C) for an hour and then kept at 55°C for an hour.

**Step - II**: The bone was then treated with 50% hydrazinehydrate for 7 hours at room temperature and kept at 55°C for an hour.

**Step - III**: The bone was treated with 75% hydrazinehydrate for twentytwo hours at room temperature and kept at 55°C for two hours.

**Step - IV**: The bone was treated with 95% hydrazinehydrate for twentyfour hours at room temperature and then kept at 55°C for two hours.

**Step - V**: The bone was treated with 50%, 75%, 87.5% and 100% ethanol for half an hour each. The bone was later dried in acetone vacuum and stored in a desiccation.
The bone was then heated at 900°C in Muffle's furnace for 24 hours to remove the volatile constituents. The resulting lumps were powdered and sieved to the desired particle size (85 BSS). Chemical composition (Calcium and phosphorous content) of the sample was determined by complexometric procedure.

2.10 ESTIMATION OF CALCIUM AND PHOSPHOROUS WITH NH₄H₂ASO₄

A convenient amount of the sample (0.4 gm) 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 was added. Phosphate was precipitated at 50°C as ammonium phosphomolybdate by adding 10 percent solution of ammonium molybdate until no further formation of yellow precipitate from the supernatant liquid was perceptible. The precipitate was filtered through IG₄ crucible and was washed several times with a solution of cromiuninitrate and 20 percent 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 a 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 an appropriate pH indicated by the blue colour of thymolblue 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 IG₄ 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 2.0 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 bule
colour changes to wine red. From the volume of magnesium sulphate used 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 was added
to maintain the desired pH. Then 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 the colour changes from yellow to purple.

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

2.11 DETERMINATION AND UPTAKE OF ARSENATE

Arsenate is determined by an indirect process based on the precipitation of
magnesium ammonium arsentate, and the subsequent titration with EDTA of the
magnesium contained in the precipitate.

To the sample containing the arsenate ion solid disodium EDTA and a slight
excess of ammonium hydroxide were added. It was diluted to 100 ml, and 0.1 M
solution of MgCl₂·6H₂O was added dropwise until a permanent turbidity occurs and
then an additional 20 ml of the magnesium chloride solution and 20 ml of conc.
NH₄OH were added. It was allowed to stand for 12 hours, then filtered and the
precipitate was washed with 1M NH₄OH containing 2-3 percent ammonium nitrate.

The precipitate was dissolved in 10 ml of conc. HCl. It was neutralised and
then made slightly acidic. Then it was cooled and 20 ml of 0.1 M disodium EDTA was
added and diluted to 100 ml. The pH was adjusted by adding an ammonium chloride
ammonium hydroxide buffer. Excess of EDTA was titrated with 0.1 M magnesium
chloride solution using Erichorome black T indicator. From the volume of magnesium chloride in the filtration the amount of arsenate was calculated. Total phosphate and arsenate in the same solution was determined by titrating with EDTA in presence of Erichrome black T at a pH of 10. The phosphate content is determined after volatilising arsenate from the same quantity of another aliquot as arsenious bromide by the treatment of a mixture of hydrobromic sulphuric acid solution. The difference between the former and later experiments gives the arsenate content.

Since arsenate is isoelectronic and isostructural with phosphate, and pure HA, Ca$_{10}$(PO$_4$)$_6$(OH)$_2$ is isomorphous with pure AsHA, Ca$_{10}$(AsO$_7$)$_6$(OH)$_2$; PO$_4$$^3$: in HA can be isomorphously substituted by AsO$_4$$^3$: present in the solution. This substitution reaction is favoured by factors like the time and temperature of equilibration of the solid HA with a solution containing arsenate and the particle size of the solid apatite.

The dependence of uptake was studied by equilibrating 0.4gm of bone (synthetic sample and natural sample) containing calcium and phosphorus and a mole ratio of Ca/PHA with 100 ml of a solution containing sodium arsenate by constant shaking. The factor effecting the uptake under investigation was changed each time while other factors were kept constant. In order to demarcate the different stages of exchange process the reacting mixtures were refluxed at about 100°C at a given set of experimental conditions. 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 was free from absorbed arsenate ions and the solid was analysed for arsenate by the method used earlier. The dependence of uptake was studied at room
temperature (28°C) and boiling temperature of the mixture at about 100°C. The concentration of the solutions were varied from 0.01 M to 0.10 M of sodium arsenate. The pH range during this investigation was between 5.0 to 7.5.

