CHAPTER II

MATERIALS AND METHODS

1. **Collection of Blood Samples**

   (i) The blood samples of human adult were obtained from the Civil Hospital, and Evengeline Booth Hospital, Ahmednagar. Blood samples of langur and bonnet monkeys were obtained from Virus Research Centre, Poona. Blood was collected by venepuncture.

   (ii) The blood samples of animals from ruminant group were obtained from the local slaughter house.

   (iii) Avian blood samples were obtained by bleeding.

   Sodium citrate, 17.1 gms/100 ml. was used as an anticoagulant for primates and ruminants and heparin 40 mg/100 ml. was used for avians throughout the blood collections. 5 ml. of Na-citrate or 3 ml. of heparin was sufficient for 25 ml. of the blood sample.

2. **Haematological Studies**

   Prior to hemolysis haemoglobin concentration, red cell count, packed cell volume and osmotic fragility were determined by standard methods (124, 138, 144).

   (i) **Estimation of Haemoglobin Concentration**

   The concentration of haemoglobin in the whole blood as well as in the hemolysate was measured in a Carl-Zeiss Spectrophotometer (PMQ-II).
0.05 ml. of the whole blood or hemolysate was mixed with 9.95 ml. of Drabkin Reagent (1.0 gm. NaHCO$_3$, 50 mg. KCN and 200 mg. K$_3$Fe(CN)$_6$ were dissolved in one litre of distilled water) and the optical density was measured at 540 μm using E$^1$ cm. = 11.5 (64). In this process the haemoglobin is oxidized by potassium ferricyanide to methaemoglobin which is further converted to cyanmethaemoglobin by potassium cyanide. The cyanmethaemoglobin has a characteristic absorption spectrum at 540 μm and remains stable for a long time.

(ii) Red Blood Cell Count (R.B.C.)

Red cells were counted in the counting chamber (Fein-Optik Gera, Germany, Improved Double-Neubauer Ruling with depth 0.1 mm. and area $\frac{1}{400}$ sq.mm) under the microscope (PZO, Warszawa). A dilution of 1:200 was made by pipetting 0.005 ml. of blood, followed by the diluting fluid in a R.B.C. pipette. The diluting fluid is prepared by dissolving 2.5 gms. sodium sulphate, 0.5 gm. sodium chloride and 0.25 gm. mercuric chloride in 100 ml. of the distilled water. The diluted blood was mixed for at least two minutes by tilting and rotating the pipette horizontally. The counting chamber was then filled with the diluted blood in one action without allowing any bubble formation in the middle or reaching the fluid to the surrounding moat. The chamber was left undisturbed for at least 2 minutes,
to allow the cells to settle. Counting should not be delayed longer, as drying makes the counting less accurate. The red cells present in five different squares (four corner squares and one middle square) were counted and always expressed in millions per cubic mm.

(iii) Packed Cell Volume or Haematocrit value (P.C.V.)

Packed cell volume was measured in a haematocrit tube (Wintrobe tube). The tube was filled with the freshly collected blood upto 100 (Calibration 1 to 110 mm.) with the help of a syringe and centrifuged at 3000 r.p.m. for 30 minutes in an IPI centrifuge. The height of the red cell column was directly taken as the measure of P.C.V. in percent.

(iv) Mean Cell Haemoglobin (M.C.H.)

The M.C.H. can be calculated from the haemoglobin content of the blood (in gms. per 100 ml.) and the R.B.C. count (in millions) as follows.

\[
M.C.H. = \frac{Haemoglobin \text{ (in gms. percent)}}{R.B.C. \text{ (in millions)}} \times 10 \text{ mgs.}
\]

(v) Mean Cell Volume (M.C.V.)

The M.C.V. is calculated from the packed cell volume and the number of red cells per cubic mm.

\[
M.C.V. = \frac{P.C.V. \text{ (in percent)}}{R.B.C. \text{ (in millions)}} \times 10 \text{ c.µ.}
\]

(vi) Mean Cell Haemoglobin Concentration (M.C.H.C.)

