

THE QUANTITATIVE ESTIMATION OF IRON STORES IN THE
BONE MARROW OF MAN

by

G.E. GALE

A thesis presented for the degree of Doctor of Medicine in the
University of the Witwatersrand.

Johannesburg,
February, 1964.

The QP
535.F4
57L



39629

This is to certify that this thesis is my own work and has not
been presented for any degree of another University.

R. E. Cole.

TO MY WIFE

ACKNOWLEDGEMENTS

It is often difficult to trace the origins of one's medical interests, so subtle is the guidance of one's approach by teachers and so easily forgotten the influence of fortuitous clinical associations. No such mystery surrounds this thesis. My interest in iron metabolism was initiated by Dr. T.H. Bothwell of the Department of Medicine of the University of the Witwatersrand, and there is no doubt that without his stimulus I should not have worked in this field. It is indeed a pleasure to thank Dr. Bothwell for introducing me to this subject, for making available to me the facilities of his laboratory and for his valued advice and constant encouragement in the work and preparation of this thesis. The work was made possible through grant AM 04912-02 from the National Institutes of Health, United States of America, and through a grant from the Council for Scientific and Industrial Research, South Africa, to Dr. Bothwell.

I should like to thank Mr. J.D. Torrance and Mrs. K.L. Hurd for their willing help in the laboratory, and the following thoracic surgeons for their kind co-operation in

allowing access to their patients: Mr. L. Fatti, Mr. P. Marchand, Mr. D. Fuller, Mr. E. Joubert, Mr. G. Katz and Mr. I. Opeskin. Necropsy material was made available through the courtesy of Professor B.J.P. Becker and Dr. C. Isaacson. I am grateful also to Mr. A.D. Joffe for help with the statistical analyses.

My thanks are due to the Photographic Unit of the Department of Medicine for the reproduction of charts and tables and to Mr. M. Ulrich of the South African Institute for Medical Research for the photomicrographs.

I am grateful to Mrs. Clayson who has done the typing with skill and forbearance.

Finally, I am deeply indebted to Professor G.A. Elliott, who, as Head of the Department of Medicine and as my Chief, has always given me great encouragement not only in this project but also in the years before ever it was contemplated; and he has always made freely available the facilities of his Department.

This thesis represents an elaboration and extension of my investigations into the measurement of the total iron stores in human bone marrow which were published in July 1963 in the Journal of Clinical Investigation.

I N D E X

Page

C H A P T E R I

INTRODUCTION

Present knowledge of iron metabolism	1
The amount and distribution of iron in the body	1
The chemical nature and function of non-storage iron compounds	3
Absorption of iron	5
Plasma iron and its turnover	7
Iron utilization	10
Iron excretion	11
Iron deficiency and iron overload	13
The scope of the present study	17

C H A P T E R II

CURRENT KNOWLEDGE OF IRON STORES

Characteristics of ferritin	20
Haemosiderin	22
Cellular distribution of iron stores	24
The regulation of iron stores	25

	<u>Page</u>
Pathological variations in storage iron	27
Quantitative aspects of total iron stores ...	28
Quantitative aspects of organ iron stores ...	29
The assessment of iron stores in the marrow	33
The clinical evaluation of iron stores	37

C H A P T E R I I I

CHEMICAL METHODS

The determination of non-haem iron fresh tissue	47
The quantitative fractionation of tissue iron compounds	48
Exsanguination methods	48
Haem extraction	49
Extraction of non-haem iron	54
The determination of non-haem iron in formalinized tissues	61
The determination of plasma iron and iron binding capacity	63

C H A P T E R I V

CLINICAL AND NECROPSY MATERIAL: METHODS AND PRINCIPLES INVOLVED

Clinical material	64
--------------------------	----

	<u>Page</u>
Procedure: isotopic techniques	65
Radiation exposure	67
Calculations	68
Body haematocrit	68
Blood volume	68
Plasma iron turnover	70
Red cell radioiron utilization	71
Total marrow storage iron	74
Weight of total body haematologically active marrow	77
Iron incorporation into haem	77
Necropsy study: material and methods ...	78
Histological methods	79

C H A P T E R V

RESULTS AND DISCUSSION

The isotopic study: quantitation of marrow stores	81
The influence of race and sex	84
The relationship of clinical diagnosis to iron stores and haematological data	91

	<u>Page</u>
The relationship of plasma iron and iron binding capacity to marrow stores	101
The correlation of marrow weight with body weight	102
Iron incorporation into haem	106
Studies on necropsy material	106
Marrow iron concentrations	107
Liver iron concentrations	111
The quantitative relationship between storage iron concentrations in bone marrow and liver	113
Histological studies	116

C H A P T E R V I I I

<u>SUMMARY</u>	128
-----------------------	-----

A P P E N D I X

I.	Method for the preparation of radioactive ferritin	132
II	Method for the preparation of radioactive haemoglobin	133
III	An aspect of iron exchange between plasma and bone marrow	134
	BIBLIOGRAPHY	140

CHAPTER I

INTRODUCTION

1. Present Knowledge of Iron Metabolism

Medical interest in iron is centuries old and the ancient Greeks held the belief that it was beneficial to persons suffering from weakness. Its identification as an essential component of haemoglobin and the recognition that its deficiency leads to microcytic hypochromic anaemia have led from this empiricism to the current rational therapeutic use of iron salts. Because iron deficiency anaemia is such a common disease and because the functions of iron in oxygen transport and cellular respiration are of such fundamental physiological importance, it has been the subject of great attention and voluminous medical literature. From the following brief summary of the state of present knowledge of iron metabolism it will be apparent that, despite outstanding recent advances in this field, there is a great deal which remains to be elucidated.

The Amount and Distribution of Iron in the Body

The total amount of iron in the adult human body is usually stated to be between 4 and 5 g. Estimates are,

however, difficult to make and these figures are based on complete chemical analyses of 4 cadavers by Widdowson et al. (1951) and on studies of iron distribution in tissues of dogs (Hahn, 1937; Drabkin, 1951). About 70 per cent of total body iron, or between 2,000 and 2,700 mg., is present in the haemoglobin of circulating erythrocytes. Myoglobin, which occurs in skeletal and heart muscle in concentrations varying from 1.2 to 6 mg. per g. wet weight (Biorck, 1949; Drabkin, 1951), represents 100 to 150 mg. of iron. Other "tissue" or "parenchyma" iron compounds are widely distributed throughout body tissues but are minute in amount, accounting for about 0.2 per cent of total body iron (Drabkin, 1951). These compounds comprise the important cytochrome oxidases, catalase, other cytochromes and iron flavoproteins. Iron is present in plasma in a concentration, in health, of between 60 and 200 µg. per 100 ml. (Ramsay, 1958), corresponding to a total of 3 to 4 mg. In addition to these fractions there exists a quantitatively more variable, metabolically less active, portion of body iron in storage depots in various organs, notably the liver, bone marrow and spleen.

This iron exists in two forms, haemosiderin and ferritin, and although quantitative estimates have varied, it is thought to comprise about 25 per cent of the total in adult males (Granick, 1958), or roughly 1 g. It can be mobilized for use in haemoglobin synthesis in case of blood loss.

The Chemical Nature and Function of Non-Storage Iron Compounds

1. Haemoglobin: Haemoglobin belongs to the group of chromoproteins and consists of a protein globin combined with the complex pigment haem. The latter consists of an atom of ferrous iron in the centre of a porphyrin ring. Each haemoglobin molecule contains four such rings and therefore four iron atoms. It is the ferrous iron which confers upon haemoglobin its ready ability to enter into reversible combination with atmospheric oxygen and so subserve its function of oxygen transportation.

2. Myoglobin: Myoglobin is a haem-protein occurring in striated muscle. It is structurally and functionally related

to haemoglobin but contains only one ferrous-porphyrin group per molecule. It has a greater affinity for oxygen than that of haemoglobin and this enables it to accept oxygen from the latter compound and serve as an oxygen reservoir in muscle.

3. Catalases and Peroxidases: The catalases and peroxidases are high molecular weight iron-pyrrol-protein enzymes which liberate oxygen from peroxides and are concerned with tissue respiration. Their iron is in the ferric state.

4. Cytochromes: The cytochromes are a group of haem enzymes present in the mitochondria of cells and provide a means of electron transfer through the ready ability of the iron atom to undergo reversible oxidation.

5. Non-haem Tissue Iron Compounds: Iron flavo proteins contain both iron and riboflavin. Succinic dehydrogenase and xanthine oxidase also contain iron but little is known of the part it plays in their enzymatic actions.

6. Transferrin: Plasma or transport iron serves as the connecting link between the other iron compartments. The metal is present in the free ionic form in very minute quantity only: almost all plasma iron is chelated to a beta-1 globulin known as transferrin or siderophilin (Surgenor et al., 1949). The iron is in the ferric form and is thought to be linked to transferrin through a hydroxamic acid group (Fiala and Burk, 1949). Eight different transferrins have been identified, each with an equal affinity for iron (Turnbull and Giblett, 1961). Normal plasma contains 240 to 280 mg. of transferrin per 100 ml., but its concentration is usually expressed in terms of the "iron-binding capacity" of plasma: this is about 300 to 400 μ g. Fe per 100 ml. (Bothwell and Finch, 1962 (a)). Normally, approximately one third of the transferrin in plasma is bound to iron or "saturated", and the remainder represents a reserve of "unsaturated iron binding capacity".

Absorption of Iron

The absorption of dietary iron is important not

only from the point of view of nutrition and the therapy of iron deficiency, but also because it appears to be the chief mode of regulation of total body iron (Crosby, 1963; Conrad and Crosby, 1963). The reason for the latter fact is that the capacity of the body to excrete iron is extremely limited. Thus there has been considerable recent interest in the mechanisms of iron transfer across the cells of the intestinal mucosa, but the exact nature of the processes involved has not yet been fully elucidated (Moore and Dubach, 1962).

Absorption takes place chiefly from the duodenum and upper jejunum. Many factors influence the efficiency of absorption of dietary iron, but in general normal subjects retain 5 to 10 per cent and iron deficient subjects 10 to 20 per cent of what is ingested. A normal person on an adequate diet containing 12 to 15 mg. of iron daily should therefore retain 0.6 to 1.5 mg. of iron per day.

The element is absorbed in the ionized ferrous form (Moore et al., 1944) and thus the presence of reducing agents such as vitamin C enhance absorption of iron ingested

in the ferric form. Other factors promoting absorption are adequate gastric acidity (Goldberg et al., 1963; Jacobs et al., 1963) and a high concentration of intraluminal iron (Bothwell et al., 1958). In addition, the availability of iron in various foodstuffs varies considerably and a high content does not necessarily imply greater retention (Moore and Dubach, 1962). Phytates or phosphates form insoluble iron salts and so interfere with absorption (Sharpe et al., 1950). The mode of regulation of iron absorption is not fully understood, but it has been established that assimilation is enhanced by accelerated erythropoiesis (Bothwell et al., 1958; Mendel, 1961), by depletion of iron stores (Dubach et al., 1948; Pirzio-Biroli and Finch, 1960) and by hypoxia (Reynafarje et al., 1959; Mendel, 1961). There is also recent evidence that anaemia per se can stimulate absorption (Mendel et al., 1963). Conversely, when erythropoiesis is depressed or iron stores are large, absorption is usually decreased.

Plasma Iron and its Turnover

Normal plasma (or serum) iron values range from

70 to 150 $\mu\text{g.}$ per 100 ml. There is considerable diurnal variation in its concentration, levels being about 30 per cent higher in the morning than in the evening (Valquist, 1941; Waldenström, 1946; Laurell, 1953). Variations may also be produced by disease; plasma iron is lowered in iron deficiency anaemia (Laurell, 1947; Kaldor, 1953; Beutler et al., 1958), in chronic infection (Cartwright and Wintrobe, 1949; Freireich et al., 1957) and in states of rapid haemoglobin synthesis (for example, following haemorrhage or in pernicious anaemia in remission) (Finch et al., 1950 (b)). In chronic iron deficiency, transferrin levels are usually abnormally high and this factor combines with the fall in plasma iron to cause a decrease in the percentage saturation of the iron binding capacity. Increased plasma iron concentrations occur when there is iron overload in the tissues (in the conditions of idiopathic haemochromatosis, transfusion haemosiderosis and Bantu siderosis) (Finch and Finch, 1955; Higginson et al., 1957; Seftel et al., 1961), in certain conditions of impaired haemoglobin synthesis (aplastic anaemias and thalassaemia) (Laurell, 1952; Erlandson et al., 1958), in rapid haemolysis

(Noyes et al., 1960), in acute hepatitis due to release of ferritin from damaged liver cells (Rumball et al., 1959) and during the exogenous administration of iron. Low transferrin levels may occur in conditions which impair the synthesis of plasma proteins, such as hepatic cirrhosis, chronic infection and severe starvation (Laurell, 1947; Lahey et al., 1958).

The intense metabolic activity of the 3 to 4 mg. of iron present in plasma is indicated by its extremely rapid turnover rate. In normal persons from 25 to 40 mg. of iron enter and leave the plasma every 24 hours (Huff et al., 1950; Bothwell et al., 1956; Pollycove and Mortimer, 1961). A standard of reference allowing for variation in blood volume between individuals expresses the turnover in mg. per 24 hours per 100 ml. whole blood, the average normal value being 0.6 mg. (Bothwell et al., 1957). This turnover represents the iron which is being internally redistributed, via the plasma, and also embodies the limited amounts derived from intestinal absorption and lost by excretion. Normally, in the state of iron balance, the latter two quantities are equal. The major

cycle of iron exchange, accounting for the movement of 20 to 25 mg. per day, is through haemoglobin synthesis, its breakdown after 120 days when red cell life span has ended, and its resynthesis. Thus the degree of erythropoietic activity has the dominant influence upon the rate of plasma iron turnover (Bothwell et al., 1957), which is rapid in iron deficiency anaemia, haemolytic anaemias and polycythaemia and slow in aplastic anaemia and uraemia.

Iron Utilization

When a tracer dose of transferrin-bound radio-iron is injected intravenously it is rapidly removed from the plasma. In 1 to 2 hours half of the injected dose has left the plasma and after 24 hours all but a minute fraction (about 1 per cent) has disappeared. The major portion is taken up by the bone marrow for haemoglobin synthesis (Huff et al., 1950; Pollycove and Mortimer, 1961) and labelled red cells start appearing in the peripheral blood within 8 hours. By the eighth to tenth day the radioactivity in circulating red cells has reached its maximum, which normally represents 70 to

100 per cent of the injected dose, and radioactivity has largely disappeared from the marrow (Huff et al., 1951; Bothwell et al., 1956; Pollycove, 1958). Of the remainder, most is found in iron-storage sites, chiefly in the liver and bone marrow, but a small portion is excreted in the urine and some is found in the extracellular fluid. Pollycove and Mortimer (1961) have postulated, on the basis of mathematical models constructed from the disappearance curve of radioiron from plasma, that about one third of the iron taken up by the marrow is not directly utilized for haemoglobin synthesis, but is returned from an "erythropoietic labile pool" to the plasma. This is further discussed in the Appendix III (page 134).

