MATERIAL AND METHODS
MATERIAL

The material consisted of thirty patients, suffering from iron deficiency anaemia who fulfilled the following criteria for inclusion in the present study:

- Hypochromic red cell morphology in peripheral blood smears.
- Mean corpuscular haemoglobin concentration of less than 20%.3
- Serum iron levels of less than 60 µg/dL.
- Serum unsaturated iron binding capacity of more than 400 µg/dL.
- Plasma iron saturation of less than 16%.7
- Complete absence of stainable reticular iron (Grade 0) in the bone marrow smears (Squash preparations).3
- Bone marrow sideroblast count of less than 10%.7
Thirty patients studied were divided into five equal groups. After preliminary investigations which included satisfying the criteria for inclusion in this study (vide supra), parenteral (intramuscular) injections of iron dextran complex were given in daily doses of 100 mg. each until the stainable iron was histochemically demonstrated in the bone marrow. For this, marrow aspirations subsequent to the start of iron therapy, were done every week beginning on the third day in subjects of group I, on the 4th day in subjects of group II, on the fifth day in subjects of group III and on the 6th and 7th days in subjects of group IV and group V respectively. By arranging the groupings of the subjects in this order, it was possible to spread the study over a continuous period beginning with the third post therapy day. As soon as the stainable iron was observed in the bone marrow smears, needle biopsy of the liver was taken to study the appearance of iron thus providing information regarding distribution of storage iron in general.

Calculations of the amount of iron deposited in the storage sites at the time it became histochemically
visible in the bone marrow smears, was done in the manner described in the following example.

**EXAMPLE:**

1) The initial haemoglobin level of the patient before iron therapy i.e. when the patient was anaemic and there was complete absence of stainable reticular iron in the bone marrow smears (Grade 0) = $H_1 \text{ gm/100 ml. of the blood}.$

2) The final haemoglobin level of the patient, at the time when iron became stainable in the bone marrow smear = $H_2 \text{ gm/100 ml. of the blood}.$

3) Blood volume of the patient (calculated on the basis of height and weight nomograms of Nadler and Hidalgo (1962) = $V \text{ ml.}$

4) The amount of parental iron injected until it became stainable in the bone marrow smears = $I_1 \text{ mg.}$

5) The amount of iron released from the injection site during the first 7 - 10 days = $I_2 \text{ mg.}$ This figure was estimated at 65% of the injected iron, based upon the studies on "imferon iron" reported by Grimes and Hutt (1957) and Evans and Ramsey (1957).
It has been assumed that the amount of iron absorbed from the injection site would either be utilized for haemoglobin synthesis or deposited in the reticuloendothelial stores. This is based upon the concept that only negligible amount of iron dextran complex are excreted outside the body (Martin, Bates, Bemford, Donaldson, Macdonald, Dunlop, Sheard, London and Rigg, 1955).

The amount of iron which is utilised for haemoglobin synthesis can be calculated from the difference of final and initial haemoglobin levels of the subject and his/her blood volume. This can be done as follows:

\[
\text{Amount of new haemoglobin synthesised during the period of study} = (H_2 - H_1) \times \frac{V}{100} = H_3 (\text{gm}).
\]

The amount of iron contained in this newly synthesised haemoglobin

\[
\text{haemoglobin} = H_3 \times 0.00338 \frac{\text{gm}}{100} \text{ or } H_3 \times 3.38 \frac{\text{mg}}{100}
\]

(1 gm. of haemoglobin contains 0.00338 gm. of iron).

Thus the amount of iron deposited in the storage organs during the period of study can be calculated from the following equation:

\[
I_2 = (H_3 \times \frac{3.38}{100}) \text{ mg}.
\]
METHODS

The various laboratory investigations mentioned above were carried out according to the following techniques:

1) Examination of the peripheral blood film for morphological type of anaemia by Leishmann staining (Dacie and Lewis, 1968).

2) Haemoglobin estimation by the cyanmethaemoglobin method (Dacie and Lewis, 1965).

3) Packed cell volume by microhaematocrit method (Dacie and Lewis, 1968).

4) Serum iron estimation and total iron binding capacity by modified Ramsay's manual method (King and Wboten, 1962). unsaturated iron binding capacity = total iron binding capacity - Serum iron.

5) Examination of bone marrow smears and liver biopsy specimens (Paraffin sections) for iron by Perl's modification of the technique of Prussian blue staining.
PERIPHERAL BLOOD FILM

Peripheral blood films were made by the usual spreading technique and stained with Leishmann's stain using the standard procedure.

HAEMOGLOBIN ESTIMATION

Principle: Measured quantity of blood is diluted in a solution (Drebbkin's solution) containing potassium cyanide and potassium ferricyanide. Haemoglobin, Methaemoglobin and Carboxyhaemoglobin are converted rapidly into cyanmethaemoglobin. Optical density of the solution is then measured against a standard in a photoelectric colorimeter.