The M.C.H.C. is calculated from the haemoglobin concentration and the packed cell volume.
M.C.H.C. = \frac{100 \times \text{Haemoglobin (in \text{gm percent})}}{\text{P.C.V. (in percent)}} \text{ percent.}

(vii) **Cell Size Measurement**

The diameter of the cell is measured with the help of a camera lucida (ATAGO, TOKYO) and an objective micrometer (NIKKEN, TOKYO).

3. **Determination of the Erythrocyte Osmotic Fragility.**

To determine the erythrocyte osmotic fragility, the concentration of sodium chloride solution at which hemolysis is just detectable and the highest concentration at which hemolysis appears to be complete are recorded. The osmotic fragility is markedly affected by the relative volumes of blood and saline, the pH of blood-saline suspension and the temperature. Since the fragility increases rapidly on keeping for a long time, always fresh blood samples were used. Also, the determination of the fragility of a sample was carried out in comparison with the standard (human) under identical conditions.

A series of test tubes containing 4 ml. of graded solutions of NaCl (from 0.1 percent to 5 percent) were taken. To each tube 0.4 ml. of the fresh blood was added. The tubes were mixed immediately, allowed to stand for 30 minutes at room temperature, remixed and centrifuged at 2000 r.p.m. Three ml. of the supernatent was transferred to a tube containing 3 ml. of 0.1 percent Na$_2$CO$_3$. After
mixing, the O.D. was measured at 540 mp in the
spectrophotometer. The highest reading was taken as the
100 percent hemolysis. A normal human blood sample was
taken as a standard each time. The graph of percent
hemolysis against concentration of NaCl was drawn. The
median corpuscular fragility (M.C.F., 50 percent hemolysis)
can be determined from the graph with more accuracy than
the minimal and maximal fragility.

4. Preparation of Haemolysates

The haemolysates were prepared according to the
method of Drabkin (69). The whole blood was centrifuged
at 5,000 to 6,000 r.p.m. for about 5 to 10 minutes to
remove plasma. The red cells were washed with physiological
saline three to four times. The washed cells were mixed
with equal volume of water and 0.4 volume of toluene and
lysed by shaking the mixture vigorously for one minute.
The mixture was kept overnight at 4°C. for complete
haemolysis. Next day, the haemolysate was centrifuged
at 12,000 to 15,000 r.p.m. for the removal of cell
debris. Cyanmeth-haemoglobin was prepared by treatment of
haemoglobin solution with few drops of 5 percent potassium
ferricyanide and 2 percent potassium cyanide in succession
at 10 minutes interval. The solution was dialyzed
against water in cold for 24 hours and then equilibrated
with appropriate buffers.
5. **Electrophoresis**

Routine survey of haemoglobins was done by paper electrophoresis. Starch gel electrophoresis was used to confirm the results obtained by paper electrophoresis.

(i) **Paper Electrophoresis**

The electrophoretic chamber consisted of a rectangular hard plastic tray (33 x 38 x 6.5 cm.) for holding the buffer solution as well as electrodes. The chamber was divided laterally into two compartments on either side with buffer holes connecting them. The platinum electrodes were fixed in the outer compartment. A special frame was prepared to hold the two Whatmann 3 MM paper strips (31 x 15 cm.) in the trough.

The paper strips were soaked in the buffer and the excess of buffer was removed by blotting. Blood samples, either in oxy- or cyanmet haemoglobin forms, were applied as streaks. The electrophoresis was run in barbital-HCl buffer, pH 8.8 \[ \frac{\gamma}{2} = 0.05 \] or Tris-EDTA-boric acid buffer, pH 8.9 at room temperature for 12 to 14 hours using 180 volts.

(ii) **Starch gel Electrophoresis**

The gel was prepared from hydrolysed starch.

**Preparation of Hydrolysed Starch (233)**

Potato starch (500 gm., BDH Holland made) was taken in one litre of 1 percent HCl-acetone mixture preheated
to 40°C. The mixture was kept without agitation for
45 minutes. The hydrolysis was stopped by addition of
250 ml. of 1 M sodium acetate. The mass was allowed to
settle and the supernatent liquid was removed. The
hydrolysed starch was kept under water for 15 hours to
remove sodium acetate and then washed repeatedly with
water, followed by washings of acetone and dried at
40°C.

