MATERIAL AND METHOD

A) MATERIAL

Fluorotic patients and experimental animals provided the necessary material for the present study. The biochemical estimations were carried out upon fluorotic patients from two districts of southern Panjab where fluorosis is in endemic form. This part of Panjab lies in a geological belt extending from Delhi to Shahpur through Hissar, Ferozepore, Kasur, Chinot, Sargodha and Sholapur on to Mianwali district of Panjab (Day 1940). The soil is sandy and the temperature in summer months rises to about 116-117°F. The population of the endemic area is predominantly agriculturist. They are well-built and hard working. Economically, there are two types of people: farmers who own agricultural land and landless tenants who work as labourers in the field.

The diet consists of bread (of wheat or maize), small amounts of pulses, vegetables, milk. Meat may be taken by some occasionally, but the average daily intake is negligible. Tea is taken in excess. Fruits are not available in Indian villages.

The soil and water samples were collected from the area under survey, when the fluoride contents were found to be abnormal, the residents were radiographed and those showing skeletal fluorosis and willing to undergo biochemical examination were included in this study. In most of the cases the excretion studies pertain to patients consuming water containing excessive fluoride. The serum studies were made on the patients of skeletal...
fluorosis admitted to the Rajendra Hospital, Patiala. These patients at the time of study were not consuming abnormal amounts of fluoride, as the water supply of Patiala contains only 0.3 ppm of water fluoride. For comparison, biochemical investigations were conducted in healthy persons of Patiala, belonging to the same age group and taking similar diet and an equal number from endemic area.

Experimental animals

For the confirmation of certain observations in fluorotic patients, guinea pigs and monkeys were used as experimental animals.

1) Guinea pigs: The experiments on guinea pigs were undertaken to find out the relation between the ascorbic acid and fluoride intoxication. These animals are incapable of synthesising ascorbic acid for the needs of the system and indispensably require an external source of it.

Each guinea pig was kept in a circular metabolic cage provided at the base with a wire gauge. The floor of cage was in the form of a funnel which was thickly coated with wax. The funnel had a pad of washed glass wool to filter any solid particles. The urine was collected in receptacle containing the required preservative. Guinea pigs were divided into four groups and the diet of each group is given below:

1. Scorbatic group: (i) Equal measure by weight, of wheat, rice, brown gram were grounded and mixed with one measure of rice bran. The mixture was mixed with sodium chloride and hydrogenated ground nut oil. The total composition of the
diet was as follows—

Pulses, cereals and bran mixture 90 per cent.
Sodium chloride 3 per cent.
Hydrogenated ground nut oil 7 per cent.

This diet contained only slight traces of vitamin C.

The water supplied was tap water from Patiala water supply containing 0.3 ppm fluoride.

2. Scorbatic fluoride group was given the same diet but in addition 50 ppm fluoride (sodium fluoride) in water supplied ad libitum.

3. The normal group was given in addition to above mentioned diet, fresh carrots and leafy vegetables. Water supplied was tap water containing 0.3 ppm fluoride.

4. The fluoride group was given the normal diet and water containing 50 ppm fluoride in water.

Comparative investigations were the object of these studies and therefore, no check on quantity of diet was made.

After ten weeks, the control group and scorbatic guinea pigs were sacrificed. In the case of fluoride group and scorbatic fluoride group, administration of fluoride was stopped, and after two days, the animal was sacrificed.

Liver, kidney and thyroid of each animal was carefully removed and its fluoride content determined.

The flesh and blood sticking to femur was removed. The bone was split open into two parts of bone and bone marrow removed. It was thoroughly washed with a strong jet of hot water, defatted, dried and fluoride and ash contents estimated. The femur was
selected for the study, because of its convenience and the fact that it has an adequate fluoride content.

ii) Monkeys - The object of this experimental work on the monkeys was to study certain abnormalities in urinary constituents which were observed earlier in human patients. No check on the quantity of diet was found necessary for this type of study. Each monkey was kept in a metabolic cage, the floor of which was thickly coated with wax. The slope of the floor was so adjusted that urine drained off easily into the special receptacle with funnel. The funnel had a pad of washed glass wool to eliminate any traces of solid matter. To guard against the contamination by the water, the drinking water tumbler was placed as near to the top as possible.

In order to eliminate any possible error due to individual variations, urinary studies were carried out before subjecting the animal to fluoride administration and then afterwards. The results were compared to find out the abnormality caused by the ingestion of fluoride. The normal diet of the animal consisted of the following:

1. Bread.

2. Wheat flour, dried milk powder 15 g., salt 12 g., cane sugar 12 g., shark liver oil 6 g., yeast 9 g. The contents were made into seven cakes. Each animal received one.