Method: 200mm. of blood was added to 5 ml. of Drebbkin's solution and tube containing solution was inverted several times and allowed to stand at room temperature for at least 10 minutes. Optical density readings were taken in a prestandardised and prescaled haemoglobinometer giving direct readings of haemoglobin in g/100 ml. of blood.

PACKED CELL VOLUME

A high speed microhaemocrit centrifuge machine was used. It provided a maximum speed of 14000 revolutions
per minute. 3 to 5 minutes centrifugation results in a constant packed cell volume.

From the sample of blood collected in an E.D.T.A. - haemogram tube a microhaematocrit capillary pipette was filled by capillary action, sealed at one end, centrifuged at high speed for 5 minutes at the end of which the reading was taken for packed cell volume on a standard scale provided with the microhaematocrit centrifuge machine.

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION

Mean corpuscular haemoglobin concentration was calculated by the following formula:

\[
\frac{\text{Haemoglobin in gm percent}}{\text{Packed cell volume}} \times 100
\]

\[= \%\]

SERUM IRON ESTIMATION (King and Wootten, 1961)

I) Principle of Technique— The serum is treated with trichloracetic acid which frees the protein bound iron and also precipitates the protein. Ascorbic acid
is added to maintain the iron in the reduced (ferrous) form so that it can react with the dipyridyl in the presence of a buffer to give a pink colour. The coloured solution is then compared in a spectrophotometer at 550 nm against a series of the working standard solutions treated in the same way.

II) Collection of Samples: A specimen of 12 ml. of blood was collected in an iron free container with an iron free syringe and a stainless steel needle. It was allowed to clot and the serum was separated by centrifugation at 3000 R.P.M. for 10 to 15 minutes. The serum samples were stored in a deep freeze (at -20°C) until the time of analysis.

III) Glassware: The entire glassware was made iron free by first washing it thoroughly followed by overnight treatment in 10% hydrochloric acid. It was then washed repeatedly first with the single glass distilled water and then with iron free deionised water followed by drying in hot air oven.

IV) Reagents: The following reagents were used. All were AR grade and the solutions were made in iron-free deionised water.
- 2 N hydrochloric acid
- 20% Trichloroacetic acid
- Saturated solution of sodium acetate
- Dipyridyl - 0.4 gms. dissolved in 5 ml. of glacial acetic acid and made up to 100 ml. with deionized water.
- Ascorbic acid was prepared fresh at each estimation by dissolving 250 mg. of ascorbic acid (AR) in 3 ml. of H₂O₂.
- Stock iron standard (5 mg. iron/ml.). 4.31 gm. of Ferric ammonium sulphate was dissolved in deionized water to which 10 ml. of concentrated hydrochloric acid was added and the volume made to 100 ml. with deionized water.
- Working standards:
  1 ml. of stock standard was diluted to 100 ml. with deionized water. This provided a working standard solution equivalent to 50 μg/ml percent. To prepare working standards equivalent to 100 μg/ml percent and 200 μg/ml percent, 2 ml. and 4 ml. of the stock standards were taken respectively and made to 100 ml.

V) Procedure:

   a) Into five different centrifuge tubes were taken (i) 4 ml. of test serum (T), (ii) 4 ml. each of the 3 working standards (S₁, S₂, S₃) equivalent to 50, 100 and 200 μg/ml percent of iron respectively (iii) 4 ml. of deionized water as blank (B).
b) 0.2 ml. of Ascorbic acid solution was added and the tubes allowed to stand for 5 minutes.

c) 0.4 ml. of 65 percent Trichloroacetic acid was then added. The tubes were immediately covered with paraffin paper and shaken vigorously for 1/2 minute. If necessary glass rod was used to mix the chunky precipitate which stuck at the bottom.

d) The tubes were left to stand for 10 minutes and then centrifuged for 20 minutes at 2500 R.P.M.

e) 2 ml. of the clear supernatant was pipetted off into separate (4" x ½") test tubes. Care was taken not to contaminate the supernatant with the precipitate.

f) To each tube 0.4 ml. of alkaline acetate solution was added to buffer the contents to the suitable alkaline pH.

g) Now the coloring agent Dipyridyl was added and the contents were mixed well after each addition and allowed to stand for 10 minutes.

h) The optical densities were then compared in a spectrophotometer at 550 m\(\mu\).