Characteristic patterns of iron turnover and red cell utilization are present in various haematological disorders and these investigations may thus be of value in elucidating clinical problems.

Iron Excretion

One of the striking features of iron metabolism is

that, once absorbed, the metal is tenaciously conserved for there is no ready route of excretion. This has been known for many years from careful balance studies (McCance and Widdowson, 1937; Little et al., 1945) and has been confirmed using isotopic techniques (Dubach et al., 1955; Finch, 1959).

In the absence of an active regulatory mechanism for the excretion of iron, there is a more or less constant loss in men and non-menstruating women which amounts to between 0.5 and 1.0 mg. per day. This occurs from desquamation from skin and mucosa of the gastro-intestinal tract, from normal red cell losses in the urine, from leucocytes and epithelial cells discharged in body secretions, and through sweat. In menstruating women an additional and considerable source of blood loss is present. It varies widely in different individuals, but an estimated average daily iron loss through menstruation is 0.8 mg. (Millis, 1951). The total loss of iron incurred through one full-term pregnancy has been estimated to be between 280 and 490 mg. (Moore and Dubach, 1962).

Iron Deficiency and Iron Overload

The figures for iron absorption and excretion make it clear that normal males are able to maintain iron balance without difficulty; but menstruating females and infants and children (who must provide for tissue growth and an expanding red cell mass) are in a precarious state of balance and their dietary iron content is crucial. A daily negative balance of a fraction of a milligram may be all that is required to produce severe iron deficiency after a number of years. It is therefore easy to understand why iron deficiency anaemia is more common in growing children and menstruating women than in other groups; and why persistent chronic haemorrhage of only minor degree may result in anaemia.

The first stage in the sequence of iron deficiency in an individual is the depletion and exhaustion of stores; there- after the usual train of events is a rise in plasma transferrin and fall in plasma iron, the appearance of hypochromic micro- cytic anaemia, and lastly the development of deficiency of

fixed tissue iron with characteristic manifestations of koilonychia, dysphagia and glossitis (Bothwell and Finch, 1962 (b)).

Conversely, excessive accumulation of iron in the body occurs when there is a long-continued excess of absorption over excretion. A positive iron balance of only 3 mg. per day would result in an accumulation of over 1 gm. per year; excess absorption does therefore not have to be great for considerable iron overload to occur after a number of years. The clinically encountered conditions of iron overload are:

- a) idiopathic haemochromatosis, b) Bantu siderosis, c) the siderosis of thalassaemia and certain refractory anaemias, d) overload following prolonged "therapeutic" ingestion of iron and e) transfusion haemosiderosis. In the first four conditions the iron enters the body by way of the gastro-intestinal tract and its distribution tends to be to the parenchymal cells of organs, especially the liver. In transfusion haemosiderosis it is parenterally introduced (in haemoglobin) and accumulates initially in reticulo-endothelial cells.

Idiopathic haemochromatosis is a relatively rare disease in which excessive intestinal absorption of iron leads to tissue iron overload associated with hepatic cirrhosis, diabetes mellitus, skin pigmentation and often with cardiac and other endocrine damage. The total body iron content in this condition averages about 20 g. but may rise to 40 g. or more (Sheldon, 1935 (a); Finch and Finch, 1955).

Tissue siderosis is common in the South African Bantu, the incidence varying in different studies between 40 and 88 per cent (Higginson et al., 1953; Wainwright, 1957; Gillman et al., 1958; Bothwell and Bradlow, 1960). All degrees of iron overload occur from slight to gross excess, the chief sites of deposition being the liver and reticulo-endothelial system. The condition first becomes manifest in late adolescence and tends to increase in severity up to the age of 50 (Wainwright, 1957); the incidence and severity are greater in males than in females. The major factor in the etiology of this form of iron overload is the very high dietary iron of the Bantu, which is derived from the iron utensils used in cooking and in the prepara-

tion of kaffir beer (Walker and Arvidsson, 1953). The incidence of tissue damage in these individuals is not high although the development of liver cirrhosis does appear to depend upon the degree of its iron overload (Bothwell and Bradlow, 1960). However, the exact relationship between iron overload and tissue damage is not clear (Cappell et al., 1957; Brown et al., 1957).

Certain anaemias which are associated with a hyperplastic erythroid marrow and ineffective erythropoiesis may in time lead to tissue iron overload by virtue of long-continued enhancement of absorption of dietary iron, which is often augmented by the administration of iron salts. These conditions include thalassaemia, "refractory anaemias", haemolytic anaemias and paroxysmal nocturnal haemoglobinuria. There is often an additional source of iron in the repeated blood transfusions administered to subjects with these anaemias.

Self-medication with oral iron preparations continuously for many years without therapeutic indication

has been reported to be associated with gross iron overload and the clinical picture of haemochromatosis (Case Records of the Mass. General Hospital, 1952; Wyatt, 1956).

In transfusion haemosiderosis the excess iron is derived from repeated blood transfusions, as may be required in the treatment of hypoplastic anaemia, thalassaemia or other refractory anaemias. Considerable quantities of iron may accrue, but cirrhosis and diabetes are not common (Cappell et al., 1957).

2. The Scope of the Present Study

The foregoing brief review of iron metabolism in general has been given in order to place in perspective the particular aspect with which this study is concerned - that of storage iron. The state of current knowledge of this subject is reviewed in the following chapter (p. 20 et seq.)

Considerable attention has in the past been paid to the role of the liver in iron storage. Although it has been

regarded as the chief storage organ, chemical analyses show that under normal circumstances it contains only up to 300 mg. of iron (Sheldon, 1935 (b); Hallgren, 1954; Morgan and Walters, 1963), or roughly one third of total stores. Significant quantities are known to be present elsewhere: variable amounts are visible in the reticulo-endothelial cells of the spleen and bone marrow, and there is some chemical evidence to indicate that appreciable amounts may be present in skeletal muscles (Roth et al., 1951). Although the spleen has a high concentration of iron, it cannot normally be regarded as an important depot because of its small size. It is common observation that quantitative differences in storable iron content of different marrows occur, but little appears to be known of the actual quantities stored therein, either in health or disease.

The present study was undertaken to determine quantitatively the amount of iron stored in the reticulo-endothelial cells of human bone marrow under normal circumstances and in various pathological states, and to define the extent to which these stores are capable of expanding when iron

overload is present: a unique opportunity for the study of iron excess is presented by its prevalence among the South African Bantu.

In order to attempt to relate these results to the major part of total body stores, the correlation between the storage-iron concentrations in marrow and in liver was then investigated. It was felt also that the methods used in these investigations might be applied towards the clarification of an incidental problem in iron metabolism, over which there has been some controversy, namely, the rate of iron incorporation into haem.

The methods used, described in detail in subsequent chapters, are a combination of biochemical, histological and isotopic techniques.

CHAPTER II

CURRENT KNOWLEDGE OF IRON STORES

Iron stored in adult tissues represents an excess which has been accumulated slowly through childhood and adolescence. It is deposited widely in the body in parenchymal cells of various organs and in the cells of the reticulo-endothelial system. It exists in two forms, ferritin and haemosiderin.

Characteristics of Ferritin

Ferritin is a water soluble iron-protein complex which occurs chiefly in the liver, spleen and bone marrow and to a lesser extent in other tissues. It was first isolated from horse spleen by Laufberger in 1937. Its iron content is variable, but is usually about 20 per cent; the nitrogen content is 10 per cent. The molecule consists of miscelles of ferric iron salts in a protein matrix, apoferritin (Rothen, 1944). It can be crystallized from tissues by cadmium sulphate, and enumeration of these crystals has been used as a semi-quantitative method of tissue ferritin determination. In solution, the substance has a reddish-brown colour. Characteristic electrophoretic migration distinguishes it from other iron-containing compounds and quantitative determinations may be made colorimetrically after paper electrophoresis and staining with Berlin blue. It may also be distinguished magnetometrically

from all other body iron (Michaelis et al., 1943).

It occurs intracellularly and may be present in dispersed or aggregate form. It is not visible by staining and conventional microscopy, but its molecules are demonstrable on ultra-microscopy and have the characteristic appearance of tetrads (Bessis and Breton-Gorius, 1957).

The mechanisms by which iron is taken up and released by ferritin have been studied by several workers. Its incorporation appears to be an energy-dependent process in which adenosine triphosphate and ascorbic acid are involved in the reduction of ferric iron to the ferrous form (Mazur et al., 1960; Mazur et al., 1961). The mobilization of iron from ferritin is incompletely understood, but it is known that reducing agents such as ascorbic acid, glutathione and cysteine may effect this release (Bielig and Bayer, 1955), and there is evidence to suggest that the enzyme xanthine oxidase may participate in the reaction (Green and Mazur, 1957; Mazur et al., 1958).

In addition to its function as an iron storage compound, ferritin possibly plays a part as a mediator of iron absorption through the intestinal mucosa (Charlton et al., 1963) and it may be involved in iron transport through the placenta (Pribilla et al., 1958). Here again, the precise mechanisms involved are not understood. Other biological effects of ferritin, which have been studied by Mazur et al., (1955), are a vasodepressor action and an antidiuretic effect.

Haemosiderin

Haemosiderin is an iron-protein complex which is thought to consist of aggregated ferritin molecules together with other elements. As an iron storage compound, it occurs in the tissues in which ferritin is found and it bears a close chemical and functional relationship to ferritin, although certain well-defined differences exist (Shoden et al., 1953; Shoden and Sturgeon, 1961). Haemosiderin is insoluble in water and may be seen both intra- and extracellularly as aggregates of golden-brown granules which take the Berlin blue stain. It evidences no migration on paper electrophoresis. The varying iron and

nitrogen contents of different preparations of haemosiderin and the presence of additional elements such as porphyrins in some preparations (Shoden and Sturgeon, 1960) indicate that what is generally regarded as haemosiderin is in fact an inhomogeneous substance. Its iron content is usually between 30 and 36 per cent.

Methods of quantitative fractionation of tissue ferritin and haemosiderin suggest that normally slightly more ferritin is present in storage than haemosiderin, but that as the stores increase, there is a progressive increase in the proportion of haemosiderin (Shoden et al., 1953).

It is evident from a number of studies that iron appears to be equally available from both storage forms (Shoden et al., 1953; Finch and Finch, 1955) and that more recent deposits are more readily mobilized (Dubach et al., 1946; Hampton, 1954).

Cellular Distribution of Iron Stores

A variety of cell-types are capable of storing iron, whose source appears to be an important determining factor at least in its initial cellular distribution. Iron derived from expired erythrocytes, whether these are endogenous or exogenous, and from parenteral administration, is stored initially predominantly in reticulo-endothelial cells; this is easy to understand in view of the phagocytic activity of the reticulo-endothelial system. There is evidence, however, that with time there is a partial redistribution of this iron to the parenchymal cells of the liver as well (Polson, 1929; Cappell, 1930; Finch et al., 1950 (a)). This distribution is seen after chronic haemolysis, and amounts to gross iron overload in the condition of transfusion siderosis. In contrast, iron storage or overload arising from enhanced intestinal absorption results in increased deposition in parenchymal cells, initially of the liver but later of other organs also, and a variable and late rise in reticulo-endothelial iron content occurs. This form of iron overload is exemplified by idiopathic haemochromatosis. In siderosis

of the South African Bantu the distribution is somewhat anomalous in that, although the excess iron is derived via intestinal absorption, the reticulo-endothelial compartment is involved early and simultaneously with hepatic parenchyma (Bothwell and Bradlow, 1960 ; Higginson et al., 1953). Thus, while the intracellular distribution of iron is of physiological interest, once equilibrium has been reached little can be deduced from it regarding its source or mechanism of accumulation. In cases of extreme iron overload, cellular histology may be obliterated and it is likely that there are large accumulations of extracellular iron.

The Regulation of Iron Stores

The ability of the reticulo-endothelial cells and of certain parenchymal cells to maintain a tremendous gradient of iron concentration across the cell membrane is of great interest and importance, since it is this attribute which makes storage possible. It is known in general terms that if iron is lost from the body in excess of that absorbed from the diet or other sources, the stores are drawn upon to maintain a constant

level of haemoglobin; and also that in continuing negative iron balance the stores will be exhausted or near-exhausted before anaemia is allowed to develop. However, the fundamental biological mechanisms controlling the release of iron from storage cells are not understood.

Rebuilding of stores, even under the most favourable circumstances, takes place slowly, and after complete exhaustion years are required before full repletion has occurred (Haskins et al., 1952). The re-accumulation of iron is made possible by enhanced intestinal absorption which occurs after depletion even in the absence of anaemia. This increased absorption is not associated with increased marrow activity (which in itself is a powerful stimulus to absorption); it has been mentioned that the level of iron stores influences the rate of its absorption independently of other factors (Dubach et al., 1948; Pirzio-Biroli and Finch, 1960). Although a humoral mechanism controlling this has failed to be demonstrated in animal experiments (Beutler and Bittenwieser, 1960; Moore, 1961), it is difficult to conceive of any other means by which

this regulation is mediated.

Pathological Variations in Storage Iron

Available methods of measuring iron stores and the content of various organs will be discussed subsequently; the following general observations relate to rough, semi-quantitative changes which have been observed in various pathological states. It should be noted in passing that iron stores may vary considerably in health.

Iron deficiency results from chronic blood loss or dietary insufficiency or a combination of both these factors. Once established it results in all degrees of diminution of stores up to complete exhaustion, after which further stages in the sequence of iron deficiency may develop.

In all anaemias not due to iron deficiency there is a greater or lesser augmentation of stores because the iron formerly in erythrocytes has no ready route of excretion. The increase may be extreme in thalassaemia (where there is blocking of haemoglobin synthesis in addition to haemolysis), in hypoplastic

anaemia or in a form of refractory anaemia in which the marrow is hyperplastic and iron absorption is therefore enhanced (Bothwell and Finch, 1962 (c)). The major source of iron in these groups is usually therapeutic administration in the form of blood transfusions, with or without additional oral or parenteral preparations, but it has been shown that subjects of these anaemias who have hyperplastic marrows may develop severe iron overload without significant contribution from blood transfusions (Wallerstein and Robbins, 1953; Ellis et al., 1954; Kleckner et al., 1955; Wyatt, 1956). The markedly increased stores of idiopathic haemochromatosis and Bantu siderosis, and following the prolonged self-administration of iron tablets, have already been mentioned. Lesser degrees of augmentation may be encountered in haemolytic anaemias, pernicious anaemia, folic acid deficiency, the anaemia of chronic renal disease and in the secondary normocytic anaemias associated with neoplasia and chronic infection. In the latter two conditions there is a block in the release of iron from the reticulo-endothelial cells, where it accumulates.