METHODS USING O-CRESOL PHTHALEIN COMPLEXONE

CPC introduced by Andereg et al., (1954) forms a deep coloured complex with calcium and magnesium in alkaline solution, due to lactone formation in the pathalein part of the molecule. In the early methods calcium was precipitated as the oxalate, which was then converted to carbonate by heat before complexing with CPC (Stern and Lewis, 1957). In the Auto Analysers method using this reagent, Kessler and Wolfman (1964) diluted the bone ash with acid to split the calcium protein complex, dialysed the calcium into an acid recipient stream and added CPC reagent before developing the colour with the diethylamine as base. Connerty and Briggs (1966) showed that interference from magnesium could virtually be eliminated by including 8-hydroxy quinoline in the CPC reagent. Gittleman (1967) introduced this into the Auto Analyser procedure which was further modified by Hall and Whitehead (1970) who incorporated the 8-hydroxy quinoline into the acid-diluent and dialysed into a CPC-hydrochloric acid sodium acetate reagent before adding the base.

Manual methods using the above reagents directly have not been entirely satisfactory but Baginski et al., (1973) described a modified CPC reagent suitable for paediatric use as it required only 20 ml solution.
AUTO ANALYSERS METHOD (GITLEMAN, 1967; HALL AND WHITE HEAD, 1970)

The sample is diluted with an acid reagent containing 8-hydroxy quinoline to minimise interference from magnesium and the calcium dialysed into a recipient stream containing CPC and acid followed by addition of base reagent to develop the colour.

Reagent:

(1) Diluent: - To about 500 ml water add 83 ml. concentrated hydrochloric acid and mix. Then add 5g 8-hydroxyquinoline mix, add 0.5 ml Brij 35 solution (250 g/l) and make to 1 litre. This can be kept at room temperature for at lest 2 weeks.

(2) Colour reagent: To about 500 ml water add 83 ml concentrated hydrochloric acid, mix and cool. Add 55mg cresol-phthalein complexone, mix thoroughly until dissolved. Dissolve 40 g sodium acetate trihydrate in about 200 ml water and mix this with the CPC solution. Add 0.5 ml Brij (250 g/l) and make upto 1 litre. This reagent is decomposed by sunlight and exposure to the atmosphere. Store at 4°C in a dark container well stoppered when not in use, for not more than 2 weeks.

(3) Base reagent: Dissolve 500 mg potassium cyanide in 500 ml water and add 150 ml diethylamine, make upto a litre and mix. Store in an airtight container to prevent loss of the volatile diethylamine.

(4) Stick calcium solution : 12.5 mmol/l (50 mg/ 100 ml).

(5) Stock magnesium solution : 40 mmol/l (97.2 mg/100 ml).

(6) Working calcium standards : All the standards contain 0.8 mmol/l magnesium (1.95 mg/100ml). Dilute the stock standards with water to make the final volume 100 ml as follows :
Final Concentrations:

Calcium: (mmol/l) 0.625, 1.25, 1.875, 2.5, 3.125, 3.75, 4.375, 5.0.
(mg /100 ml) 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0

Magnesium: (mmol / l) 0.8, 0.8, 0.8, 0.8, 0.8, 0.8, 0.8.
(mg /100 ml) 1.95, 1.95, 1.95, 1.95, 1.95, 1.95, 1.95.

Stock Calcium Solution: (ml) 5, 10, 15, 20, 25, 30, 35, 40.

Stock Magnesium Solution: (ml) 2, 2, 2, 2, 2, 2, 2.

Technique:

Place the mixing coils and delay coils in the water bath, preferably enclosed, of 5 to 10 litre capacity. Run at 40 samples/h using a samplers with 2:1 sample.