A solution of 10.7 percent hydrolysed starch in
Tris-EDTA citric acid buffer, pH 8.6 (174) was heated
briskly over a low flame. After a gelling point was
reached, the solution was degassed and poured on clean,
dry glass plates fixed with frames of the dimensions
17.5 x 6 x 0.3 cm. The gel was allowed to set overnight.
Narrow insertions were cut in the gel by a scalpel along
a line parallel to the lateral sides. The paper strips
(10 x 3 mm.) soaked in the haemoglobin samples were
blotted lightly and placed inside the gel by forceps.
The glass plate along with the frame holding the gel was
placed inside the electrophoretic chamber. Whatmann paper
wicks (15 x 6 cm. three fold), soaked in the electrophoretic
buffer and blotted, were fixed on both sides of the gel,
thus making a bridge between the buffer and the gel. To
prevent evaporation, the gel was covered with a polythene
sheet, the inner side of which was coated with paraffin.
The electrophoresis was run in barbital buffer, pH 8.6
\( \frac{V}{2} = 0.05 \) at 100 V. for 10 to 12 hours at room
temperature.

6. Alkaline Denaturation

The rate of denaturation of oxyhaemoglobin by
alkali was measured by the method of Jonxis et al. (125).
In this method, haemoglobin solution (0.05 ml.) was mixed
with 0.06 N NaOH (10 ml.) containing two drops of
10 percent NH₄OH. Decrease in extinction \( E_T \) was measured
at every one minute after mixing for a period of
15 minutes. Simultaneously, the remaining solution was
placed in a water bath at 40°C for 15 minutes to denature
the haemoglobin completely. The extinction of this
solution was also measured \( E_B \). The extinction of
undenatured haemoglobin \( E_B \) was obtained by mixing 0.05 ml.
haemoglobin solution with 10 ml. of water containing two
drops of NH₄OH. The percentage of undenatured haemoglobin
at a certain time was calculated from the relation:

\[
\text{Percent of undenatured Hb} = \frac{E_T - E_E}{E_B - E_E} \times 100. \]

The logarithms of the percentage of undenatured
haemoglobin were plotted against time.

7. CM-Cellulose Chromatography

The component haemoglobins were isolated by CM-cellulose
chromatography by stepwise pH elution as well as pH gradient elution.

The method was essentially the same as that developed by Huisman et al. (105). In the absence of a cold room, CM-cellulose columns of final height of not more than 5 cm. were used and operated at room temperature. Whenever necessary, column chromatography could also be performed inside a refrigerator.

(1) Preparation of CM-Cellulose

Cellulose derivative was prepared from cellulose powder No. 123 (Carl-Schleicher and Schull) according to the method of Peterson and Sober (197).

One hundred and twenty grams of cellulose powder was taken in a glass trough of 5 litre capacity. It was stirred thoroughly with small amount of 400 ml. of 45 percent NaOH solution. The mixture was cooled in ice bath for 30 minutes with occasional stirring. Monochloroacetic acid solution (60 g. in 20 ml. of water) was added with stirring. The reaction was allowed to proceed for about 25 minutes on a water bath at 60°C with occasional stirring. The mixture was cooled in ice bath and mixed with one litre of 10 percent acetic acid. It was further diluted with 2 litres of water. The yellow mass was allowed to settle and was given repeated washings with water to remove the colour. Later, it was acidified
with glacial acetic acid to lower the pH to about 3. Cellulose derivative appeared as a white, swollen mass. The pH was raised to 5.5 by giving frequent washings of water. The ion exchanger was stored as a slurry in 0.01 M sodium phosphate buffer, pH 6.3.

(ii) **Buffer Solutions**

Phosphate buffers of varying pH values were prepared by mixing solutions of 0.01 M NaH$_2$PO$_4$·2H$_2$O, pH 6.3 and 0.01 M Na$_2$HPO$_4$·12H$_2$O, pH 9.2. Each solution contained 100 mg. KCN per litre. All the pH measurements were performed with Beckman pH meter, H2 Model.