Quantitative Aspects of Total Iron Stores

Little attention appears to have been given to the quantitation of iron stores in man. Hahn (1937) found storage iron in dogs to be about 20 per cent. of total body iron; extrapolation of this result to man would place the normal human value at about 800 mg. On the basis of repeated small bleedings over a period of 136 days, Hynes (1949) calculated the iron reserve in a normal man of 37 years of age, weighing 63 kg., to be 600 mg. In a series of ingenious experiments, Finch et al. (1950 (b)) and Haskins et al. (1952) measured the available iron stores of normal subjects and of patients with iron deficiency and with haemochromatosis. These workers observed the rate of haemoglobin synthesis when their subjects were exposed to measured iron loss by repeated phlebotomy for several weeks. A fall in the rate of synthesis was reflected by a fall in the haemoglobin level, and this indicated that available stores had been exhausted. In the normal adult male, iron stores measured by this method are 1,000 to 1,500 mg.; only one female (who had haemochromatosis) was studied.

In a protracted isotopic dilution study with a 4-year follow-up, Finch (1959) measured the rate of loss of radioactive iron from the red cells in normal humans. He found an initial exaggerated fall of activity lasting about a year; thereafter the rate of loss became constant. The initial phase implied a mixing component and from it was calculated the total miscible storage iron. The mean values obtained were 600 mg. in 6 males and 380 mg. in 18 females - appreciably lower than those of the previously outlined method. The discrepancy is presumably to be explained by the fact that the second method measures miscible rather than totally available stores - the implication being that only a portion of stores are normally miscible even over a prolonged period. The lower value obtained in females is significant and is in keeping with their more ready tendency to iron deficiency.

Quantitative Aspects of Organ Iron Stores

Usual estimations of iron concentration in an organ reflect both tissue storage iron and iron present in haem. Unless the former form is far in excess of the latter (as

happens in states of iron overload) the total iron content of an organ does not provide an accurate quantitative estimation of its storage iron. More meaningful measurements of iron stores in individual organs have been made on the chemical basis that all or nearly all of the non-haem iron in tissue represents stores. (This is an adequate working hypothesis in view of the nature and distribution of body iron discussed in Chapter I, and is borne out by the very low non-haem iron content of the liver and spleen in iron deficiency anaemia when the available stores have been exhausted). The estimation involves chemical differentiation of haem and non-haem iron for which a number of methods are available (p.47 et seq.). Another method of making this differentiation is the postmortem perfusion of organs (other than muscle which contains haem in the form of myoglobin) to remove the blood as the source of haem iron. This has been found to be unsatisfactory (Hahn and Whipple, 1936); viviperfusion is likewise difficult to perfect and in any case is applicable only to experimental animals. In the case of certain organs e.g. kidney (Brückmann and Zondek, 1939) and skeletal muscle

(Hahn, 1937), there is some evidence that all the non-haem iron is not available for haemoglobin synthesis and therefore does not represent true storage iron.

An immunochemical technique for the quantitative analysis of tissue ferritin (Mazur and Shorr, 1950) has the advantage of far greater accuracy than chemical methods, but does not itself quantitate iron stores because it does not measure haemosiderin.

In quantitative terms, the amount of iron present in the liver is well documented. Sheldon (1935 (b)) regards the normal range as between 0.05 and 0.1 per cent dry weight, which in a liver weighing 1,500 g. corresponds to a total content of 190 to 375 mg. The normal figure for storage iron in the liver is of the same order and has been found by various authors to be between 220 and 390 mg. (Finch and Finch, 1955; Hallgren, 1954; Morgan and Walters, 1963). This represents only one quarter to one third of the total body stores as determined by the methods previously outlined. The spleen normally

contains only 15 to 20 mg. of storage iron (Roth et al., 1951; Hallgren, 1954; Morgan and Walters, 1963).

As previously indicated, there is evidence that muscle contains significant amounts. Thus Hahn (1937) states that in a perfused dog of 20 kg. body weight, the total bulk of striated muscle may contain from 65 to 195 mg. non-haem or "parenchyma" iron. According to this author these are inviolate "stores" and are not drawn on even in severe anaemia. The latter view is at variance with the findings of Roth et al. (1951) that the mean muscle non-haem iron was 530 mg. in 19 haematologically normal human subjects and 195 mg. in 9 subjects with iron deficiency anaemia. Hallgren (1954) found a mean non-haem iron concentration in human psoas muscle of 1.2 mg. per 100 g. If even distribution throughout remaining muscle is assumed, this would correspond to a total muscle content of 360 mg. in an average adult male.

The low concentration of non-haem iron in muscle throws doubt on the accuracy of its chemical determination, and immunochemical methods provide more reliable results. Using such a method

Mazur and Shorr (1950) found a mean ferritin-iron concentration of 10 $\mu\text{g.}$ per gram in skeletal muscle of dogs. In an animal of 20 kg. this would correspond to a total muscle ferritin-iron content of 65 mg. and, since there is no visible haemosiderin in muscle, it may be assumed that this figure represents the value for non-haem iron. Applied to humans this figure would give a value of 170 mg. It is, however, not known to what extent muscle non-haem iron can serve as true storage iron, i.e. to what extent it can be mobilized when the need arises.

The brain is also known to contain a significant amount of non-haem iron (Tingey, 1937; Hallgren, 1954) and very small quantities are present in the lungs and kidneys. The significance of this iron is not known.

The Assessment of Iron Stores in the Marrow

The total iron concentration in human red marrow has been estimated to be between 400 and 500 $\mu\text{g.}$ per g. of fresh tissue (Cavayé Hazen, 1949). The concentration of non-haem iron, representing stores, was determined by Hallgren

(1954), who obtained a mean value of 148 $\mu\text{g. per g.}$ in 41 adult subjects dying from a variety of causes. The value in 3 cases of sudden traumatic death was 133 $\mu\text{g. per g.}$ On the basis of a total red marrow weight of 1,500 g. in the adult (see p. 102) these figures would place total marrow storage iron at approximately 220 mg. Quoting the higher estimate of 3,000 g. for total red marrow (Whitby, 1954), Morgan and Walters (1963) suggest that iron reserves in the marrow are of the order of 300 mg. There are, however, no available data indicating that these stores have been measured directly.

Their usual assessment is by qualitative histological means, and although it is well known that the iron content of marrow is subject to considerable individual variation there is no information on what actual quantities the visible iron represents. Rath and Finch. (1948) stained marrow fragments for haemosiderin and graded their specimens according to the density of iron staining. In 16 cases of iron deficiency no appreciable amounts of iron were seen, and increased

amounts were seen in pernicious anaemia, uraemia, hepatic cirrhosis, chronic infections and haemochromatosis. The histological grading was thus found to parallel the expected state of iron stores in all groups of cases, and was considered to provide a fairly accurate qualitative indication of the state of the iron stores of the marrow and usually also of the body as a whole. Stevens et al. (1953) subsequently evaluated 298 marrows with similar results, and found that in females the visible iron was less than in males. Further work has confirmed the clinical value of this method of assessment, especially in the detection of iron deficiency (Davidson and Jennison, 1952; Hutchison, 1953; Beutler et al., 1954).

The iron-containing reticulo-endothelial cells are to be found almost exclusively in the solid marrow particles, and unless especial care is taken too little marrow tissue to allow for accurate assessment may be present on smears. For this reason, fixed sections of marrow have been found to be especially satisfactory (Davidson and Jennison, 1952; Hutchison, 1953; Beutler et al., 1954). Artefacts may arise

during the preparation of specimens, but with experience it is usually possible to distinguish these from the granular deposits of true tissue iron.

A further histological method which has been used in the assessment of marrow iron is the examination of haemosiderin-containing red cell precursors. These cells are known as sideroblasts and normally comprise from 24 to 81 per cent of the total number of normoblasts (Douglas and Dacie, 1953; Kaplan et al., 1954). In the vast majority of cases with iron deficiency the sideroblast count is less than 1 per cent (Kaplan et al., 1954). However, the absence of stainable iron in bone marrow particles does not necessarily mean that sideroblasts will be absent; nor is their absence pathognomonic of iron deficiency; and furthermore, increased stores in haemochromatosis or after repeated transfusions are not associated with greater numbers of sideroblasts or any obvious increase in the size of their iron granules (Douglas and Dacie, 1953; Beutler, 1957). An increased count is characteristically found in conditions where iron incorporation into haemoglobin is "blocked",

such as thalassaemia (Morse, 1955). McFadzean and Davis (1947) have found smaller numbers of sideroblasts in normal marrows than other authors; a possible explanation for this discrepancy is difference in staining technique. In summary then it would appear that while the sideroblast count is of value in differentiating iron deficiency anaemia from other types of anaemia and in the elucidation of states of blocked haemoglobin synthesis, it cannot be regarded as a direct measure of stored iron.

The Clinical Evaluation of Iron Stores

The correct evaluation of the state of iron stores may be of major importance in clinical medicine. It affords a means of distinguishing between iron deficiency anaemia and other anaemias; in cases of hypochromic microcytic anaemia the *diagnosis is not always clear, and evaluation of the stores will differentiate those with chronic infection or blocks in haemoglobin synthesis from the commoner category of iron-deficiency anaemia. It also provides an assessment of the patient's degree of vulnerability to chronic blood loss.*

Further, it is essential for the diagnosis of haemochromatosis and other states of iron overload. The importance of early diagnosis of iron overload cannot be overstressed in view of the extensive tissue damage which may be done to the liver and other organs (notably the pancreas, heart and pituitary gland) by excessive iron deposits. This is of more than academic interest, since in anaemias associated with gross iron excess, where venesection as a method of removing iron is obviously not applicable, the recent use of chelates to promote iron excretion has opened up a promising line of therapeutic attack.

These drugs prise the iron from transferrin and cause it to be mobilized from the storage sites; the iron-chelate complex is then excreted in the urine. Chelating agents which have been used for this purpose are ethylenediamine tetraacetic acid, diethylene triaminepentaacetic acid (D.T.P.A.), ethylenediamine di-*o*-hydroxyphenyl acetic acid and desferrioxamine (McMahon, 1956; Fahey et al., 1961; Cleton and Finch, 1961; Bannerman et al., 1962; Lancet, 1962). Desferrioxamine and

D.T.P.A. are currently the most efficient and in an iron-overloaded individual cause a daily excretion of iron of the order of 15 to 50 mg.; a 24 hour loss as high as 108 mg. has been reported. In the treatment of haemochromatosis, even this amount compares unfavourably with a single venesection, which removes about 250 mg. of iron; furthermore, the administration of intravenous chelates may give rise to renal damage (Seven, 1960).

The methods currently employed in the clinical evaluation of iron stores have been reviewed by Beutler (1957). These include:

- 1). The plasma iron turnover calculated by the isotopic dilution technique.
- 2). The determination of the serum iron and iron-binding capacity.
- 3). Repeated phlebotomy until stores are exhausted (applicable only to subjects with haemochromatosis).
- 4). The iron tolerance curve.
- 5). Histological demonstration of haemosiderin in

bone marrow particles and sideroblasts.

6). Histological assessment of iron content of liver biopsy sections.

7). The measurement of urinary iron following the administration of iron chelates.

The plasma iron turnover depends primarily upon erythropoietic activity and has proved unsatisfactory in the evaluation of iron reserves (Bothwell et al., 1957; Beutler, 1957).

The determination of the serum iron and iron binding capacity causes little inconvenience to the patient, but it provides only an indirect assessment of stored iron and may be difficult to interpret. In the sequence of changes found in iron deficiency (which has been discussed on pages 13 and 14), low plasma iron values are found after exhaustion of mobilizable stores has occurred. Thus degrees of storage depletion short of exhaustion are not reflected in the plasma iron. Furthermore, when erythropoiesis is much accelerated, as after specific therapy of pernicious anaemia, the plasma

iron may fall temporarily (Hawkins, 1955) in the presence of normal stores because the latter cannot be mobilized rapidly enough to keep pace with the increased rate of erythropoiesis. Low plasma iron values are also found not uncommonly in chronic infection, carcinoma and uraemia, when stores are increased. The unsaturated iron binding capacity, however, is usually decreased in the latter conditions and is increased in iron deficiency (with resultant decrease in the percentage transferrin saturation), although there may be overlap between the two groups. Furthermore, the capacity may be elevated in pregnancy (Ventura and Klopfer, 1951) and hepatitis (Brendstrup, 1953), presumably independently of iron stores.

Conditions of iron excess are reflected by an elevation of plasma iron and a fall of the unsaturated iron binding capacity. These changes become diagnostic of severe iron overload when they are extreme and the transferrin saturation approaches 100 per cent.

All in all, these parameters, even in combination,

are of limited help in the assessment of iron stores, and may in particular be unreliable in the detection of iron deficiency.

The iron tolerance curve measures the response of the serum iron to a large dose of inorganic iron given orally; it is dependent upon the absorptive capacity of the gut and the rate of removal of iron from the plasma by the bone marrow. A high curve has been shown to occur in iron deficient subjects (Moore et al., 1939). Jasinski (1949, 1950) has used the test extensively to gauge iron stores. Roth et al. (1951) found a good correlation in 12 cases between the iron tolerance curves and iron stores estimated chemically after death. However, there appear to be no constant criteria for evaluating results, and some authors have even found flat curves in iron deficiency (Crawley, 1952; Thedering, 1953). Fallacious results due to unrelated sources of malabsorption, such as steatorrhoea, may occur. Furthermore, erythropoietic activity influences iron absorption to an appreciable extent independently of iron stores (Bothwell et al., 1957). Thus, while a case may be made out for the value of the iron

tolerance curve as an indication of the state of the body's iron reserves, particularly when they are low, there is evidence that conclusions drawn from it in this regard do not always hold; and it cannot provide a quantitative measure.

One of the most informative and simple methods is the histological assessment of the amount of visible iron in marrow tissue, as previously described. Iron deficiency may readily and accurately be thus diagnosed, and established overload easily recognized. Early overload is not always reflected, since marrow demonstrates reticulo-endothelial cell-contained iron, and in the earlier stages of oral iron overload parenchymal cells (of liver) contain the greater amount.

Sections of liver biopsy, suitably stained for iron, provide a more valuable indication of stores than marrow assessment, since both parenchymal and reticulo-endothelial deposits may be demonstrated. The added risks inherent in liver biopsy are a drawback.

Recently, attempts have been made to use the iron chelates ethylenediamine tetraacetic acid (EDTA), diethylene triaminepentaacetic acid (DTPA), ethylenediamine di-o-hydroxy-phenyl acetic acid (EDDHA) and desoferrioxamine in evaluating iron stores. Fahey et al. (1961) found a well-marked increase in urinary iron following parenteral administration of DTPA to subjects with iron storage disease, whereas control subjects showed no such increase. The amount of iron excreted could be correlated roughly with the degree of iron overload as assessed by other methods. This work was confirmed qualitatively by Bannerman et al. (1962) using DTPA and desoferrioxamine, although they did not attempt to correlate the degree of overload with the amount of iron excreted. A similar study of chelate administration to iron-overloaded children with thalassaemia was conducted by Smith (1962), who found an increased iron excretion which could be correlated with the degree of transferrin saturation, the latter being regarded in these subjects as a measure of iron stores.