VI) Calculations:

\[
\text{Serum iron} = \frac{T - B}{S - B} \times \text{concentration of standard} \\
\text{gms/100 ml.}
\]

\[
T = \text{Test} \\
S = \text{Standard} \\
B = \text{Blank}
\]
ESTIMATION OF TOTAL IRON BINDING CAPACITY

I) **Principle**: Iron is transported in the serum in a combination with a specific protein called Transferrin or Siderophilin. An excess of iron is added to the serum to saturate the iron binding proteins. The left over unbound iron is removed by adsorption with light magnesium carbonate and the iron remaining bound with transferrin is estimated as described already for serum iron.

II) **Collection of Sample**: Blood is collected in the same way as described under determination of serum iron.

III) **Glassware**: All the glassware used is iron free.

IV) **Solutions**:
   a) **Stock Ferric Chloride** - was prepared by dissolving 145 mg. of ferric chloride in 100 ml. of 0.5 N hydrochloric acid.
   b) **Working Ferric Chloride** - Stock ferric chloride was diluted 1 in 100 with demineralised water.
   c) **Magnesium Carbonate**: Hydrated basic light laboratory reagent. The preparation supplied by British Drug House was used.

V) **Method**:
   a) 3 ml. of the test serum was taken in a iron free
chloride solution was added to it. After mixing thoroughly it was allowed to stand for 5 minutes.

b) About 300 mg. of light magnesium carbonate was added.

c) Tubes were covered with paraffin paper and agitated thoroughly at frequent intervals for 30 - 60 minutes.

d) It was centrifuged for 5 minutes at 3000 R.P.M. and the contents were agitated to wash down the crust that formed on the surface and centrifuged again for a period of 5 minutes.

e) 4 ml. of the supernatant (T) and 4 ml each of two standards (S1 and S2) solution equivalent to 200 Ugm and 400 Ugm per cent and deionised water (B) were treated in the same way as for the estimation of serum iron.

Serum Total Iron Binding Capacity:

\[
\text{SBI} = \frac{T - B}{S - B} \times \text{Concentration of } S \text{ standard}
\]

T = Test

S = Standard

B = Blank
UNSATURATED IRON BINDING CAPACITY (U.I.B.C.)

U.I.B.C. = Serum total iron binding capacity
- Serum iron.

TRANSFERRIN SATURATION

Transferrin saturation was calculated in each case.

\[
\text{Plasma iron saturation} = \frac{\text{Serum iron}}{\text{T.I.B.C.}} \times 100
\]

Iron STAINING OF BONE MARROW

Prussian blue staining of the bone marrow smears was done in each case to evaluate the status of storage iron and to examine for the sideroblast count.

A) Procedure of Bone Marrow Aspiration - Marrow aspiration was done from the posterior superior iliac spine from most of the cases except in very fatty subjects in whom sternal puncture was performed. For obtaining the marrow material from the posterior superior iliac spine the
subject was placed in a lateral position and the hip and knee joints were flexed to obtain the maximum prominence of the selected area of the iliac spine. The area was cleaned thoroughly first with tincture of iodine and then with savlon. Under aseptic condition the front was anaesthetized with 1% xylocaine up to periosteum with a 23 gauge needle. More than 2 ml. amount of xylocaine were hardly ever required. After 2 - 3 minutes the area was tested for local anaesthesia.

With the help of a Salah's sternal puncture needle the skin and subcutaneous tissue were pierced. When the needle reached up to the periosteum the guard on the needle was adjusted to allow further penetration of 5 mm. The needle was then pushed with a boring motion until a feeling of 'Give in' was experienced. The stilette was removed and 0.5 ml. to 1 ml. of bone marrow was aspirated with a well fitting 10 ml. injection syringe. The peripheral blood was sucked with the help of 23 gauge needle attached to a 2 ml. syringe until the marrow bits were visible on the slide. 4 - 5 smears were prepared on the dust free glass slides. Apart from the smear made with the usual spreading technique at least 2 smears were prepared by squashing the bits of marrow between two glass slides.
The slides were allowed to dry and fixed in Methanol for 10 minutes.

B) Staining Procedure

I) Solutions:

a) 2% solution of potassium Ferricyanide - prepared fresh each time by dissolving 2 gms. of potassium ferricyanide in 100 ml. of deionized water.

b) 2% Hydrochloric acid - prepared by adding 2 ml. of concentrated Aalar Hydrochloric acid to 98 ml. of deionized water.

c) Equal quantities of 2% Potassium Ferricyanide and 2 percent hydrochloric acid were mixed just before the time of staining.

d) 1% neutral red solution prepared by dissolving 5 gms. of neutral red powder in 500 ml. of distilled water.

II) Method:

a) Methonal fixed bone marrow smears were placed in an iron free coplin's staining jar. Freshly
prepared Prussian blue stain was added to the jar which was then placed in a water bath at a temperature of 56° for a period of 15 minutes.

b) Slides were washed several times with deionized water and were left in it for 10 - 15 minutes.

c) After another wash the slides were counterstained with one percent neutral red for 30 - 45 seconds.

d) Slides were washed again with deionized water and then with tap water and allowed to dry.