The calorimeter has a 15 mm flow cell and interference filter of 580 nm. Pump the reagents until a steady base line is achieved and then introduce the standards in ascending order. Follow the last standard by a 2.5 mmol / l standard and then the serum samples. Repeat the 2.5 mmol, standard every tenth sample to correct for any baseline drift.

Note: As calcium may be absorbed on to auto Analysers cup; specimens for analysis should be transferred to the cups shortly before assaying. It should not be stored in capped cups overnight.
Auto Analysers flow diagram for determination of calcium by cresolphthalein complexone has been given in (Fig. 9). The method is that of Hall and Whitehead (1970). The broken outline represents a water bath at room temperature containing a single mixing coil (SMC) double mixing coil (DMC) and a delay coil of 6m length.

**Method**:

O-Cresol phthalein complexone, without deproteinization.

**Test Principles**:

Ca\(^{2+}\) forms a violet complex with O-Cresol phthalein complexone in an alkaline medium.

**Preparation and Stability of Solutions**:

R\(^1\)

1. Buffer - use solution as supplied. Stable up to the expiry date specified when stored at +15 to 25°C.

R\(^2\) (Starter reagent)

2. Chronogen: use contents are supplied. Stable up to expiry date specified when stored at +15 to 25°C.

**Sample material**:

Samples are stable for ten days at +20 to 25°C, ten days at +4°C and eight months at -20°C.

**Calibration**:

Calibrantor was used for automated system. Values were expressed in mg / dl. Multiply expected value (in mmol / l) by factor 4.
Fig. 9

FLOW DIAGRAM OF AUTO ANALYSER

WASTE

WASTE

Single Dialyser

SMC

DMC

37° C

SOLVA FLEX

SOLVA FLEX

37° C

SOLVA FLEX

WASTE

WASTE

Sampler II

40/h

Sample wash ratio - 2

Flow rate

Reagent ml/min

2.0 Water

0.16 Sample

2.0 Reagent I

1.6 Air

2.5 Reagent II

2.0 Air

2.0 Reagent III

2.5 F/C

Colorimeter

15 mm Flow cell

580 nm

Recorder
Instrument Settings:

Temperature: 30°C/37°C

Program 6 Chemistry Parameters.

Test Code: 9 (CA)

Assay Code (End point): 1

Sample Volume (ml): 10

R1 Volume (ml): 350 - No.

R2 Volume (ml): 350 - σ - No.

Boehringer Mannheim GmH Instrument Depositment Hitachi 705:

R³

Wave length 1: 660 NM

Wave length 2: 546 NM

RGT. BLK. ABS.: -

RGT. BLX. CONC.: 0

STD. CONC.: σ σ σ

Factor: -

STD. ABS. Allowance: 10%

Normal Range (mg / dl): 8.1

Normal Range H (mg/dl): 10.4

Abs. Limit (Rate): 0

Control ID. No.: σ - σ - σ

Program 7 Channel Setting

Channel No.: σ

Test Code: 9 - 9

σ Date entered by operator

- Determined by instrument
Limitations of method:

If the calcium concentration exceeds 15 mg/dl (3.75 mmol/l), then mixing requires 100 ml of sample with 100 ml of redist water (result × 2).

Normal values in Serum²:

8.10 - 10.4 mg/dl (2.02 - 2.60 mmol/l)

Quality Control:

For control of accuracy: Precinorm® U, Precinorm® S, Precipath® U, Precipath® S.
For Precision Control: Precinorm® UPX.

Initial Concentrations of Solutions:

1. 2-Amino-2methylpropanol - (1) 3.5 mol/l pH 10.7.
2. 0-Cresolphthalein Complexone:
   0.16 mmol/l; 8-hydroxy quinoline: 6.89 mmol/l; hydrochloric acid: Ca 60 mmol/l.

Concentration in the test:

2. 2-Amino-2methylpropanol - (1) 1.7 mol/l pH 10.7
0-Cresolphthalein Complexone: 0.08 mmol/l;
8-hydroxy quinoline;
3.4 mmol/l; Hydrochloric acid: Ca 30 mmol/l

Phosphorus - UV

Catalog No. - 749087

Principle: Inorganic phosphate reacts with ammonium molybdate in an acidic solution to form ammonium phosphomolybdate with a formula of (NH₄)₃[P(MO₃O₁₀)₄]₁. The ammonium molybdate is quantified in the ultraviolet range (340 nm).
Specimen Collection:

Serum:

Specimens are stable up to 8 hours at room temperature, one day in refrigerator and if frozen at 20°C for up to one year.