(iii) **Column Operation**

The slurry of CM-cellulose, previously equilibrated with 0.01 M sodium phosphate buffer, pH 6.3, was poured into a glass column (20 x 3 cm.) to produce a final height of 4.5 cm. Haemoglobin solution (100 to 150 mg.), previously equilibrated with 0.01 M sodium phosphate buffer, pH 6.3, was added to the column. After passage of about 50 ml. of the same buffer to remove any excess of haemoglobin and non-haem proteins, gradient elution with 0.01 M sodium phosphate buffer, pH 6.6 was started. The higher pH buffer was taken in a reservoir of 500 ml. capacity and was allowed to pass into a mixer containing 100 ml. of starting buffer. The elution was carried out at a flow rate of 16 to 20 ml. per hour. Four to

* For primates and avians pH 6.8.*
five ml. fractions were collected per tube. The reservoir buffer was successively replaced by phosphate buffer of pH 6.9 and 7.4. Optical density of the eluted fractions was measured at 415 μm and the graph plotted.

(iv) Estimation of Component Haemoglobins

The percentage of each haemoglobin component was estimated by diluting the corresponding fractions to a constant volume and measuring the optical density at 415 μm.

8. Preparation of Globin

The component haemoglobins isolated through CM-cellulose columns were concentrated either by pervaporation below 10°C. or by freezing and gradual thawing when the concentrated haemoglobin could be decanted out. Globin was prepared according to the method of Anson and Mirsky (7A). One volume of concentrated haemoglobin solution was added slowly and with constant stirring to 20 volumes of acetone containing 0.5 percent concentrated HCl at -20°C. The precipitated globin was washed 3 to 4 times with ice cold acetone to remove haem completely, dissolved in a minimum amount of ice cold water and reprecipitated as above. The globin was dried under vacuum over sodium hydroxide pellets.

9. Subunit Separation by Electrophoresis

The separation of polypeptide chains of globins was
done in urea-veronal buffer, pH 8.1 (59) as well as on starch gel in sodium formate buffer, pH 1.8 (177). However, paper electrophoretic procedures in 6 M urea using the following buffer system was found to be effective for primate and ruminant globins, while starch gel electrophoresis was found to be suitable for avian globins in the present work.

(i) **Paper Electrophoresis in Urea-veronal buffer, pH 8.1**

This procedure was adapted from the urea-starch gel electrophoretic technique of Chernoff and Pettit (59).

**Preparation of Buffer**

Diethyl barbituric acid (18.4 g.) was added to about 600 ml. of boiling water. Sodium salt was prepared by adding 50 ml. of 1N NaOH. The solution was allowed to attain room temperature. The pH of the solution was checked and adjusted to 7.7 by adding more amount of 1N NaOH if necessary. The volume of the solution was made to 1 litre. In this 0.1 M veronal buffer, urea was dissolved to the final concentration of 6 M. The resulting pH of the buffer was 8.0 ± 0.1.

**Electrophoresis**

Five milligrammes of freshly prepared globin was dissolved in 0.2 ml. of urea-veronal buffer containing mercaptoethanol (50 μl per 5 ml. of buffer). Electrophoresis was carried out at 4°C. as described in the earlier procedure.
Staining of papers

The paper strips were dried at 80°C. and developed with bromphenol blue (0.1 percent in ethanol containing 10 percent HgCl₂). The excess of dye was removed by giving washings of 5 percent acetic acid.

(II) Starch Gel Electrophoresis in Sodium-formate buffer, pH 1.8

Avian globins were separated by the method of starch-gel electrophoresis in Na-formate buffer, pH 1.8 developed by Muller (177).

Fourteen percent starch gel in 1.4 M sodium formate buffer, pH 1.8, μ = 0.02 was prepared as described earlier.