In the recognition of depleted iron stores, Korman (1960) showed that the intravenous injection of EDDHA-bound radioiron

is followed by significantly lower radioiron excretion in iron deficient subjects than in normals. In the same study, the difference between the urinary iron-chelate and radioiron-chelate was taken to represent the iron exchange between the chelate and the labile pool of the stores; this had a positive value in normals but was virtually nil in iron deficient subjects who presumably had no labile iron pool.

Chelates may thus be used to determine marked excess or depletion of storage iron, but further experience with them is needed before it can be established whether they can accurately indicate finer variations than these.

In summary, then, the investigations of most value in the clinical assessment of iron stores are the direct methods of liver and marrow biopsy. The combination of plasma iron and iron-binding capacity estimations may be informative when applied with circumspection; and the measurement of iron excretion following the administration of chelating agents is of value although its exact position

remains to be defined. None of these methods yields a quantitative estimation.

CHAPTER III

CHEMICAL METHODS

The Determination of Non-haem Iron in Fresh Tissue

Tissue non-haem iron is represented almost entirely by storage iron and its measurement provides, for practical purposes, an accurate quantitative assessment of the stores. Four types of procedure have been employed in the quantitative determination of this iron. Firstly, attempts have been made to exsanguinate organs completely by means of perfusion and their residual iron content, representing non-haem iron, is determined after ashing. Secondly, various chemical methods have been used to extract haem from tissues and the residual non-haem iron has then been measured directly or by difference between total iron and haem-iron contents. Thirdly, the non-haem iron itself may be quantitatively extracted and measured directly. Fourthly, a method has been described for the quantitative fractionation of tissue ferritin, haemosiderin and haem.

Details of procedures vary considerably within the first three of these groups and it was from this array of possible methods that one had to be selected for application to the present study. They were therefore carefully reviewed and

validation experiments were done with a view to choosing the most satisfactory technique.

1. The quantitative fractionation of tissue iron compounds
(Gabrio et al., 1953)

Ferritin and haem are separated from haemosiderin by virtue of their water-solubility and the haem fraction is then precipitated by heating to 75° C. The quantitative overall accuracy is to within 5 per cent, but the fractionation of ferritin and haemosiderin is not germane to the present study and the method is too cumbersome and time consuming.

2. Exsanguination methods (Bognaird and Whipple, 1932; Wakeham and Halenz, 1936; Austoni, Rabinovitch and Greenberg, 1940).

Carefully conducted viviperfusion of experimental animals (Copp and Greenberg, 1946; Hallgren, 1954) has made it possible to eliminate virtually all haemoglobin from the body.

Postmortem perfusion of organs, however, never

renders them wholly blood-free (Hahn and Whipple, 1936; Hallgren, 1954), so that there is always some degree of contamination with haem iron. In the case of muscle there are large amounts of residual haem in the form of myoglobin. The method is particularly unsuited to bone marrow, in which it is impossible to even approach complete exsanguination; in addition there is the possibility of significant quantities of non-haem iron being washed out.

3. Haem extraction

a) Kooyman (1949) incubated tissue with hydrochloric acid and extracted the acid haematin thus formed with a mixture of ether and alcohol; non-haem iron was determined in the residuum. The spectroscopic validity tests of the method appear to be inadequate and some of the figures arrived at for non-haem iron concentrations are so extraordinarily high (260 µg. per g. of psoas muscle; 90 µg. per g. of heart muscle) that they are almost certainly due to technical errors. Furthermore, during my own preliminary attempts to apply this technique to the quantitative separation of a

mixture of solutions of ferritin and haemoglobin, it soon became obvious that the extraction solvent was removing appreciable amounts of ferritin as well as haem.

Similar methods based on acid haematin extraction or precipitation are described by Scott (1945) and by Kaldor (1954 and 1958).

b) Labbe and Nishida (1957) used an extraction solvent containing strontium chloride, glacial acetic acid and acetone to prepare pure haem from "blood or a similar haem containing preparation". In order to establish whether haem and non-haem iron could be quantitatively differentially extracted from bone marrow in this way, the method was tested in the following experiment:

Method: A stock solution of 2 per cent strontium chloride hexahydrate in glacial acetic acid was prepared by mixing freshly one part of the stock solution and three parts of acetone. Twelve ml. extraction solvent was added to each of (i) 1 g. fresh marrow plus 0.5 ml. Fe^{59} labelled ferritin

solution containing approximately 1.2 mg. Fe per ml. ^{*},
(ii) 1 g. fresh marrow plus 0.5 ml. Fe⁵⁹ labelled haemo-
globin solution containing approximately 0.5 mg. Fe per
ml. ^{*}, in separate centrifuge tubes. The mixtures were
then heated to boiling point and allowed to stand at room
temperature for 30 minutes, with occasional stirring. After
centrifugation at 4,000 - 5,000 r.p.m. for 20 minutes the
supernatant fluid was decanted and the extraction procedure
repeated once on the residue. The radioactivity in the
combined supernatant fluid was determined by counting an
aliquot and the total amount of radioactivity in the residue
was also counted.

Results: These appear in Table 1. In seven separate
experiments a mean of 95.7 per cent (range 94 - 98 per cent)
of the total ferritin remained in the residue, which contained
a mean of only 4 per cent (range 2 - 7 per cent) of the total
haemoglobin.

* See Appendix I and II for the methods of preparation
of radioactive ferritin and radioactive haemoglobin.

T A B L E I

The Quantitative Fractionation of Haem and Ferritin by the Strontium
Chloride Method

Substrate	Per cent total counts in deposit	Per cent total counts in super- natant fluid
Marrow + radioactive ferritin	98 97 95 94 96 94 96 Mean <u>95.7</u>	2 3 5 6 4 6 4 <u>4.3</u>
Marrow + radioactive haemoglobin	7 7 2 3 4 2 3 Mean <u>4</u>	93 93 98 97 96 98 97 <u>96</u>

Discussion: The requirements of this method are that the non-haem iron should not be liberated and that all the haem iron should indeed be extracted. These requirements have been satisfied to a reasonable degree of accuracy. The quantitative differentiation between the two forms of iron can be expected to hold for marrows likely to be encountered clinically, since the amount of ferritin used corresponds with that found in a moderately heavily overloaded marrow, and the amount of haem is well in excess of that present in 1 g. of marrow. On these validation data, however, it would have to be assumed - not unreasonably - that haemosiderin behaves identically to ferritin under the conditions of this method.

There were, however, technical disadvantages in its application to the study. Counting of the radioactivity of the residue by available equipment necessitated its transference to a narrower tube, with inevitable loss; and further transference to a Kjeldahl flask for digestion prior to iron estimation was necessary. It was apparent therefore that another method should be sought.

4. Extraction of non-haem iron

(a) Hydrochloric acid extraction. The earliest procedure for direct extraction of non-haem iron from tissues appears to be that of Starkenstein and Weden (1928). They heated tissue fragments in 5N hydrochloric acid and found that haem iron was not extracted in appreciable quantity. Other authors have used similar methods (McFarlane, 1932; Sherman et al., 1934). Buffered extraction solutions and more dilute hydrochloric acid have been found to be unsatisfactory as they do not remove all the non-haem iron (Sherman et al., 1934).

(b) Reducing agents have long been used to release iron from organic compounds. Ramsay and Campbell (1954) found that dipyridyl reagent in the presence of the reducing agent sodium sulphite would selectively measure tissue non-haem iron if both the tissue suspension and the reagents were first saturated with carbon monoxide in order to convert haemoglobin to the stable carboxyhaemoglobin. This method was used by Kerr (1957) in estimating marrow storage iron. Although it appears to be accurate, it is time-consuming and

rather too cumbersome for application to the present study, which ideally requires that haem and non-haem iron should be separated in order that each fraction may be studied.

(c) In 1935 Tompsett reported a method in which he extracted "inorganic" or non-haem iron by means of sodium pyrophosphate solution. The exact mechanism of extraction is not clear, but it is apparent that a stable iron-pyrophosphate anion is formed which is soluble in acids - in contradistinction to haem, which may then be precipitated by trichloroacetic acid. The method was modified twice by Brückmann and Zondek (1939, 1940) who claimed that Tompsett's time was too short for complete extraction. Although it had not been subjected directly to validity tests, the modified method gave results for non-haem iron concentrations which were in close agreement with other established procedures and thus seemed likely to be reliable.

In the present study, the following modification of this method was tested.

Pyrophosphate Method for the Quantitative Determination of
Tissue Non-Haem Iron

To a known volume (approximately 1 ml.) of tissue is added sufficient saturated solution of sodium pyrophosphate to make the volume up to 5 ml. Five ml. 20 per cent trichloroacetic acid are added and the mixture is heated for 10 minutes in a water bath at 80° C., with intermittent stirring. After filtration an aliquot of the filtrate is used for the non-haem iron determination by the thioglycollic acid method of McCance et al. (1936).

The validity of the method was tested as follows:

Ferritin solutions of varying concentrations and a haemosiderin suspension were prepared according to the respective methods of Mazur and Shorr (1948) and Shoden and Sturgeon (1960). The iron concentrations in each preparation and in a blood specimen were determined after digestion. Recovery experiments were then conducted, applying the method to aliquots of:

- i) 0.5 ml. ferritin solution containing between 1,250 and 2,000 µg. iron per ml.

- ii) 0.5 ml. blood containing 476 $\mu\text{g.}$ iron per ml.
- iii) 0.5 ml. each of ferritin solution and blood.
- iv) 0.5 ml. each of radioactive ferritin solutions and non-radioactive blood, with 0.5 g. fresh marrow.
- v) 0.5 ml. each of radioactive blood and non-radioactive ferritin solutions with 0.5 g. fresh marrow.
- vi. measured volumes from 0.4 to 14 ml. of haemosiderin suspension of iron concentration 660 $\mu\text{g.}$ per ml.

Results: These are detailed in Tables II and III.

Only 5.1 per cent (range 3 - 8 per cent in 14 observations) of haem iron appeared in the filtrate. whereas the mean recovery of ferritin iron from solutions containing up to 1,000 $\mu\text{g.}$ was 103.6 per cent (range 100 - 106 per cent in 22 observations) (Table II). The mean recovery of haemosiderin iron was 103.7 per cent (range 97 - 111 per cent in 14 observations) over a range of iron contents between 70 and 9,900 $\mu\text{g.}$ (Table III).

Discussion: A colorimetric estimation of the amount of haem in 1 g. marrow showed that it was roughly equivalent to the haem contained in 0.3 ml. of the blood used, which corresponds to

T A B L E II

Haem and Ferritin Recoveries by the Pyrophosphate Method

Method	Per cent recovery of ferritin iron in filtrate	Per cent recovery of haem iron in filtrate
Chemical Method (ferritin or blood alone)	103	8
	100	8
	103	3
	103	4
	103	4
	103	4
	103	8
	103	4
Chemical Method (ferritin and blood mixed)	104	
	104	
	104	
	105	
	106	
	106	
	103	
	105	
Isotopic Method (fresh tissue added)	104	6
	103	6
	105	4
	104	4
	101	4
	104	4
MEAN	103.6	5.1

T A B L E III

Haemosiderin Recovery by the Pyrophosphate Method

Volume of haemosiderin suspension (ml.)	Iron Content (μ g.)	Per cent recovery of iron in filtrate
0.1) 0.1)	70	111
0.25) 0.25)	165	107
0.5) 0.5)	330	105
0.75) 0.75)	480	101
1.0) 1.0)	660	105
2.0) 2.0)	1300	103
10.0	6600	97
15.0	9900	101
		Mean 103.7

196 $\mu\text{g.}$ of iron. On the data of the present validation experiment, 5.1 per cent of the haem iron (i.e. 10 $\mu\text{g.}$ per g. marrow) contaminates the ferritin iron extracted from marrow. This amount is small and in fact proved to be negligible in relation to the quantities of storage iron later found to be present in marrow, with the exception of those marrows whose stores were virtually exhausted. The explanation for the ferritin and haemosiderin recovery rates being almost consistently higher than 100 per cent is probably that during heating some degree of evaporation takes place from the test tube; for this reason the temperature was not allowed to exceed 80°C. However, here again the experimental error is small, and is less than the error from other sources encountered later in the study. The amounts of ferritin and haemosiderin completely recovered are well in excess of those normally expected to occur in 1 g. of marrow, and the marrow of highest iron content encountered in the subsequent study in fact contained less iron than the 9,900 $\mu\text{g.}$ which was successfully recovered by this method. It was not considered necessary to use ferritin solutions containing more than 1,000 $\mu\text{g.}$ of iron because in marrows of very high iron

concentration the major storage compound is haemosiderin.

It may be concluded, therefore, that the pyrophosphate method quantitatively extracts non-haem or storage iron in the presence of haem and marrow tissue. The method is quick, neat and accurate and does not possess the technical disadvantages of the strontium chloride haem-extraction procedure. For these reasons it was adopted as the method of choice for the present study.

The Determination of non-haem Iron in Formalinized Tissues

The foregoing discussion has dealt with the quantitative extraction of non-haem iron from fresh tissue, specifically bone marrow. To enable the convenience of tissue storage, a method for the same determination on formalinized tissue is necessary. The procedure evolved for the present study for the estimation of storage iron in formalinized liver (Gale et al., 1963) is as follows:

An aliquot of tissue is blotted dry and weighed. Its total iron concentration is determined by the thioglycollic acid

method after digestion with a 2:1 mixture of concentrated sulphuric acid and nitric acid.

Approximately 1 g. of the same specimen is blotted dry, weighed, cut into slices and placed in a 50 ml. volumetric flask, 0.5 ml. of the detergent sodium alkyl sulphate is added (in order to give a clear final solution) and the flask made up to volume with N sodium hydroxide. The flask is then placed in an oven at 56°C . for 18 hours. After cooling the clear solution is then filtered and the optical density of the filtrate is determined using an Evelyn photoelectric colorimeter with a 540 filter. The result in mg. haem iron is obtained from a standard curve prepared by using haemoglobin solutions of known iron contents.

The non-haem iron concentration is obtained by subtracting the value for haem iron from that for total iron.

The validity of this technique is supported by the fact that concentrations of haem iron were in the same range as those obtained by other methods on fresh liver specimens (Gillman et al., 1945; Roth et al., 1951). However, because of the chemical

similarity between haem and bilirubin, it was felt that the possibility of the estimated haem concentration being influenced by the amount of bile present in the liver should be excluded. The method was therefore applied to 10 heavily jaundiced livers of rats in whom obstructive jaundice has been surgically induced. The estimated haem-iron concentration was actually found to be lower in these specimens than in 6 normal controls, the respective mean values in the two groups being 0.017 and 0.028 $\mu\text{g. per g.}$ It may thus be concluded that the method is an accurate one for haem iron determination in formalinized liver; its importance is critical in the present study only in those livers of low iron content.

The Determination of Plasma Iron and Iron Binding Capacity

The respective methods used for these estimations were those of Bothwell and Mallett (1955) and Bothwell et al. (1959). The radioactivity of the serum or plasma on which the iron binding capacity estimations were done was not sufficient to significantly alter the values obtained.