Reagents:

The phosphorus UV reagents are intended in vitro diagnostic use.

The components of the phosphorus UV system pack for HITACHI 705 include:

1. **Specimens Blank Reagent**:

   Non reactive ingredients: Sodium Chloride, Sulphuric acid, Surfactant.

   **Precautions**: Warning - Irritant, never pipette by mouth. In case of contact, flush affected areas with copious amounts of water. Requires immediate medical attention for eyes.

   **Storage**: Store tightly closed at 20 - 25°C.

   Reactive ingredient.

   2.0 mmol/l Ammonium molybdate.

   Non reactive ingredients.

   Sodium chloride, Sulphuric acid.

   **Precaution**: As before.

**System pack for Hitachi® 705 for approximately 1542 (3x514) tass®**:

**Preparation**:

1. For $R_1$ working solution, Bottle 1 specimen Blank reagent was used as supplied. No preparation is required. The $R_1$ working solution is stable at 20 - 25°C until the expiration date printed on the bottle.
2. For $R_2$ working solution, Bottle 2 (Phosphorus UV Reagent) was used as supplied and proceed as before.

**Instrumental setting** : 37°C

**Channel setting** : (CRT Page 7)

**Channel No.**
- Test Code : 29 - 29
- Chemistry Parameters : (CRT Page 6)
- Test Code : 29 (phos)
- Assay Code : (End point)
- Sample Volume : 9 ml
- $R_1$ Volume : 350 ml - N
- $R_2$ Volume : 350 ml - N
- $R_3$ Volume : -------
- Wave length 1 : 376 nm
- Wave length 2 : 340 nm
- RGT, BLK, ABS : 0
- RGT, BLK, CONC : 0
- STD. CONC : -------
- FACTOR : 0
- STD. ABS. Allowance : 10%
- Normal Range L : 30 mg / dl
- Normal Range H : 4.5 mg/ dl
- ABS Limit (Rate) : 0
- Control ID No. : -------

- Donestes use or instrument specific settings.
CALIBRATION:

Calibrator system pack for HITACHI® 75 catalog no. 608432 (20 x 3 ml). The Hitachi® 705 microcomputer use the absorbance measurements to calculate phosphorus concentration as follows:

\[ C_x = K (A_x - A_b) + C_b. \]

Where  
\( C_x \) = Concentration of sample.

\( K \) = Concentration of factor.

\( A_x \) = Mean of absorbance of sample + \( R_1 \) + \( R_2 \) read at positions 30 and 31 minus mean of absorbance of blank + \( R_1 \) read at position 14 and 15.

\( C_b \) = Concentration of reagent blank.

............ Corrected for reagent / sample volume by \( K = \frac{(\text{Sample volume} + R_1)}{(\text{Sample volume} + R_1 + \text{IR})} \).

Linearity : 2.00 mg/dl.

Expected Values 3. : 3.0 - 4.5 mg/dl.

Quality control:

Precinorm U. Catalog No. 171743 (20x5 ml).

Precipath U. Catalog No. 1717778 (20x5 ml).

Specific Performance Characteristics:

Precision : Precision studies using the package reagents and control sera on the Hitachi® 705 yielded the following results:
## Within Run:

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<th>SD</th>
<th>CV</th>
<th>Mean Value</th>
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<tbody>
<tr>
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<td>0.11 mg/dl</td>
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<tr>
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## Run to Run:

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<tr>
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<tr>
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<tr>
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<td>1.4%</td>
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<td>0.14 mg/dl</td>
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**Accuracy (method comparison):** A comparison of this method on the Hitachi® 705 with the manual procedure results in the linear regression equation of $Y = 1.00x + 0.06$ and a co-relation of 0.997.
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** Next page **