Application of the samples

Globins were dissolved in the formate buffer to give solutions of 10 mg. per ml. Rectangular pieces of filter paper (Whatmann 3 MM, 10 x 3 mm.) were soaked in the globin solutions and introduced into slots of the same dimension in the gel.

After the application of the samples, the gel was coated with melted paraffin and placed inside the electrophoretic chamber. Formate buffer, twice as concentrated as the buffer used for the gel, was introduced into the buffer compartments. It was observed that discontinuity of the buffer concentration improved the separation. A contact of the gel with the buffer compartment was established by means of a paper wick.
Electrophoresis was carried out for 5 hours in the cold applying a voltage of 100 V.

**Staining**

The gel was stained by dipping for 5 minutes in a saturated solution of Amido Black 10 B in methanol : water : acetic acid (50:50:1 by volume). Excess of dye was removed from the gel by several washings in a methanol-acetic acid solution (9:1 by volume).

10. **Isolation of Polypeptide Chains**

Polypeptide chains were isolated by CM-cellulose column chromatography (60).

**CM-Cellulose Column Chromatography**

(a) **Buffers**

Starting Buffer: 8 M urea containing 0.05 M 2-mercaptoethanol and 0.005 M Na$_2$HPO$_4$·12H$_2$O. The pH was adjusted to 6.5 with phosphoric acid.*

Gradient Buffer: 8 M urea containing 0.05 M 2-mercaptoethanol and 0.03 M Na$_2$HPO$_4$·12H$_2$O. The pH was adjusted to 6.5 with phosphoric acid.

(b) **Preparation of CM-cellulose Column**

Five grammes of CM-cellulose (Bio Rad, 0.69 m. equiv./g) was taken without any pre-treatment and suspended in 100 ml. of starting buffer. The pH of the suspension was adjusted to 6.5 with 1N NaOH and was allowed to stand for 15 minutes. The supernatant

* For primates pH 6.8.
containing fine particles was removed and the ion exchanger was resuspended in starting buffer. After the removal of unsettled particles, the CM-cellulose was suspended in large amount of starting buffer to give a fine slurry. This slurry was poured into a glass column (20 x 0.9 cm.) to produce a final height of 10 to 12 cm.

(c) **Column Chromatography**

Seventy milligrams of globin was dissolved in 7 ml. of starting buffer. The globin solution was transferred to a dialysis bag and dialysed against 2 to 3 changes of a 50-fold excess of the same buffer for two and a half hours at room temperature.

The dialysed globin solution was applied to the previously equilibrated CM-cellulose column. About 50 ml. of starting buffer was passed to remove any unadsorbed protein. This was checked by measuring the optical density at 280 μm of the eluted buffer solution. Elution with gradient buffer was started. The gradient buffer was taken in a reservoir of 500 ml. capacity and was allowed to pass into a mixer containing 150 ml. of starting buffer. The elution was carried out at a flow rate of 4 ml. per 5 minutes. Fractions of 4 ml. in each tube were collected. The optical density was measured at 280 μm.
11. **Aminoethylolation**

Globins as well as polypeptide chains were converted to S-aminoethyl derivatives using ethyleneimine (121). The procedures of aminoethylolation are described separately for (A) globins and (B) polypeptide chains.

(A) **Aminoethylolation of Globins**

**Solutions required**

(a) 5 percent EDTA solution
(b) Tris buffer, pH 8.6
(c) 0.2 M Acetic acid

One hundred milligrams of globin was mixed with 3.61 g. of deionised urea, 0.3 ml. of 5 percent EDTA solution and 3 ml. of Tris buffer. The mixture was diluted to 7.5 ml. with water. The solution was treated with 0.1 ml. of 2-mercaptoethanol and kept for 30 minutes. Ethyleneimine (0.2 ml.) was added in excess and the solution was kept for another 30 minutes. Aminoethylolated protein was freed from urea and other low molecular weight substances by passing the solution through a column of sephadex G-25 (55 x 1.5 cm.) equilibrated with 0.2 M acetic acid. The rate of elution was 60 ml. per hour and 4-5 ml. fractions were collected. The optical density of the eluted fractions was measured at 280 mp.