CHAPTER IV

CLINICAL AND NECROPSY MATERIAL: METHODS AND PRINCIPLES INVOLVED

Clinical Material

The total iron stores present in bone marrow were estimated in 61 patients undergoing thoracotomy. Forty-one were white and the remainder were Bantu; the age range was 14 to 74 years, only 3 subjects being under 20 years. The individual diagnoses are shown in Table IV. The cases of pulmonary sepsis comprised non-tuberculous bronchiectasis and empyema.

T A B L E IV

Clinical Diagnosis in 61 Patients Subjected to Thoracotomy

Diagnosis	Number of patients			
	White		Bantu	
	Male	Female	Male	Female
Mitral stenosis	7	9	0	0
Intrathoracic neoplasm	8	0	1	0
Pulmonary tuberculosis	3	0	9	3
Pulmonary sepsis	0	4	5	2
Hiatus hernia	6	4	0	0
Total	24	17	15	5

Procedure: Isotopic Techniques

In order that tracer studies of iron metabolism should accurately reflect existing ferrokinetics when the radioactive iron is introduced intravenously, it is essential that it should all be bound to transferrin upon its introduction into the circulation. This may be achieved by its prior incubation with plasma (Huff et al., 1950; Bush et al., 1956) or Cohn's fraction IV (Elmlinger et al., 1953), but it has been demonstrated that complete binding occurs immediately after injection if the iron is given in a 0.1 per cent solution of sodium citrate (Loeffler et al., 1955; Bothwell et al., 1957). A further requirement is that the quantity of iron added to the plasma should be small enough to not significantly affect the amount or concentration of iron already present in any body compartment. This is achieved through the use of a minute quantity of radioactive iron of high specific activity.

Each subject was given between 5 and 10 $\mu\text{c.}$ high specific activity ($20 \mu\text{c. per } \mu\text{g.}$) Fe^{59} citrate intravenously between 18 and 24 hours pre-operatively. The dose of radio-

activity administered was determined by relating the weight of the injected solution to the counts present in a weighed aliquot of the same solution, which was set aside as a standard. A well-type scintillation counter was used. Blood samples were collected at timed intervals during the hour following the injection (usually 10, 20, 30, 45 and 60 minutes) and these were counted to determine the rate of clearance of radioiron from the plasma. The plasma iron and iron-binding capacity were measured and the venous haematocrit was determined. These data enabled the blood volume and plasma iron turnover to be calculated (page 68 et seq.)

The rib removed at operation was squeezed dry and an aliquot of about 1 ml. of the expressed marrow was collected in the bottom of an iron-free test tube. It was then weighed and counted for radioactivity and its non-haem iron content was determined by the pyrophosphate method described on page 56. The total non-haem radioactivity was determined by counting an aliquot of the filtrate.

A blood sample was collected 10 to 14 days after the injection of radioiron and this was counted and corrected for physical decay after recounting of the standard solution. The total percentage of injected radioactivity in circulation at this time (the red cell utilization) was then calculated as described on page 71. In the 8 cases in which it was either impossible to collect this blood sample (due to death or early discharge of the patient) or the counts would have been an inaccurate reflection of red cell utilization (as after considerable postoperative haemorrhage), an arbitrary utilization of 80 per cent of the injected dose was assumed. (The mean figure for the calculated group was 83 per cent).

Particular attention was paid in the history of each patient to the assessment of dietary iron intake and possible sources of iron loss such as gastro-intestinal haemorrhage, menorrhagia and repeated blood donation.

Radiation Exposure

The doses of Fe^{59} used in this study were within the maximum permissible dose of 13 μc . recommended for

control subjects by the International Commission on Radiological Protection (1955). Tissue irradiation to subjects receiving Fe^{59} has been calculated by Bothwell and Finch (1962 (d)). The marrow, which is assumed to be the critical area, is exposed to a total dose of 3.5 rads when 1 $\mu\text{c.}$ Fe^{59} per kilogram body weight is administered. The total blood dose from this amount of Fe^{59} is 4.2 rads and the total body irradiation is 1.5 roentgens per gram of body weight. On this basis, no subject of the present study received more than 0.5 rads to bone marrow, 0.6 rads to blood and 0.2 roentgen per gram of tissue total body irradiation.

Calculations

Body Haematocrit

The venous haematocrit was corrected by a factor of 0.96 to obtain the true body haematocrit at the Johannesburg altitude of 5,740 feet (Metz et al., 1962).

Blood Volume

This was calculated from radioiron dilution in the

circulation (Bothwell, et al., 1956). The initial exponential disappearance of bound radioiron from the plasma enables the amount of radioactivity present in a plasma sample at zero time to be derived by extrapolation. By relating this value to the total amount of radioactivity injected, the blood volume may be calculated as follows:

$$\text{Blood volume = (ml.)} = \frac{\text{counts injected} \times 100}{(100 - \text{body haematocrit}) \times \text{counts per ml. plasma at zero time}}$$

Blood volumes estimated by this method are accurate providing there is no initial loss from the circulation of injected radioiron due to an appreciable amount of it being unbound to transferrin. Such an error would give a falsely high blood volume. The value obtained for blood volume was checked in each case by comparison with the figure calculated on the basis of body weight. The agreement on the whole was good and in only 3 out of the 61 cases did the value obtained by the dilution technique exceed 80 ml. per kg. body weight. The mean figure

was 68.2 ml. per kg. (S.D. 10.7). This is in keeping with complete binding having occurred which has indeed previously been shown to be ensured by the use of Fe^{59} citrate (Loeffler et al., 1955; Bothwell et al., 1957).

Plasma Iron Turnover

In order to permit valid comparison of iron turnover rates between individuals of varying weights and red cell masses, a simplified standard of reference has been evolved by Bothwell et al. (1957). This is based on the formula of Huff et al. (1950) but relates the turnover to 100 ml. whole blood. This approach was used in the present study, the calculation being as follows:

$$\begin{array}{lcl} \text{Plasma iron turnover} & \text{Plasma iron} & (100 - \text{haematocrit}) \\ (\text{mg./day/100 ml.} & = (\mu\text{g./100 ml.}) & \\ \text{whole blood}) & \frac{\quad}{T_{\frac{1}{2}}} & \times \frac{\quad}{100} \\ & (\text{minutes}) & \end{array}$$

where $T_{\frac{1}{2}}$ = time for clearance of 50% of radioactivity from plasma.

Red Cell Radioiron Utilization

This is a measure of the amount of radioiron present in the total circulating red cells at a time when it is maximal. This occurs from about the 8th day after radioiron injection onwards, until the labelled population of red cells is destroyed or lost. The red cell utilization may be expressed as a percentage of the injected radioiron, and was calculated from the formula

$$\text{Red cell utilization} = \frac{\text{B.V.} \times 4 \text{ ml. corrected counts}}{\text{C.l.}} \times 25$$

(%)

where

B.V. = blood volume in ml.

4 ml. corrected counts = number of counts in 4 ml. whole

blood withdrawn on the 10th to 14th day after the

Fe⁵⁹ injection, corrected a) for physical decay

by relation to the decay of the standard, and

b) by a factor of 0.96 (the body/venous haematocrit ratio).

C.l. = number of counts injected.

It is postulated that the value obtained for red cell utilization reflects, to the degree of accuracy demanded by the present study, the amount of radioactivity present in the total haematologically active bone marrow from 18 to 24 hours after the injection. This assumption is the basis for Suit's quantitative estimation of marrow cellularity (1957), and it is relevant to examine in some detail the evidence for its truth.

It is established that bound radioiron is rapidly removed from the plasma largely to bone marrow (Huff et al., 1950), and that after 24 hours 1 per cent or less remains in the plasma. The subsequent fall of activity in marrow, which takes place normally over 8 to 10 days, by which time counts have dropped to virtually nil, occurs at the same time as, and reciprocally to, the appearance of activity in circulating red cells. The latter activity reaches a maximum about the 8th or 10th day (Pollycove and Mortimer, 1961; Huff et al., 1951). The inference is clear that all the radioactivity present initially in the bone marrow has appeared in the circulating red cells. This amounts normally to between 70 and 100 per

cent of the injected dose. Of the remainder, a small portion is excreted, some is found in the extracellular fluid and the largest fraction (the amount depending on the size of the stores) is found in the storage iron - mainly of the liver, but also of the reticulo-endothelial tissues elsewhere (including bone marrow) (Pollycove and Mortimer, 1961). Presuming that this marrow fraction is not quickly utilized for red cell production, a source of error is thereby introduced into the calculation; however, this inaccuracy is offset by the error incurred through approximately half of the activity at first taken up by the liver and spleen being discharged from these sites for red cell utilization by the 10th day (Huff et al., 1951). The error from these sources must therefore be small. A further inaccuracy might arise through the radioiron in the marrow at 18 - 24 hours not being fixed in erythropoietic tissue but a fraction of approximately one-third being in the "erythropoietic labile pool" previously mentioned. This iron, however, would rapidly re-enter plasma and would be continuously recycled through the marrow so that a progressively smaller proportion of it would escape utilization for erythropoiesis. Thus most would, by the 10th day, be

present in the circulating red cells, and the error from this possible source is also small.

Donohue et al. (1958), on the basis of direct carcass analysis of skeletal radioiron in animals, have estimated that at the time when blood radioactivity is minimal, 66 per cent of the injected radioiron is present in the bone marrow in man. Figures obtained in the present study for red cell utilization were usually higher than this, but as may be appreciated from the foregoing discussion, and because the present method of calculation allows for individual variations, it would seem to provide a more rational and more accurate assessment of the original marrow uptake. In any event, the magnitude of the inaccuracies incurred in this assessment is not considered to be critical.

Total Marrow Storage Iron

Because the red cell utilization is a measure of the total amount of radioactivity in all the erythropoietic marrow at the time of rib resection, the total (erythropoietic) marrow storage iron content is given by the formula:

$$\begin{array}{rcl}
 \text{Total marrow} & \text{Non-haem iron in} & \text{Red cell} \\
 \text{storage iron} & \text{marrow aliquot} & \text{utilization*} \\
 \text{(mg.)} & \text{(}\mu\text{g.)} & \text{X} \\
 = & \frac{\text{Counts in marrow aliquot}}{\text{Counts in marrow aliquot}} & \text{X } 10^{-3}
 \end{array}$$

*Expressed as counts corrected for physical decay

This calculation is based on the assumption that iron is evenly distributed throughout the active marrow of the body. This would appear to be a reasonable assumption in that experience has shown that histological examination of even minute marrow particles from different sites demonstrates fairly even haemosiderin distribution. In a preliminary attempt to validate the assumption, non-haem iron estimations were made on marrow aliquots of about 1 g., obtained from each of two different sites in 16 necropsy subjects; for technical reasons the sites used were ribs. The results of this investigation are shown in Table V. Over a wide range (15 - 4,476 $\mu\text{g. per g.}$), the average difference from the mean of the iron concentrations in each pair of observations was 5.9 per cent. The range was 0 - 23 per cent, although in only two cases was the difference greater than 8 per cent. These figures confirm the

T A B L E V

Storage Iron Distribution in Two Marrow Sites

Case	Non-haem iron concentration (µg. per g. marrow)		Per cent difference in iron concentrations in sites 1 & 2 from mean
	Site 1	Site 2	
1.	460	288	23
2.	102	92	5
3.	416	450	4
4.	309	342	5
5.	307	266	7
6.	32	31	1.5
7.	419	383	4
8.	194	198	1
9.	14	15	3
10.	18	18	0
11.	298	320	4
12.	50	44	6
13.	1527	980	22
14.	4520	4432	1
15.	1882	1880	0
16.	448	529	8
Mean			5.9

even distribution of iron and suggest that a 1 g. aliquot is adequate to permit meaningful conclusions to be drawn with regard to the total body marrow. The even distribution of radioactivity in the marrow is assumed, as it has been by others (Suit, 1957; Donohue et al., 1958).

Weight of Total Body Haematologically Active Marrow

This was calculated by dividing the red cell utilization (expressed as counts corrected for physical decay) by the number of counts per g. of marrow aliquot.

Iron Incorporation into Haem

The amount of haem radioiron present in the marrow aliquot was arrived at by subtracting the counts present in the pyrophosphate extraction filtrate from those in the whole marrow aliquot. This figure was expressed as a percentage of the total radioactivity in the marrow sample, and it provided an assessment of the distribution of radioiron in the marrow 18 to 24 hours after its first appearance there.

Necropsy Study: Material and Methods

Tissue was obtained from random necropsies performed at Baragwanath Hospital, Johannesburg, on 58 male and 45 female Bantu subjects. Their mean age was 45 years (range 15 - 89 years). Material from 25 male and 28 female white subjects was obtained from random necropsies at the Johannesburg General Hospital. The mean age in this group was 65 years (range 41 - 90 years). In both groups there was a wide unselected variety of causes of death, the subjects having been derived from medical, surgical and gynaecological wards.

In each case a liver sample was placed in a 10 per cent formol phosphate buffer solution for at least 48 hours to ensure thorough formalinization, and then subjected to the method previously described (page 61) for the determination of non-haem iron content. A marrow sample of approximately 1 g. was obtained in each case by expression from one or two ribs. The non-haem iron content per gramme of marrow was determined by the pyrophosphate method described on page 56.

Histological Methods

Marrow particles were obtained in 206 cases from operative and necropsy material by suspending aliquots of marrow samples in saline. Squash preparations of these particles were made on slides, and these were fixed and stained for iron by Dry's method (1945). The fixed criteria for histological grading of their visible storage iron content were as follows:

- Grade 0: No visible iron under oil immersion (magnification X 720).
- Grade 1+: Small iron particles just visible in reticulum cells under oil immersion (magnification X 720).
- Grade 2+: Small sparsely distributed iron particles visible under low power (magnification X 80).
- Grade 3+: Numerous small particles present in reticulum cells throughout the marrow particles.
- Grade 4+: Larger particles throughout the marrow with a tendency to aggregate into clumps.

Grade 5+: Dense, large clumps of iron throughout the marrow.

Grade 6+: Very large deposits of iron obscuring cellular detail of the marrow.

In order to obviate bias, grading was done in each case before the chemical concentration of iron was known.

Examples of the 7 grades are illustrated by photomicrographs on pages 119 to 125.