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6. Chemistry Parameters

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<td>20 **</td>
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</tbody>
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Stand - BY
Temp 37.0
S.No. 144

**
MULTI-ANALYSERS- HITACHI 705
The New Generation of Multi-analysers Systems

1. Front View (Fig. 10)
   - Compact selective multi-analyser
   - a new operating principle
   - easy "push-button" operation

Fig. 10
HITACHI 705 (MULTI-ANALYSING SYSTEMS)
2. Sampler + Sampler Disk

Routine samples, stat samples, standard solutions and control sera were loaded on the sample turnable. The sampling proved aspirated the serum and pipetted it into each reaction cuvette on the reaction disk. (Fig. 11)
3. Cooling Unit + Pipetting System

The reagent pipetting unit aspirates the required volume from a container in the built-in cooling unit. The reagent was brought to reaction temperature before being pipetted in to the reaction cuvette.

(Fig. 12 and 13)
Operating Principle Diagram

- sampler
- mixer
- reagent dispensing system
- refrigerator
- photometer
- washing unit
- reaction rotor
- electrodes (Na, K, Cl)
- sample disk
- incubation bath

Fig. 13
4. Reaction Disk + Washing Unit (Fig. 14)
After each sample had been measured, the reaction cuvette was thoroughly washed. Distilled water was pipetted into the cuvette and the absorbance was measured. This water was aspirated and the cuvette was then ready for the next test.

5. Level Sensor (Fig. 15)
Both the reagent and sampling systems were equipped with a level sensor. Advantages: a 5 to 10 ml vacutainer could be placed directly on the sample disk. The amount of reagents in the reagent container could always be requested.
Rotation: 13 sec.; Stop: 7 sec.
Rotation-interval: 20 sec.
Test remains in the cuvette: 31 rotations = 10 minutes
Preparation of the cuvettes for the first test: 11 rotations = 3 min. 40 sec.
Reading for "One point": after the 30. + 31. rotation
Reading for "Two point": after the 14., 15., 30. + 31. rotation
Reading for "Rate II": after the 19. up to the 31. rotation (programmable)
For linearity-check: at least 5 measuring-points = 4 $\Delta$ ABS
6. Photometer Position (Fig. 17, 18)
Absorbance were measured directly in the reaction cuvette at two wavelengths (bichromatic measurement). The disk holding the reaction cuvettes rotated so that each cuvette passed through the optical path of the spectrophotometer. Measurements were taken at 20 second intervals over a period of 10 minutes. If necessary a second reagent might be added to the reaction mixture during this time.
Fig. 18

Concave grating
Rowland circle
11 Photodetectors, silicon cells
Light source
Converter
LOG Converter
Wave Length Selector
A/D Converter
Micro-Computer
Printer
7. Display (Fig. 19)

13 programs could be visually checked at any time on the display.

8. Operating Panel (Fig. 20, 21)

The system had been designed for easy operation. Loaded the samples on the sample disk and key in the requested tests for each sample. The microprocessor does the test.
2.12 ANALYTICAL PROCEDURE

(a) Disodium EDTA: It is a white, crystalline compound occurring with two molecules of water of hydration. The sodium salt of EDTA (ethylene diamine tetra-acetate) has the composition $\text{Na}_2\text{H}_2\text{C}_{10}\text{H}_{12}\text{O}_6\text{N}_2\cdot2\text{H}_2\text{O}$ and is represented by the abbreviated formula $\text{Na}_2\text{H}_2/2\text{H}_2\text{O}$. Its molecular weight is 372.10. This compound is obtained in such purity that standard solution can be prepared by direct weighing and a standardisation procedure is not necessary. Dilute of convenient concentration was prepared from the stock solution.

(b) Buffer Solution of a pH about 10: Many of the EDTA titrations are carried out in solution buffer to a pH 10. For the most part, ammonium chloride, ammonium hydroxide buffers are used. The most widely used buffers are as follows:

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

(c) Magnesium Sulphate Solution: About 22.60 g of Mg SO$_4$7H$_2$O was dissolved and made up to 1 litre to give an approximate 0.1 M solution. This was subsequently standardised by titrating complexometrically against 0.1 M EDTA solution. Solution of desired concentration were prepared by dilution of the stock solution.