3. Gram (unhusked)

4. Source of vitamin C. The vitamin was not given in the form of ascorbic acid but fresh fruits and vegetables e.g. mango, banana, carrot etc.

5. Water ad libitum (Patiala water supply containing 0.3 ppm of fluoride).

After the necessary estimations on the normal diet,
monkeys were given water containing 50 ppm to drink. The same estimations were then carried out on the same monkeys after the expiry of ten weeks.

B) METHODS

Nutritional survey

A nutritional survey was carried out in the already specified area of endemic fluorosis. The various constituents of diet for a whole year was noted and average of it was taken to determine the dietary pattern. The nutritive values of Indian foods were then found out from tables of Aykroyd (1963).

Estimation of fluoride in various constituents is as follows:

Estimation of fluoride in water

The fluoride was estimated in water by method of Association of Official Agricultural Chemists (1954) with slight modification.

According to the original procedure, 100 ml. of the sample was made alkaline and evaporated to 20 ml., before distillation. In view of excess of fluoride present in the water from endemic area, this step involving the concentration of fluoride has been found to be superfluous and directly 20 ml. of water was taken in the claisen flask for distillation. 20 ml. of 60% perchloric acid (made free of fluoride) was added and the contents steam distilled. The temperature of the solution during distillation was kept at 137°C ± 3. The amount of fluoride was determined by back titration.

In recovery experiments, the values obtained were 99%± 4.
Estimation of fluoride in urine.

The fluoride in the urine was determined by the method of Smith and Gardner (1955) with slight modification.

Smith and Gardner (loc.cit.) used solid calcium oxide of low fluoride content for ashing, but we made fluoride-free lime suspension. The procedure adopted was as follows: 50 g of calcium oxide of low fluoride content was carefully shaken with about 250 ml of water and 250 ml of 60% perchloric acid slowly added with stirring. Few glass beads were added and the contents boiled down to copious fumes of acid. It was cooled and 200 ml of water added and boiled again. The boiling to copious fumes was repeated again, it was cooled and diluted considerably. It was then filtered through fritted glass filter. The clear solution was stirred into a solution of NaOH (10%). The precipitate was allowed to settle and supernatant liquid decanted. The sodium salts were removed by washing five times. The precipitate was then shaken into suspension, made to 2 litre and stored in paraffined bottles. 10 ml of this suspension gave no appreciable blank, when evaporated distilled and fluoride estimated in it. The suspension was shaken before use.

To 25 ml of urine was added 10 ml of lime suspension and contents stirred. A drop of phenolphthalein added to ascertain that the sample was alkaline. If not, more suspension was added to make it alkaline and its volume noted, in order to use the same volume of the suspension for blank determination.
The sample was evaporated, dried and fluoride determined by titration with standard thorium nitrate.

In recovery experiments the values obtained were 99.5 ± 5 of the theoretical values.

**Estimation of fluoride in blood**

The fluoride in blood was determined by method of Smith and Gardner (1951).

All the reagents were the same which were used in the determination of fluoride in urine. In addition to these, fluoride-free sulphuric acid was prepared by diluting the acid with the same volume of double distilled water and it was boiled down to original volume. The process was repeated once more and the acid diluted with equal volume of double distilled water.

5 ml. of the oxalated blood was taken in an ordinary 100 ml. distillation flask 20 ml. of sulphuric acid, 0.2 g of silver sulphate added along with glass beads and distilled at \(137^\circ C \pm 3\). The distillate was kept alkaline to p-nitrophenol indicator and 200 ml of it collected. The distillate was then evaporated along with fixative (lime suspension). The residue was ashed, distilled and fluoride determined in the same way as urinary fluoride.

In recovery experiments the values were 98.7% ± 5.2 of the theoretical values.

**Estimation of fluoride in bone.**

After removal of soft tissue, the bone was cut to suitable size and all the traces of blood and marrow were removed by hot water spray. The bones were brushed carefully with ethanol and ether to remove adhering fat. It was dried at 105°C and powdered.
with a dental burr. 1-2 gms of dried mass was accurately weighed and its fluoride contents determined by the same method used in blood fluoride determination (Smith and Gardner 1951).

In recovery experiment the values were 100% ± 4 of the theoretical values.

**Estimation of fluoride in soft tissues.**

The procedure was the same as used in blood fluoride determination.

**Estimation of fluoride in various food stuff**

The procedure was the same as used in the blood fluoride determination.