(B) **Aminoethylolation of Polypeptide Chains**

Fractions corresponding to a specific polypeptide
chain isolated by CM-cellulose column chromatography were pooled together. Solid tris was added to a final concentration of 1 M and pH adjusted to 9.2 with concentrated HCl. Ethyleneimine was added to a final concentration of 0.5 M. Reaction mixture was kept for 2.5 hours. The pH was lowered down to 3 with concentrated HCl. The solution was passed over Sephadex G-25 column (55 x 1.5 cm) equilibrated with 0.5 percent formic acid. The fractions were collected as above. The optical density was measured at 230 nm.

The fractions corresponding to a specific protein, either a globin or polypeptide chain, were pooled together and transferred to a dialysis bag. The solution was dialysed for 24 hours under running water and finally against 2 to 3 changes of glass distilled water. The precipitated protein was dried by lyophilization.

12. Peptide Mapping

S-aminomethyl derivatives of globins as well as of polypeptide chains were subjected to tryptic digestion followed by fingerprinting according to the methods of Ingram (116).

The complete units for tryptic( and chymotryptic) hydrolysis and fingerprinting were fabricated locally which included the following accessories:
(a) Tryptic digestion unit consisting of toluene bulb thermostat, pH meter and micropipette.

(b) High voltage power pack (0 to 1,500 volts and 100 mA.) supplied by Malwa E.E. Co., India.

(c) Chromatographic chambers, with glass plates on all sides and at the bottom, held by wooden frame. The joints were coated with paraffin wax (E. Merck).

(i) Tryptic Digestion

Enzyme Solution

Enzyme used to carry out hydrolysis was 2 x crystalline, salt free trypsin obtained from Mann chemicals (U.S.A.). Further purification of the enzyme was not necessary. Five milligrammes of trypsin was dissolved in 0.001 M HCl (1 ml.) and the solution was preserved at 0°C.

Protein Solution

Globin or polypeptide chain (5 to 100 mg.) was dissolved in 3 ml. of water and taken in a small beaker of 10 ml. capacity. The pH of the solution was adjusted to 8 with 0.01 N NaOH. Protein in the native state was completely denatured by heating at 80°C. for 7 minutes when a flocculent precipitate was obtained. The solution was kept in a thermostat at 37°C. with constant stirring by a magnetic stirrer.
Protein Digestion

Trypsin was added to protein solution in a ratio of 1 to 200. There was a rapid decrease in the pH due to the liberation of protons during enzyme action. The pH was adjusted to 8 by addition of 0.01 N NaOH. After a period of about one hour, there was only a slight intake of alkali. The digestion was continued for two hours. The enzyme action was stopped by lowering the pH to 6.5 with 0.05 N HCl. The trypsic hydrolysate was kept at -20°C. overnight. Trypsin was destroyed by heating trypsic digest in boiling water bath for one minute. The solution was centrifuged and filtered to give a clear trypsic hydrolysate. It was preserved in a frozen state.

(ii) Fingerprinting

It involves high voltage electrophoresis in one direction followed by paper chromatography in a perpendicular direction.

Samples:

A specific amount of trypsic digest corresponding to about 5 mg. of protein was taken in a clean, dry porcelain tile. The solution was evaporated to dryness in a vacuum desiccator. For comparison of peptid pattern two samples were dried at a time.
Electrophoresis

Two Whatmann 3 MM papers were cut of identical size and shape (31 x 31 cm. with a pair of sleeves of 13 cm. length and 5 cm. breadth). The papers were soaked in Michl's volatile buffer (pyridine, acetic acid, water, in the proportion 10:0:4:90 by volume, pH 6.4 (170) Excess of buffer was removed by blotting the papers. The two papers were placed on a horizontal glass plate (68 x 38 cm.). The two samples were dissolved in about 30 μl. of water and applied separately on each paper in succession with the help of micropipettes. A glass plate of identical size to that of first was placed above the papers carefully. The sleeves on either side of the two papers were dipped in Michl's buffer taken in glass troughs. The two platinum electrodes were also fixed in the glass troughs. After a period of about ten minutes, the electrophoresis was run at 1,000 volts for 2.5 hours. The sleeves were cut off and the papers were removed from the glass plates and dried in a blast of air.