(d) Indicator Solution:

(i) Murexide Indicator: This compound is the ammonium salt of purpuric acid ($\text{C}_8\text{H}_5\text{N}_5\text{O}_6$). 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.
(ii) Eriochrome Black T: This compound is one of the most widely used metal indicator in EDTA titrations and is sold under various trade names, such as Erichrome Black T, Erichrome schwartz T, Pentachrome Black T, Pelting Black C, Diamond Blue Black - EBS, Omega Chrome Blacks and chromagen Black special ETOO (Russian). It is no. 203 in the "colour Index". Chemically it is sodium 1-(1-hydroxy-2-napthylazo)-5-nitro-2-naphthol-4-sulphonate. A fresh solution of it is prepared for each set of titration. A stable (at least for 7 months) and covenant form of Erichrome Black T indicator was prepared by dissolving 0.2 g of indicator in 100 ml triethanol amine.

(iii) Phthalein Complexcne Indicator: Other names applied to this indicator are metalphthalein, O-cresoI phthalein complexone, phthalein purple and phthalein purpur. Phthelein complexone is almost colourless and is insoluble in pure water. A stable form of indicator was prepared by dissolving in triethanol amine.

2.13 X-RAY DIFFRACTION ANALYSIS

The formation and homogeneity of the solid solutions of CaHA and AsHA (synthetic and natural) can be confirmed by X-ray diffraction studies of the samples. Since AsHA is known to exist in naturally occurring minerals mostly in the form of mixed apatites, the possibility of PO\textsuperscript{3-} - AsO\textsubscript{4}\textsuperscript{3-} exchanges reaction in synthetic samples was considered. The isoelectronic and isostructural considerations of the interacting ions is the factor which facilitates the formation of such solid solutions, though the ionic sizes of the participating ions are also important factors. When PO\textsubscript{4}\textsuperscript{3-} is isomorphously substituted by AsO\textsubscript{4}\textsuperscript{3-} in HA.
The X-ray diffraction patterns of the samples were recorded by using a Debey Schoerrerr Unicam camera of 9 cm dia, with a rotating sample exposed to Cu. Ka (Nickel filtered) radiation with a graphite monochromator using a Siemens powder Diffractometer, whose voltage, current strength, and exposure time being 34 KV, 15 ma, and 5 h respectively. The distance between each line on Debey Schoerrerr pattern and the point where the transmitted beam would strike the film was measured. Sharp peaks separated by less than 0.1 degree (2q) can be resolved with a 0.006 inch entrance slit at a scanning speed of 1 degree (2q) per minute for all the samples by a micrometer scale from which the values for 'd' was calculated. In most of the cases only a few reflection planes could be indexed. Knowing the lattice parameters, the unit cell volume was obtained using the formula \(\frac{a^3}{2}\) for the hexagonal apatite lattice.

The X-ray density of the samples was calculated by using the formula \(\frac{MZ}{V} \times 1.6602 \text{ g cm}^{-3}\), where \(M\) = the molecular weight of the sample, \(Z\) = the number of the formula units within the unit cell, \(V\) = the unit cell volume. 1.6602 is the conversion factor. A relationship between X-ray density and the compositions of the solid solution between the end members was studied. From this relationship the formation of the ideal solid solution in the thermodynamic sense or otherwise was investigated by Donald et. al., (1971). The d-spacing and the relative intensities of the investigated materials were theoretically calculated using available programmes by the well known Azaroff method (Azaroff, 1952).
The unit cell mass was calculated by knowing the number of various atoms present in the accepted unit cell, and the number of formula units within the unit cell. The calculated cell mass, \( M_g \), the weight in grams per unit cell is expressed by the following equation:

\[
M_g = \frac{M}{6.023 \times 10^2}
\]

Where, \( M \) = molecular weight in gram corresponding to the chemical formula of the solid solution.

A set of three planes conveniently indexed were chosen and tested for their zone conditions by applying Weiss Zone Law (1971) using the relationship \( U_h + V_k + W_l = 0 \) where \( U, V, W \) are indices of the point of intersection of \( hkl \) plane. The tests were done to understand whether the chosen set of faces were parallel or vice versa.