CHAPTER V

RESULTS and DISCUSSION

1. The Isotopic Study: Quantitation of Marrow Stores

A comprehensive table of results relating to 61 subjects of this study appears in fig. (1). Since each patient was subjected to thoracotomy, all diagnoses were confirmed at operation. The necessary selection of cases results in a bias toward conditions which may influence iron metabolism, so that the subjects of the study cannot be regarded as representative of the general population. Random sampling would indeed have been contrary to the scope of the study, which is directed towards subjects with abnormal as well as those with normal iron metabolism. It is considered, however, that although individual values must certainly have been modified, in white subjects the case selection has in fact exerted little or no significant effect on overall results for iron stores. Justification of this view is detailed on page 91. It should be noted that the haemoglobin values do not in every case represent the patient's own level, since some cases received pre-operative blood transfusions. The marrow samples were not weighed in six early

Case No.	Race	Sex	Age	Wt. (kg.)	Hb (g.%)	PCV (%)	Plasma Fe (µg.%)	Plasma UIBC (µg.%)	% Saturation of I.B.C.	Blood volume (ml. per kg. body weight)	"T _{1/2} " (mins)	Plasma Fe turnover (mg/24 hrs/100 ml. blood)	Rbc utilization %	Marrow weight (g.)	Marrow storage Fe concentration (µg. per g.)	Marrow Fe stores (mg.)	Marrow histology	24 hour 59 Fe incorporation into haem (% injected dose)	Diagnosis
1	W	M	59	89	16	47	105	290	27	71	87	0.64	89			515		72	M.S.
2	W	M	28	66	18	51	89	475	16	57	56	0.79	68			188		72	M.S.
3	W	F	26	48	13	38	80	221	27	76	72	0.69	83			181	+		Sep.
4	W	M	49	85	17	50	48	264	15	67	49	0.48	89			287	3+	86	H.H.
5	W	F	34	50	15	50	107	267	29	74	77	0.69	85			38	0	81	M.S.
6	W	M	65	61	13	41	37	298	11	78	35	0.62	86	1335	125	167	2+	75	Neo.
7	W	M	56	84	13	37	63	210	23	77	50	0.79	99	1694	98	166	+	75	Neo.
8	W	M	51	76	18	52	118	215	35				94			244	3+	95	H.H.
9	W	M	57	62	14	39	102	209	33	74	56	1.11	85	1545	147	227	2+	83	M.S.
10	W	M	43	77	16	47	86	234	27	68	67	0.68		1463	281	411	3+		T.B.
11	W	M	20	55	16	48	92	292	24	65	37	1.29	82	1056	10	10	0	79	M.S.
12	W	F	15	43	15	49	101	369	21	85	49	1.06	79	863	25	21	0	80	M.S.
13	W	M	44	66	16	49	67	242	22	52	88	0.39	87	1091	362	395	3+	72	M.S.
14	W	M	74	64	13	42	78	303	20	83	65	0.69		1329	17	22	0		H.H.
15	W	F	22	57	13	40	57	340	14	58	62	0.55	71	626	183	115	2+		M.S.
16	W	M	45	65	16	50	70	311	18	74	68	0.51	72	1680	239	400	3+	91	T.B.
17	W	F	35	53	14	39	54	384	12	60	31	1.06	85	1266	19	24	0	89	M.S.
18	W	M	28	53	16	49	67	226	23	78	52	0.66	82	1097	135	148	+	75	M.S.
19	W	M	63	69	13	48	48	432	10	84	39	0.63	83	1373	13	17	0	82	H.H.
20	W	F	59	68	12	43	46	230	17	78	54	0.49	87	1136	158	179	2+	79	Sep.
21	W	F	50	70	15	45	75	298	20	44	47	0.88	79	719	110	79	2+	96	H.H.
22	W	M	57	81	11	42	55	298	16	61	30	1.06	81	1473	146	216	2+	84	Neo.
23	W	F	46	66	14	43	89	305	23	61	54	0.94		913	176	161	3+	82	H.H.
24	W	F	14	48	13	36	54	196	22	72	30	1.15		968	210	204	2+	76	Sep.
25	W	F	20	52	15	36	27	363	7	72	38	0.45	100	965	25	24	0	88	M.S.
26	W	F	58	77	14	40	41	297	12	56	56	0.44	100	1315	137	180	+	100	H.H.
27	W	F	33	80	13	47	29	400	7	63	37	0.42	100	962	52	50	0	89	H.H.
28	B	M	39	56	16	50	49	438	10	75	44	0.56	65	1338	3474	4820	6+	74	Sep.
29	W	F	16	51	15	42	74	298	20	59	31	1.40	83	1164	16	19	0	91	M.S.
30	W	F	56	78	14	43	77			70	30	1.49	84	1740	34	68	0	75	M.S.
31	W	F	42	43	14	45	172						70	952	85	81	0		M.S.
32	W	M	54	68	18	48	40	231	15	65	40	0.52	97	1340	341	457	3+	60	H.H.
33	B	M	43	47	16	45	120	105	57	69	63	1.05	63	820	2016	1654	5+	71	T.B.
34	W	M	35	74	18	50	135	106	56	67	78	0.87	75	1810	94	169	+	66	T.B.
35	B	M	27	66	16	46	46	169	21	71	44	0.56	99	1197	374	393	3+	78	T.B.
36	B	M	35	54	15	44	220	102	68	64	67	1.84	75	690	495	342	3+	72	T.B.
37	B	M	46	67	17	50	219	131	63	75	82	1.34	64	1046	1778	1443	6+	36	T.B.
38	B	F	43	60	11	35	60	360	14	73	63	0.62	100	908	486	400	3+		Sep.
39	B	M	50	57	12	39	36	330	10	72	24	0.98	96	1010	760	767	4+		Sep.
40	B	M	38	53	14	45	106	310	25	75	61	0.96	55	824	1563	1287	5+	78	T.B.
41	W	M	38	77	17	54	83	269	24	63	69	0.55	87	1456	205	298	2+	78	M.S.
42	B	M	42	62	14	36	137	105	57	59	71	1.23		1391	2593	2949	6+	67	T.B.
43	B	M	45	49	13	30	57	191	23	70	35	1.16		1680	2723	4579	6+		Neo.
44	W	M	59	66	15	46	78	230	25	85	34	1.24	72	1700	490	833	4+	85	Neo.
45	B	M	49	61	15	47	73	206	26	65	59	0.66	81	1081	547	591	4+	82	T.B.
46	W	F	44	66	15	47	63	316	17	56	47	0.72	99	1200	198	237	2+	80	M.S.
47	B	M	30	59	11	35	67	147	31	77	27	1.64	90	966	1723	1663	5+	92	Sep.
48	B	M	38	60	15	44	81	190	30	77	60	0.76	79	1071	2333	2499	6+	81	Sep.
49	W	M	66	67	17	46	51	383	12	54	56	0.49	100	1711	247	422	3+	91	H.H.
50	W	F	24	56	13	39	42	404	9	85	34	0.76		1156	24	28	0	92	Sep.
51	W	M	60	61			35	347	9		38		73	1469	260	382	3+	80	Neo.
52	B	F	43	59		43	71	272	21	61	70	0.58		835	530	355	4+	79	Sep.
53	W	M	55	77	16	45	75	284	21	62	58	0.71	92	1361	181	213	2+	84	Neo.
54	B	M	42	61	14	46	80	168	32	56	60	0.72	77	960	317	304	3+	82	T.B.
55	B	M	23	56	15	46	65	200	25	64	39	0.90	81	782	563	440	4+	84	T.B.
56	B	F	21	38	13	40	45	239	16	83	39	0.69	75	646	87	56	+	79	T.B.
57	W	M	69	82	15	41	60	210	22	56	52	0.68	87	1261	482	608	4+	89	Neo.
58	W	M	69	57	12	39	41	191	18	61	29	0.88	79	1221	98	120	+	82	Neo.
59	B	F	31	49	14	43	78	252	24	52	64	0.69	79	476	122	58	+	70	T.B.
60	B	F	40	53	16	52	86	186	32		46	0.90	77	956	817	781	4+	55	T.B.
61	B	M	21	59	14	40	70	135	34	56	37	1.12	80	712	995	708	4+	68	Sep.

P.C.V. = Packed cell volume
 U.I.B.C. = Unsaturated iron binding capacity
 I.B.C. = Iron binding capacity
 "T_{1/2}" = Time for clearance of 50% of radioactivity from plasma

M.S. = Mitral stenosis
 Sep. = Pulmonary sepsis
 H.H. = Hiatus hernia
 Neo. = Intrathoracic neoplasm
 T.B. = Pulmonary tuberculosis

Fig. 1: Haematological data and values obtained for marrow iron stores in subjects of the isotopic study.

cases before the study was fully under way.

Values for red cell utilization were not obtained in 8 cases, for various reasons. In cases 42 and 43 there was considerable delayed postoperative haemorrhage and the loss of labelled red cells would have rendered inaccurate the calculation of total red cell utilization of the injected radioiron. Cases 10, 23, and 50 were discharged from hospital and lost before completion of the 10-day period required for the maximal re-appearance of radioactivity in the blood. Cases 14 and 52 died during this period. In these instances, where the value for red cell utilization could not be determined, an arbitrary value of 80% was assumed for the purposes of calculations. This approximates to the mean value of 83% for the calculated group.

The only blood donors were cases 7, 22, 25, 30 and 51, the respective total amounts donated being 64, 30, 2, 5 and 22 pints.

The mean value for marrow iron stores in all subjects was 585.6 mg. There was tremendous individual variation between 10 and 4,820 mg. Analysis of the factors

affecting iron stores and breakdown of the subjects into corresponding groups make these results more meaningful.

The influence of race and sex

When the results are viewed according to race and sex, a striking correlation between iron stores and these factors is apparent (Fig. 2). The mean value in 17 white females was 99 mg. (range 19 to 237 mg.) and in 24 white males was 288 mg. (range 10 to 833 mg.). In Bantu subjects the figures were considerably higher. The 5 Bantu females studied showed a wide scatter, with values ranging between 56 and 781 mg. The mean value in 15 Bantu males was 1,629 mg. (range 304 to 4,820 mg.).

The well-marked sex difference is, as has been mentioned, probably a reflection of the cumulative losses of iron through years via menstruation and pregnancies in females. These results are in keeping with the generally accepted tendency for females to become iron deficient more readily than males, and with the smaller stores found by other workers

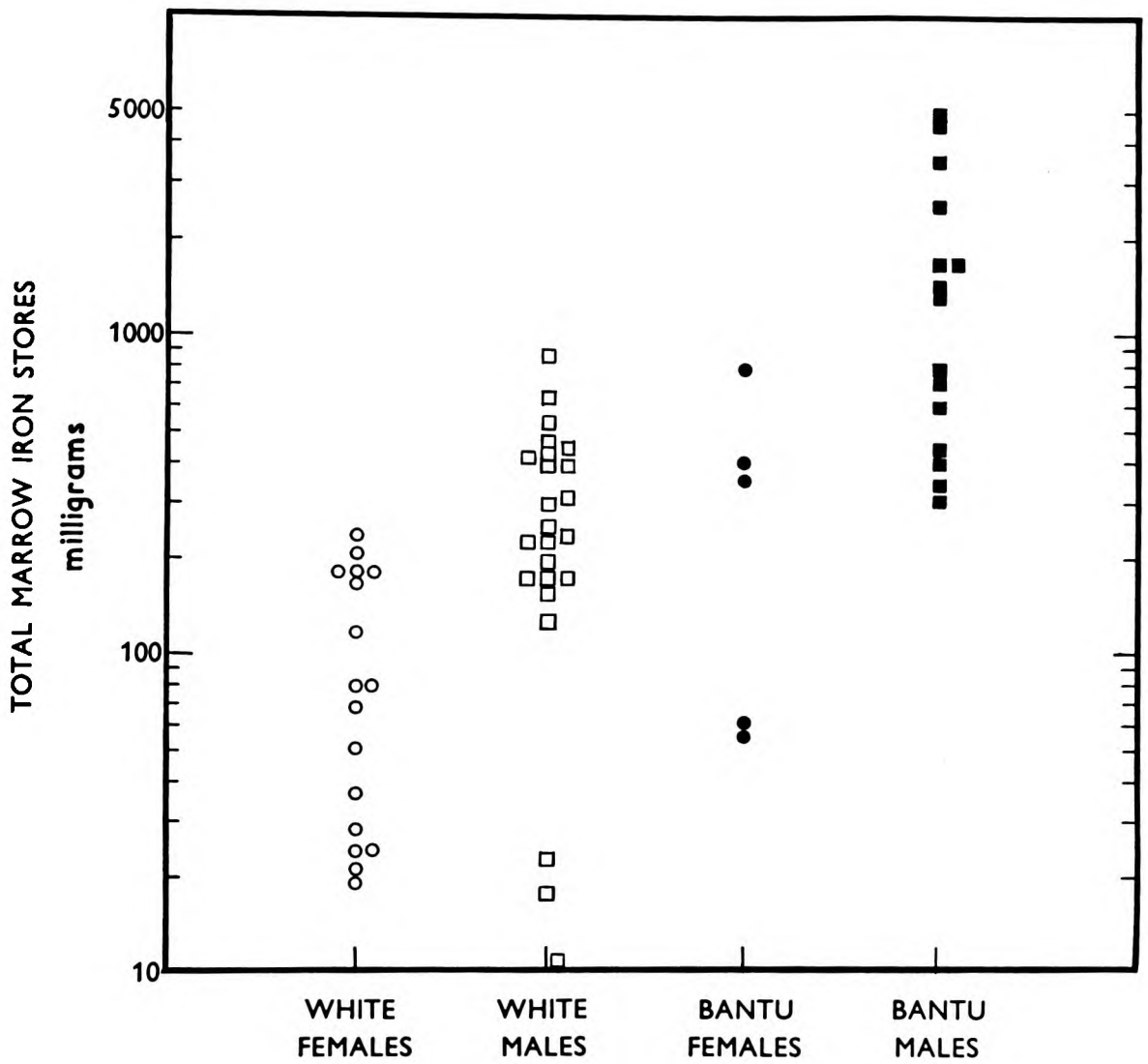


Fig. 2: The calculated values for total marrow iron stores in 61 human subjects.

in marrows and livers of females (Roth et al., 1951; Finch, 1959).

An attempt was made to correlate the iron stores in females with their degree of menstrual blood loss and number of full-term pregnancies. This analysis is shown in Table VI. The interpretation thereof raises a number of difficulties. Assessments of the amounts of menstrual loss based on patients' histories, however carefully taken, are notoriously unreliable, and it was felt that even after careful appraisal nothing would be gained by a more precise categorization of menses than "normal" or "heavy". Bantu subjects were excluded because of doubts about the accurate interpretation of their histories.

Furthermore, despite excessive iron loss, adequate stores may be established and maintained through enhanced intestinal absorption - providing that there is no dietary deficiency. Estimations of the dietary iron intake of these subjects was impossible, but in no case was there a history of obvious deficiency. Finally, the number of cases studied is small (17).

T A B L E VI

Relationship of Marrow Iron Stores to Menstrual Loss and
Number of Pregnancies in 17 White Subjects

Marrow Iron stores (mg.)	Menstrual loss	Number of full-term pregnancies	Age	Diagnosis
19	"normal"	0	16	M.S.
21	"normal"	0	15	M.S.
24	"normal"	0	35	M.S.
24	"heavy"	0	20	M.S. *
28	"heavy"	1	24	Sep.
38	"heavy"	0	34	M.S.
50	"heavy"	3	33	H.H.
68	"normal"	2	56	M.S. **
79	"heavy"	5	50	H.H.
81	"normal"	5	42	M.S.
115	"normal"	1	22	M.S.
161	"normal"	0	46	H.H.
179	"heavy"	3	59	Sep.
180	"normal"	?	58	H.H.
181	"normal"	1	26	Sep.
204	"normal"	0	14	Sep.
237	"heavy"	2	44	M.S.