III) Grading of Iron:

Slides were studied for the presence of iron particles under oil immersion. At least 2 squash smears were studied in each case. Only the iron particles attached to the reticuloendothelial cells were considered, the free particles were ignored as artifacts. The grading was done according to the criteria of Lundin et al (1964) as given below:
Grade zero - No haemosedrin granules in the smear in the whole preparation.

Grade Traces - One or a few granules in the whole preparation.

Grade I - Fine granules in about every third or fourth oil immersion field.

Grade II - Rather more heavy granule in about every second or third oil immersion field.

Grade III - Haemosedrin granules in every oil immersion field in one or more cells.

Grade IV - Massive haemosedrin deposits with clumped heavy granules.

SIDEROBLAST COUNT

The ordinary smears (not squash preparation) were examined for the sideroblast counts. At least 300 normoblast were examined from two smears. The percentage of these which showed the presence of iron granules in their cytoplasm was calculated. This provided the percentage of sideroblast count of the subject.
IRON STAINING OF LIVER BIOPSY SPECIMEN

Prussian blue staining of the liver biopsy sections was done in each case to evaluate the status of storage iron.

Liver biopsies were done with the Vim-Silverman's needle by the aseptic technique at the usual site of this procedure.

**Staining Procedures**

1) **Solutions:** The same solutions as described earlier for the iron staining of bone marrow smears were used.

All the solutions, including the different dilution of alcohol were prepared in iron free deionized water and all the glass were used was made iron free beforehand.

II) **Methods:**

a) Paraffin sections of liver biopsy tissue were brought down to deionized water deparaffinization in xylene and hydration through rapid changes in descending series of alcohol (absolute, 90%, 70%, 50% and 30%).
b) The sections were kept overnight in demineralized water. This modification in the original method was found to be helpful in avoiding the artifacts.

c) Slides were placed in an iron free coplin's staining jar. Freshly prepared Prussian blue stain was added to the jar which was then placed in a water bath at a temperature of 50°C for a period of 30 minutes.

d) Slides were washed several times with demineralized water and were left in it for another 30 minutes.

e) After another wash the slides were counterstained with one percent neutral red for 3-5 minutes.

f) Now the slide could be washed with running tap water without producing artifacts.

g) Slides were quickly treated with absolute alcohol (Excessive treatment with alcohol or treatment with changes through ascending series of alcohol results in excessive removal of neutral red stain).

h) Slides were cleared in xylene for 30 - 45 minutes and mounted with D.P.X.
III) Precaution observed during the process of staining of liver biopsy specimens—This simple procedure of staining for hemosiderin iron needs all the care to avoid the appearance of artifactual iron deposits in and on the sections. Unless due precautions are taken, these often unavoidable artifacts create immense difficulty in interpretation of the results. Although the precautions outlined below were strictly adhered to, yet as many as 40% of the sections stained in this study needed restaining to obtain satisfactory section devoid of artifacts.

a) It was necessary to deparaffinize the sections very thoroughly, without which loss of artifactual iron deposits stuck around on the sections as well as the microslides used. This was achieved by leaving the paraffin sections for long periods (1–2 hours) in xylene, after repeating the process of blotting several times on the filter paper sheets.

b) It was observed that a thorough wash of the sections in demineralized water before treating these with potassium ferrocyanide helped considerably in avoiding the artifacts.

c) The temperature at which the treatment with
potassium ferrocyanide in 0.1 N hydrochloric acid was

carried out, was also found to be critical. Heating tempera-
tures higher than 58°C and particularly beyond 60°C was
invariably fraught with artifacts.

d) It was found necessary to make all the
glass-wares iron free (by prior overnight treatment with 10 N
HCl followed by thorough washing with deionised water and to
prepare all the solutions, including various grades of
absolute alcohol in deionised water. However, this precaution
was necessary only up to the stage of washing the sections
after treatment with potassium ferrocyanide. Thereafter single
distilled water and even tap water could be used).

IV) Grading of Iron. Grading of the reticuloendothelial
tissue iron was done according to the criteria of Lundin
et al (1964) as given below:

Grade zero  - No iron other than obviously extracellular
factors.

Grade traces  - Isolated very fine granules in the sections
but difficult to define whether intracellular
or extracellular.

Grade I  - Definite intracellular granules in single
or several cells.
Grade II - Moderately heavy granulation on average in one or several cells in the same field.

Grade III+ - Abundant iron (also in clumps) in several cells in the same field.

Grade IV+ - Very abundant iron and in clumps or coarse crusts.

Note: Positively stained granules outside the reticular cells were ignored unless it was possible to differentiate these from the often unavoidable artifacts of Prussian blue staining.