**2.14 INFRA RED SPECTRAL STUDIES**

The infra red spectra of the samples were recorded as KBr Pellets using PERKIN- ELMER Model 137E Spectrophotometer. The assignment of major frequencies observed were made by Nakamato (1971). The observed changes in the peak length ratios and shift of the vibrational peaks were correlated with the changes in the composition of the solid solution.

The degree of splitting of certain IP 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 solution, in addition to the static field, the dynamic interaction between the ions also has strong influence on the internal
vibrations of PO$_4^{3-}$. This results in the alteration in the position and shape of infrared spectra corresponding to the phosphate ion. The shift in vibration of frequency of phosphate ion depend upon the binding energy and the atomic mass of the subsequent, in the lattice structures according to the equation of Barnes et al., (1944).

This gives a relationship between frequency, atomic mass and force constant.

The vibration frequency is dependent on the reduced mass $m$ of the participating atoms and 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.

2.15 ELECTROMICROSCOPIC INVESTIGATIONS

Electromicroscopic investigations were primarily intended to supplement the X-ray diffraction results and to confirm the homogeneity of the solution prepared. The absence of the extraneous phases present in the samples could also be confirmed in the electromicroscopic scanning as a test for the purity of the samples.

The electromicroscopic scannings of all the samples were done using Siemens Elminskop 1, No. 591, with water as dispersion medium and carbon as background. The suspense of the solid hydroxylapatite in water was subjected to ultrasonic dispersion and the supernatant liquid was used for scanning. The samples used were only acetone washed and air dried. From each micrograph the length and breadth of a few individual crystals of varied dimensions were measured by the help...
of a precision travelled microscope and their average dimensions were calculated. Considering the crystals to be minute cylinders, surface area of the samples were calculated. The specific surface areas of the samples were given by the formula:

\[
\frac{2r^2 + 2\pi rh}{\pi r^2 h}
\]

Where \( r = 0.5 \) times the average breadth
\( h = \) average length
\( p = \) density of the sample.

**Thermographical Studies:**

Thermographical studies of the samples include TGA, DTA, and DTG. The instrument used for these analysis is Derivatograph (Optical works, Budapest, Hungary). A convenient weight of each of the sample powdered to 200 mesh size was heated upto about 980°C. The heating was done in air under dynamic conditions at one atmospheric pressure. The activation energies in each case were calculated from the TGA curves using the equation of Freeman and Carrol (1958) as given below.:

\[
\frac{-E / 2.3 R x D (1 / T)}{\log W_r} = \frac{-X + D \log (dw/dt)}{\log W_r}
\]

Activation energies for the same chemical reactions were also calculated from DTA curves by Krishnan & Etalo (1976). The results obtained by both the method were compared. The kinetics of the process of dehydration was also studied.
2.16 SOLUBILITY EQUILIBRIA

The studies on the solubility equilibria with the solid solution of AsHA over the entire compositional range mainly concerned with the accurate analytical determinations of calcium in presence of phosphate and arsenate in the solvent used for the equilibration. The studies were undertaken by equilibrating a convenient weight of the dried samples within a close range of particle size at 37± 0.5°C. The calcium content in convenient volume of the solvent was determined by Singh et. al. (1968) and the solubilities were calculated in terms of calcium in mg/l of the solvent. The investigations were intended to understand the following aspects:

(i) Dependence of solubility of a given solid solution with the increase in pH of the medium between physiologically important range of 5.0 to 7.5.
(ii) The arsenate content in the solid solution at a given pH.

Method:

About 0.4 g of each sample powdered and sieved to 200 mesh (B.S.S) was equilibrated with 100 ml of the solvent in air tight polyethylene containers by shaking at a regulated speed using a microid glass shaker at 30 ±0.5°C. The time required for attainment of saturation was determined in preliminary experiments by analysing the calcium content in the filtrate obtained by filtration through a IG₄ crucible at the end of each convenient time of equilibration. It was found that the saturation was attained within about 2 h of equilibration in both acidic and alkaline regions.