**Serum calcium** was determined by the method of Kramer-Tisdall with modifications by Clark-Colllip (Hawk, Oser and Summerson 1954). The recovery experiments gave values 100% ± 2.5 of the actual amount added to the serum.

**Serum inorganic phosphorus** was determined by the method of Fiske and Subba Row (Hawk, Oser and Summerson 1954).

Values 99.5% ± 3.1 of the actual amount of inorganic phosphorus were found in recovery experiments.

**Alkaline phosphatase** in the serum was determined by the method of King and Armstrong (King and Wootten 1959).

**Protein bound iodine** was determined by the method of Barker Humphrey and Soley as modified in the Laboratory of Clinical Medicine, Hospital of the University of Pennsylvania (Simmons and Gentzkow 1956).

In recovery experiments values 98% ± 5.4 of the actual amount added to the serum were obtained.
Total protein in the serum was determined by the method of Kjeldahl (King and Wootton 1959).

Albumin was determined by Kjeldahl's method (King and Wootton 1959).

Globulin fraction in the serum was found by subtracting the amount of albumin from total amount of proteins.

Haemoglobin was determined by Sahli Hemoglobinometer.

Total leucocyte count and differential leucocyte count were determined in Neuberg counting chamber.

Urinary calcium was determined by the method of Shohl and Fedley (Hawke, Oser & Summerson 1954). In the recovery experiments 99.5 ± 1.5 calcium added to the urinary could be estimated.

Urinary inorganic phosphorus was determined by the method of Fiske and Subba Row (Hawke, Oser & Summerson 1954). In the recovery experiments 98.8 ± 1.3 of the added inorganic phosphorus could be recovered.

Acid soluble ester phosphate by the method given by Hawk, Oser and Summerson (1954).

To test for the presence of different chromogens in the urine the following compounds were tested by the methods given by Harrison (1958):

1) Melanogen.
2) Homogentisic acid.
3) Porphyrin
4) Uroerythrin
5) Urobilin and urobilinogen.
6) Bile pigments.
7) Indican
8) Urochrosein

The presence of blood was tested by the method described by Hawk, Oser and Summerson (1954).

The phenols were tested by the method of Bray and Thorpe (1954).
The quantitative estimation of phenols was made according to the method of Volterra (Hawk, Oser and Summers 1954).

The tyrosine and its metabolites were estimated by the method of Folin and Ciocalteau (1927). Into 15 ml. centrifuge tube 2.5 ml. of urine and 2 ml. of 15% mercuric sulphate in 6N sulphuric acid was added. Contents were allowed to stand for 30 minutes, centrifuged and poured into 50 ml. volumetric flask. One ml. of 2% sodium nitrite added and contents diluted to the volume. The optical density was then read in a photometer. 98% ± 2 of added tyrosine to the urine could be estimated in recovery experiments.

Total nitrogen in the urine was determined by a Microkjeldahl's method (King and Wootten 1959). 99% ± 1.4 of the amount added to the urine was obtained in the recovery experiments.

Amino-acid nitrogen in the urine was estimated by Fornal titration method (Varley 1958). In recovery experiments 98% ± 1.5 of the amounts added to the urine were estimated.

Amino-acid nitrogen in the blood was determined by method of Folin (Varley 1958). In recovery experiments 98 ± 3.3 of the amounts added to the blood were obtained.

Hydroxy proline was estimated by method of Prockop and Undenfriend (1960). In recovery experiments values obtained were 92% ± 7.6.

Blood urea was estimated according to the method given in King and Wootten (1959). The normal values by this method ranged from 12-25 mg per 100 ml. In recovery experiments 99% ± 1.5 of the added urea was obtained.
Urinary urea was determined by hypobromite method (King and Wootton 1959). 98\% ± 1.8 of urea added to the urine was estimated in the recovery experiments.

Urea clearance test was estimated by the method of Moeller, McIntosh and Van Slyke (King and Wootton 1959).

Composition of bone - The following estimations in the bone were carried according to the method of Eastoe and Eastoe (1954).

1) Ash contents.
2) Collagen.

Citric acid was determined according to the method of Natelson, Pincus and Lugovoy (1948). In recovery experiments 95.4 ± 5.8 citric acid could be recovered.

Method of determination of inorganic constituent in bone ash were the same as used in serum determination.

Separation of enamel was according to the method of Manly and Hodge (1939).

Vitamin C was determined according to the method of Bessey (Hawk, Oser and Summerson 1954). In the recovery experiments 95.4 ± 5.2 ascorbic acid could be recovered.