Chromatography

The two papers were suspended in the chromatographic chambers, prepared according to the size of the papers and coated inside by the paraffin, containing n-butanol, pyridine, acetic acid and water (30:20:6:24) as a
chromatographic solvent (20). Whenever necessary, pyridine, isoamylalcohol and water (35:35:27) was also used (12). After a saturation period of about two hours, chromatography was carried out overnight for about 15 hours in the corresponding solvent. The papers were removed from the chromatographic chambers and dried as before.

Staining

The peptide spots were made visible by staining the papers in ninhydrin solution (0.5 percent in acetone) and drying in air at room temperature. Purple coloured spots appeared after 10 to 20 minutes of development.

(iii) Colour Tests

The fingerprints were colour tested for certain amino acids like tryptophane, tyrosine, arginine, histidine and sulphur containing amino acids. These colour tests are very useful in locating a specific amino acid in different peptides and pointing out differences if any, in the two haemoglobins.

Colour tests for Tryptophane (232) and Sulphur (241)

The two colour tests can be performed on the same fingerprint papers in succession.

For identifying tryptophane containing peptide, the papers which were already developed with ninhydrin were dipped in 1 percent solution of p-dimethyl-aminobenzaldehyde in 10 percent concentrated HCl in acetone.
On drying at room temperature, the appearance of purple violet spots in certain areas revealed the presence of tryptophane.

The same papers were further dipped in a solution of the following composition:

- $K_2PtCl_6$, 0.002 M - 20 ml.
- KI, 1 M - 1.25 ml.
- HCl, 2 N - 2.0 ml.
- Acetone - 330 ml.

On drying, white bleached spots appeared against yellow greenish background indicating the presence of sulphur containing amino acid.

The colour tests for tryosine, histidine and arginine were carried out after decolourising the ninhydrin developed papers with 10 percent HCl in acetone. The papers were dried in a current of air to remove HCl fumes, the presence of which interferred with the colour development.

**Colour test for Histidine (Baldridge and Lewis) (18)**

- **Solution A** - Sulphanilic acid - 5 g.
  - Hydrochloric acid 12 N - 40 ml.
  - Water - 460 ml.

- **Solution B** - Sodium nitrite - 3.5 g.
  - Water - 500 ml.

- **Solution C** - (Freshly prepared)
  - Sodium carbonate - 50 g.
  - Water - 500 ml.
All the solutions were kept at room temperature. Before use, Solutions A and B were mixed in equal volume. After a few minutes, the diazotised sulphanilic acid was sprayed lightly on both sides of the fingerprint papers. The papers were dried at 60°C. for 5 minutes. Then the papers were sprayed with Solution C. Histidine was indicated by bright red spots on a yellow background.

**Colour test for Arginine (Sakaguchi reaction)** (1)

Solution A - Urea - 25 g.
- α-naphthol - 50 mg.
- Alcohol - 500 ml.

Solution B - (Freshly prepared)
- Potassium hydroxide (5 percent) - 500 ml.
- Bromine - 0.7 ml.

Solution A was kept at room temperature or better freshly prepared.

To solution A, some KOH pellets (about 30) were added and the mixture was shaken to dissolve some pellets. The papers were dipped in the solution and dried for a few minutes at room temperature. The solution B was next sprayed on the papers.

Red spots against pale pink background indicate the presence of arginine.

**Colour test for Tyrosine** (1)

Solution A - α-nitroso - β-napthol - 500 mg.
Ethanol (96 percent) - 500 ml.
Solution B - (Freshly prepared)

Nitric acid (concentrated)  - 10 ml.
Acetone  - 90 ml.

Solution A was kept at room temperature. The papers were dipped in solution A and dried in air for 10 minutes. Then the papers were dipped in solution B and heated over a low flame until the colour appeared. The red spots against green background indicate tyrosine, while tryptophane was indicated by a greyish-brown spot.