M.S. = Mitral stenosis; Sep. = Pulmonary sepsis; H.H. = Hiatus hernia.

* Blood donor, total 2 pints

** Blood donor, total 5 pints.

In view of these considerations it is not surprising to find that this group shows no correlation between iron stores and degree of menstrual loss as judged on patients' histories. The anomalous impression that nulliparity tends to be associated with low iron stores carries little weight on account of the paucity of cases. However, since many years are required for the accumulation of stores, it is possible that this apparent trend simply reflects the younger ages of the subjects with less than 50 mg. of marrow storage iron - their mean age is 26 years and that of the remainder is 41 years.

More striking than the sex difference is the influence of race on marrow iron stores. The mean figure of 1,304 mg. in Bantu subjects was approximately 6 times the mean value in whites and the highest figure obtained was 23 times greater.

Since all the Bantu subjects were suffering from chronic infection or neoplasia, conditions often associated with the increased reticulo-endothelial iron deposits of relative haemosiderosis, it is relevant to consider to what extent results

may have been influenced by these conditions. Finch and Finch (1955) have stated that providing there is no blood replacement, the total excess in tissue iron of the relative haemosiderosis of various anaemias can be no greater than 1 or 2g. However, a severe secondary anaemia of say 8 g. haemoglobin per 100 ml. would imply that (at this altitude) approximately 350 g. of haemoglobin had left the blood and its 1,200 mg. of iron had entered reticulo-endothelial stores. Because of distribution to other organs, it is probable that less than half of this amount would be stored in the bone marrow. Thus, since iron absorption in these cases may be impaired or normal but not increased (Gubler et al., 1950; Saylor and Finch, 1953), the maximum estimate for iron shunted to marrow in sepsis and neoplasia is likely to be between 500 and 600 mg. In the cases studied the amounts were probably considerably less than this because anaemias were unlikely to have been as severe as 8 g. haemoglobin per 100 ml. The degree of anaemia actually present in each case could unfortunately not be determined with certainty because of an unknown number of cases had received pre-operative blood transfusions.

It is clear therefore that the relative haemosiderosis which may have been present in a number of subjects could not have raised marrow iron stores by more than twice normal, and probably had less effect than this. The very high values obtained in the Bantu therefore represent an absolute increase in the body iron content. This finding correlates with several previous studies which have demonstrated a high incidence of varying degrees of iron overload in the adult Bantu population of Southern Africa (Strachan, 1929; Gillman et al., 1945; Higginson et al., 1953; Wainwright, 1957; Bothwell and Bradlow, 1960; Bothwell and Isaacson, 1962). The excess iron in these subjects is largely derived from the containers used for the preparation of alcoholic beverages (Walker and Arvidsson, 1953; Bothwell et al., in press) which are widely consumed by the Bantu but not by whites. In most affected individuals the iron is stored almost exclusively in the liver and reticulo-endothelial system (Higginson et al., 1953; Bothwell and Bradlow, 1960).

The Relationship of Clinical Diagnosis to Iron Stores and Haemato-
logical Data

In view of the possible modifying effects upon iron metabolism of the diseases from which the subjects were suffering, the results obtained for marrow iron stores should be considered in relation to the clinical diagnosis. This relationship is summarised in Table VII.

The mean figures for mitral stenosis and hiatus hernia, in which groups there were no Bantu subjects, show no significant difference from the means for all white subjects. The cases of mitral stenosis may be regarded as having normal iron metabolism, since none had pulmonary siderosis or a history of severe haemoptyses and rheumatic activity had been excluded in all. Therefore the similarity of results in the 16 subjects with mitral stenosis (65 mg. for females and 253 mg. for males) to those in the whole group of white subjects (99 mg. for females and 288 mg. for males) indicates that, despite the necessary case selection involving patients suffering from a variety of illnesses, the overall results are probably a fair

T A B L E VII

Marrow Iron Stores According to Diagnosis

Diagnosis	Number of Cases			Mean marrow iron (mg.)		
	Male	Female	Total	Male	Female	Total
Mitral stenosis	7	9	16	253	65	150
Hiatus hernia	6	4	10	238	117	190
Pulmonary tuberculosis	12	3	15	882	298	746 328 *
Intrathoracic neoplasm	9	0	9	799	-	799 336 *
Pulmonary sepsis	5	6	11	1815	225	947 148 *

* These figures exclude Bantu cases.

reflection of the findings in the general population.

This impression is borne out by analysis of the results obtained in the other diagnostic groups. The normal figures for hiatus hernia are perhaps surprising since this condition is commonly associated with overt or occult blood loss. Even in the absence of a history of obvious haemorrhage, the subjects in this group might be expected to have some depletion of stores. (In fact, in only 3 out of the 10 cases of hiatus hernia could a history of overt blood loss be obtained). Despite the normal mean value, it is probably significant that 2 of the 3 males with very low storage values (22 and 17 mg. respectively had long histories of symptoms attributable to hiatus hernia.

The increased stores associated with chronic infection and neoplasia, from which many of the subjects were suffering, may be considerable, but, as has been indicated, on theoretical grounds would not be expected to represent more than 600 mg. of iron. The very high values obtained in these 2 groups are almost certainly a reflection of the fact that over

70% of these cases were Bantu. If the Bantu cases are excluded, the results do not differ significantly from those for the other groups of white subjects. The differences in the figures for whites with pulmonary tuberculosis (328 mg.) or intrathoracic neoplasm (336 mg.) and pulmonary sepsis (148 mg.) could be related to the sex distribution, for the former two groups comprised only males and the latter one only females. Thus on the present data there appears to be no more than a trend towards the expected augmentation of marrow iron stores in these conditions. The increase would doubtless have been greater if more of the subjects had been anaemic: their mean haemoglobin was 14 g. per cent (S.D. 2) and only 3 of the 15 had haemoglobin levels below 13 g. per cent.

Individual storage iron and haematological values are grouped according to diagnosis in Figs. (3) to (7). The generally low levels of plasma iron are due, certainly in part, to the time of collection of blood samples, which was almost without exception late afternoon. An afternoon fall in plasma iron concentration by about 30 per cent of its morning level has

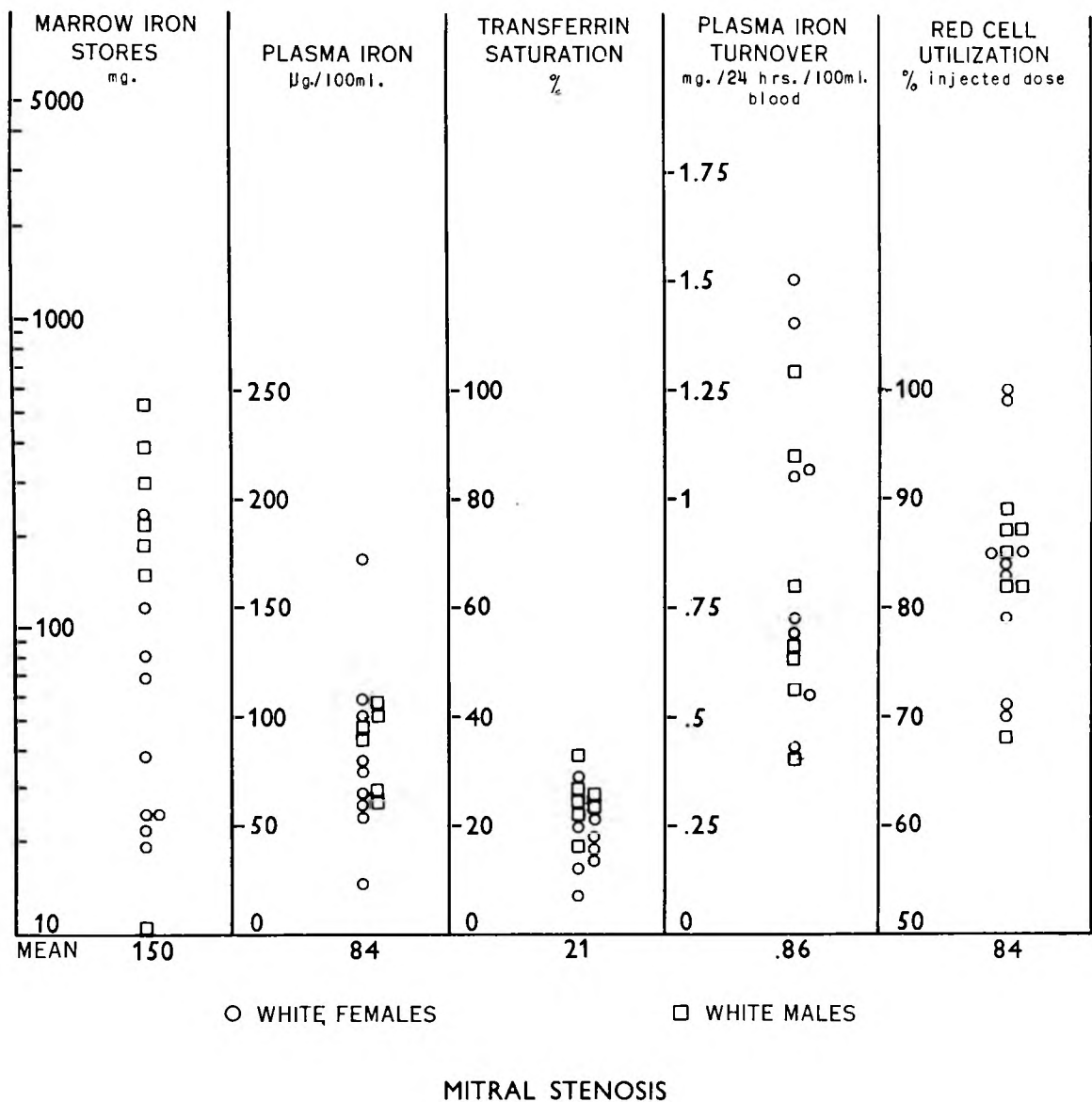


Fig. 3: Marrow iron stores and haematological values in 16 subjects with mitral stenosis.

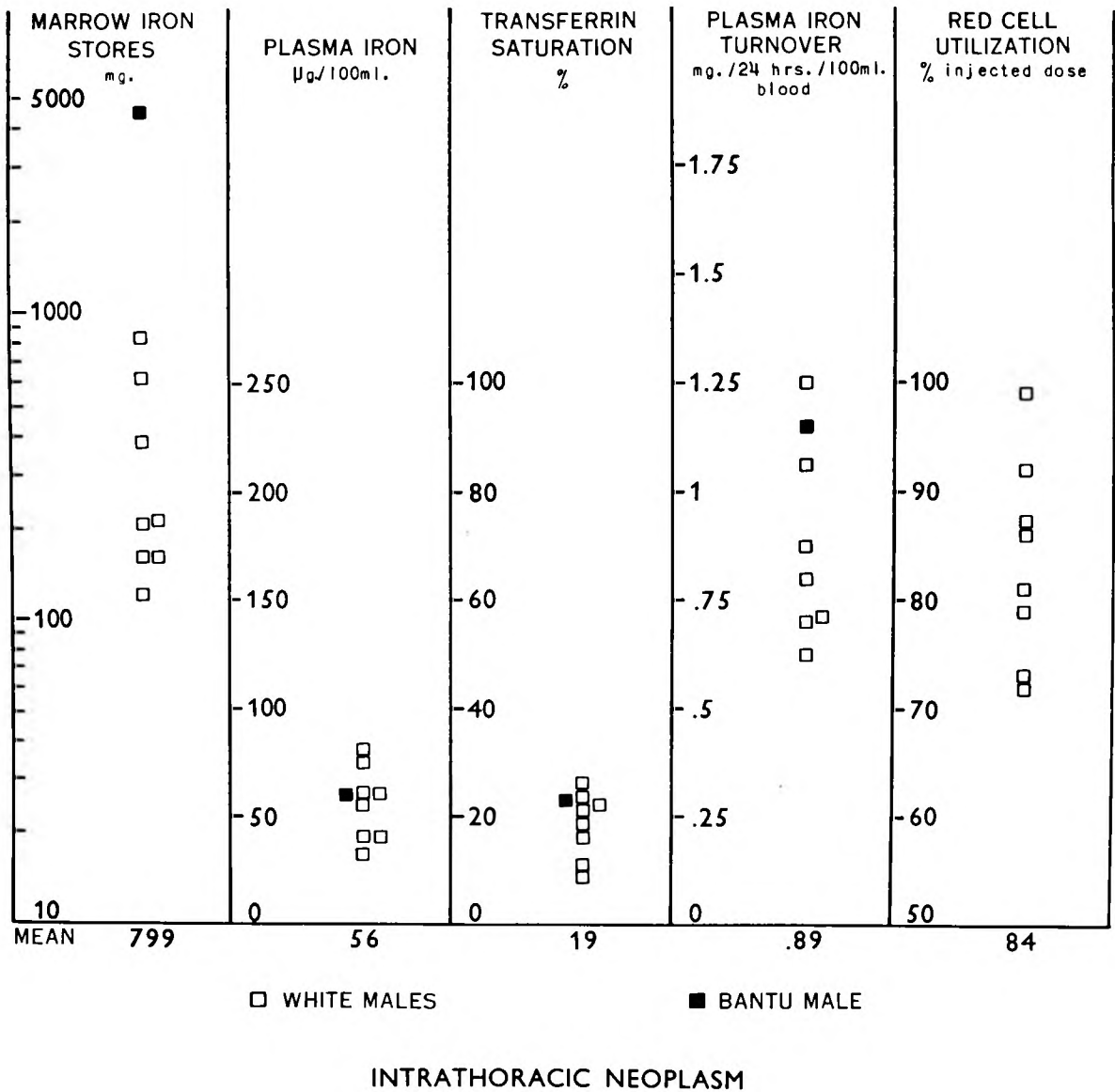
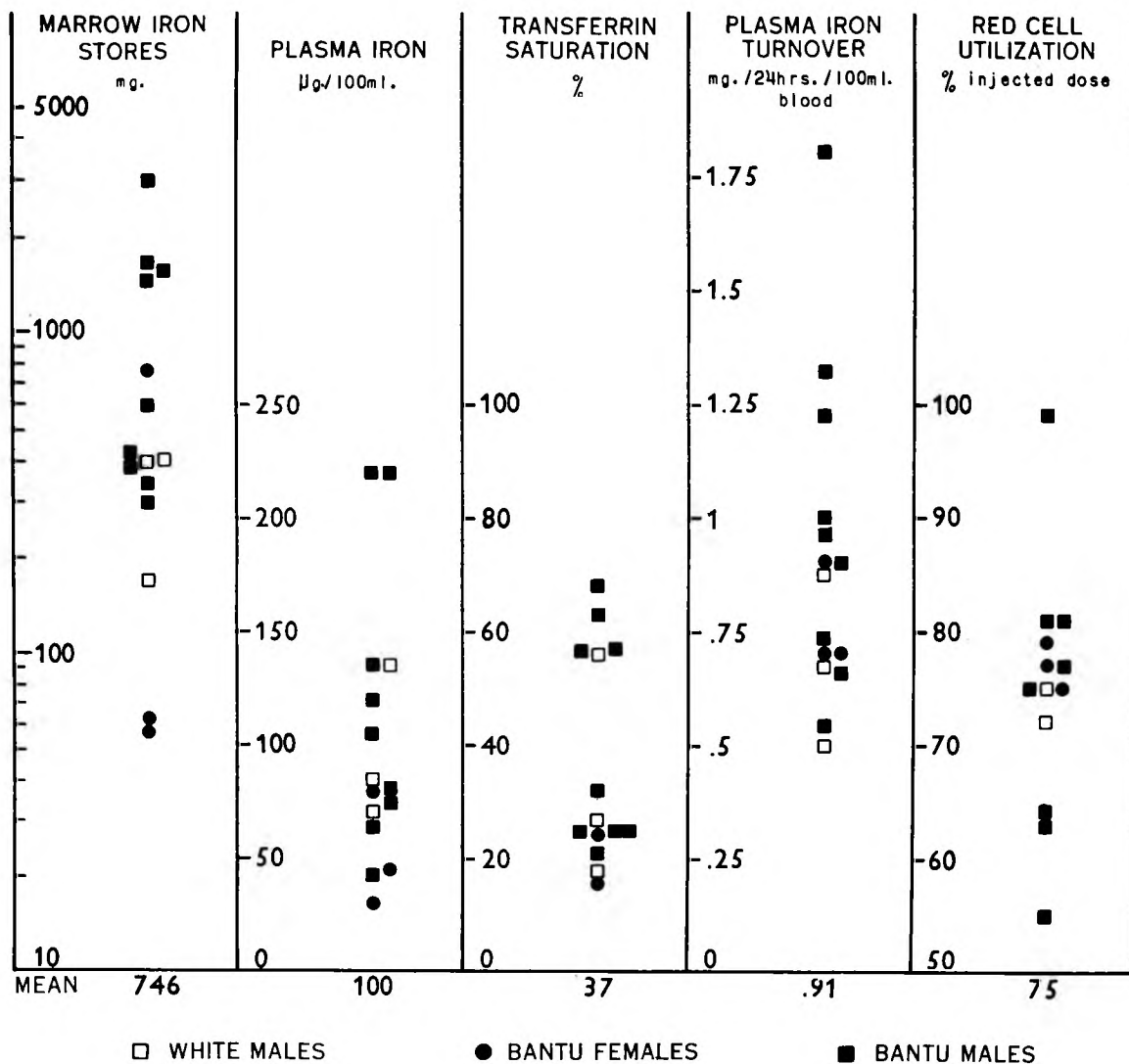
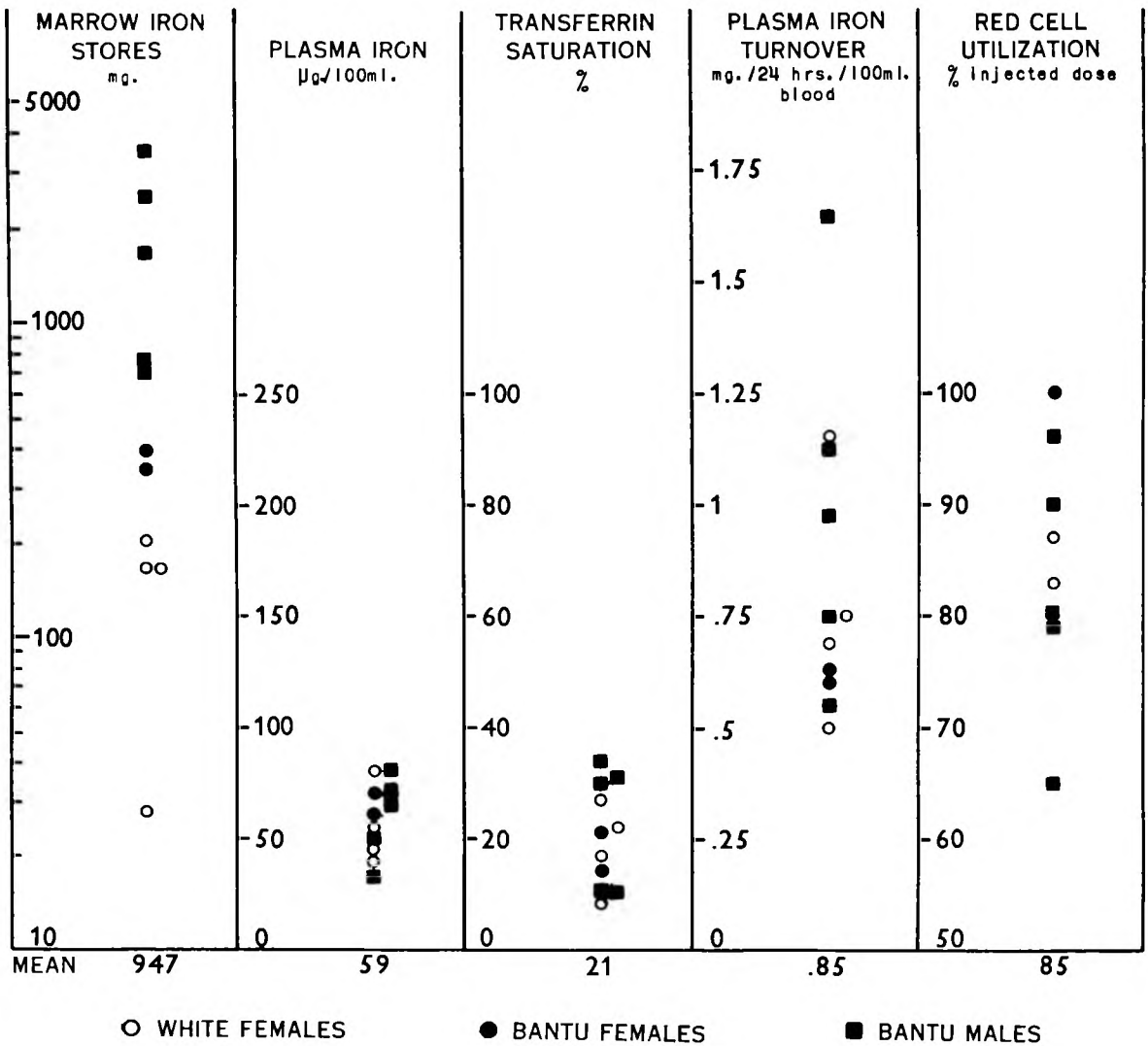


Fig. 4: Marrow iron stores and haematological values in 9 subjects with intrathoracic neoplasm.



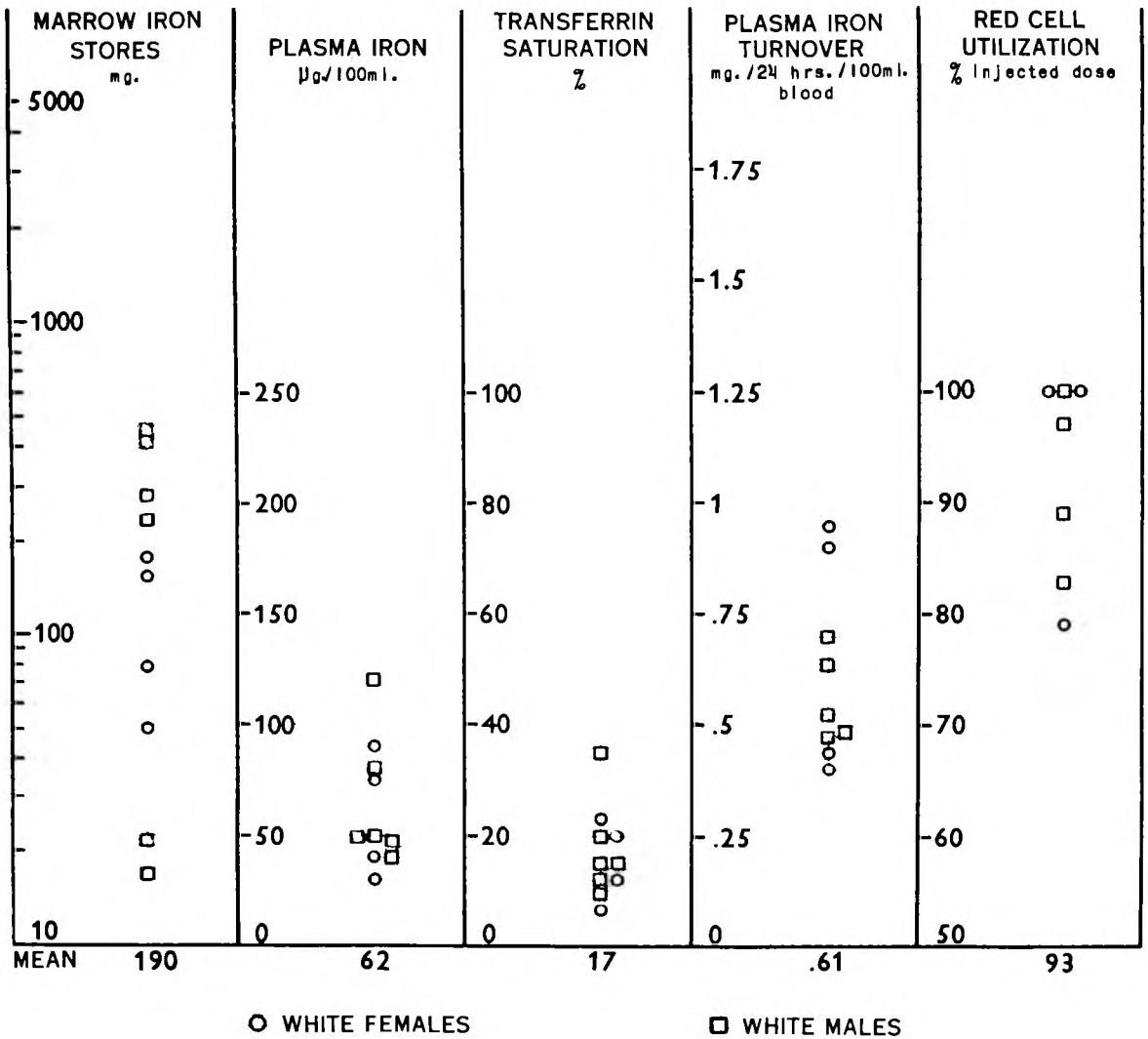
PULMONARY TUBERCULOSIS

Fig. 5: Marrow iron stores and haematological values in 15 subjects with pulmonary tuberculosis.



PULMONARY SEPSIS (NON-TUBERCULOUS)

Fig. 6: Marrow iron stores and haematological values in 11 subjects with non-tuberculous pulmonary sepsis.



HIATUS HERNIA

Fig. 7: Marrow iron stores and haematological values in 10 subjects with hiatus hernia.

been found consistently by numerous workers (Valquist, 1941; Waldenström, 1946; Johnston, 1947; Laurell, 1953), and in one series (Hamilton et al., 1950) the mean fall was as much as 57 per cent. Allowance for this diurnal variation places most individual values and mean values for all diagnostic groups well within the normal range. The trend towards high values in the pulmonary tuberculosis group, in which low plasma iron levels might be expected on account of chronic infection, is explained by the high proportion of siderotic Bantu, in whom plasma iron levels may be high even in the presence of infection (Higginson et al., 1957; Hathorn et al., 1960). The subjects in whom disease-activity was presumably greatest - those in the pulmonary sepsis and neoplasia groups - had low normal values even allowing for diurnal variation. This drop in plasma iron in infection has been shown to be due to an impaired release of iron from reticulo-endothelial stores (Freireich et al., 1957; Noyes et al., 1960; Quastel et al., 1961). This would result in a significant increase in the latter iron compartment only if the infection were long-standing.

The values for red cell utilization in each diagnostic group are within the normal range of 70 - 100 per cent and the mean for the whole study was 83 per cent. Moderate depression of the red cell utilization was present, however, in several individuals suffering from infection. This mild depression has previously been shown to occur in rabbits (Keiderling, 1958) but was not found in man by Bothwell et al. (1956). Earlier studies (Finch et al., 1949; Yuile et al., 1949) showing profound depression of red cell utilization in subjects with infection are probably invalid since large doses of radioiron were used and incomplete binding to transferrin must have occurred. It is possible that haemolysis or post-operative haemorrhage resulted in the false impression of depression of red cell uptake in some subjects of the present study.

The Relationship of Plasma Iron and Iron Binding Capacity to Marrow Stores

The limited value of the plasma iron level and the percentage transferrin saturation as parameters of iron storage

has been mentioned. Their appraisal in relation to the values obtained for marrow iron stores in the present study confirms that it is not possible to predict from them the state of the iron reserves. There is a trend towards higher plasma iron and transferrin saturation in subjects with more than 1,000 mg. of marrow iron, but there is overlap between this group and all others when results are arranged according to marrow iron content (Table VIII). The low transferrin saturations have been previously commented upon.

The Correlation of Marrow Weight with Body Weight

Estimations of the size of the red bone marrow in normal adults have varied somewhat. Figures given by Best and Taylor (1961) and by the International Commission on Radiological Protection and on Radiological Units (1950) are 1,400 ml. and 1,500 g. respectively. Whitby (1954) states that the active bone marrow in an average adult weighs some 3,000 g. and occupies a volume of 4,000 ml. Mechanik, cited by Wintrobe (1961), considered that the red marrow of an adult could vary with body weight between 800 and 1,800 g.

T A B L E VIII

Relationship of Marrow Iron Stores to Plasma Iron and Transferrin Saturation

Marrow iron stores (mg.)	Plasma iron (μ g. per 100 ml.)		Percent transferrin saturation	
	Mean	Range	Mean	Range
0 - 49 (9 subjects)	69	27 - 107	17	9 - 29
50 - 99 (4 subjects)	57	29 - 78	17	7 - 24
100 - 199 (11 subjects)	68	37 - 135	22	11 - 56
200 - 299 (9 subjects)	74	55 - 118	24	16 - 35
300 - 499 (11 subjects)	75	51 - 220	24	9 - 68
500 - 999 (7 subjects)	73	36 - 105	25	10 - 34
1000 and over (8 subjects)	104	57 - 219	37	10 - 63

The present investigation provided an opportunity of calculating by the method indicated on page 77 the mass of haematologically active marrow in living subjects. It was thought that it would be of interest to attempt to correlate marrow weight with body weight and thus establish a ready means of predicting the former in an adult individual.

Ten subjects were excluded from this study because of unavailability of marrow sample weights or on account of obesity, which would have given a false impression of physiological body weight. The 51 subjects studied comprised 34 males and 17 females with body weights ranging from 38 to 84 kg. (mean 62 kg., S.D. 10). Their mean marrow weight was 1,182 g. (S.D. 288, range 645 to 1,740 g.). The effect upon this value of the illnesses from which these patients were suffering is not known. A fairly good positive correlation between body and marrow weight is shown to exist for this number of subjects ($r = + 0.66$, $p < 0.001$) (Fig. (8)). However, the 95, 95 tolerance limits indicate that, according to the present data, the relationship between the two quantities is

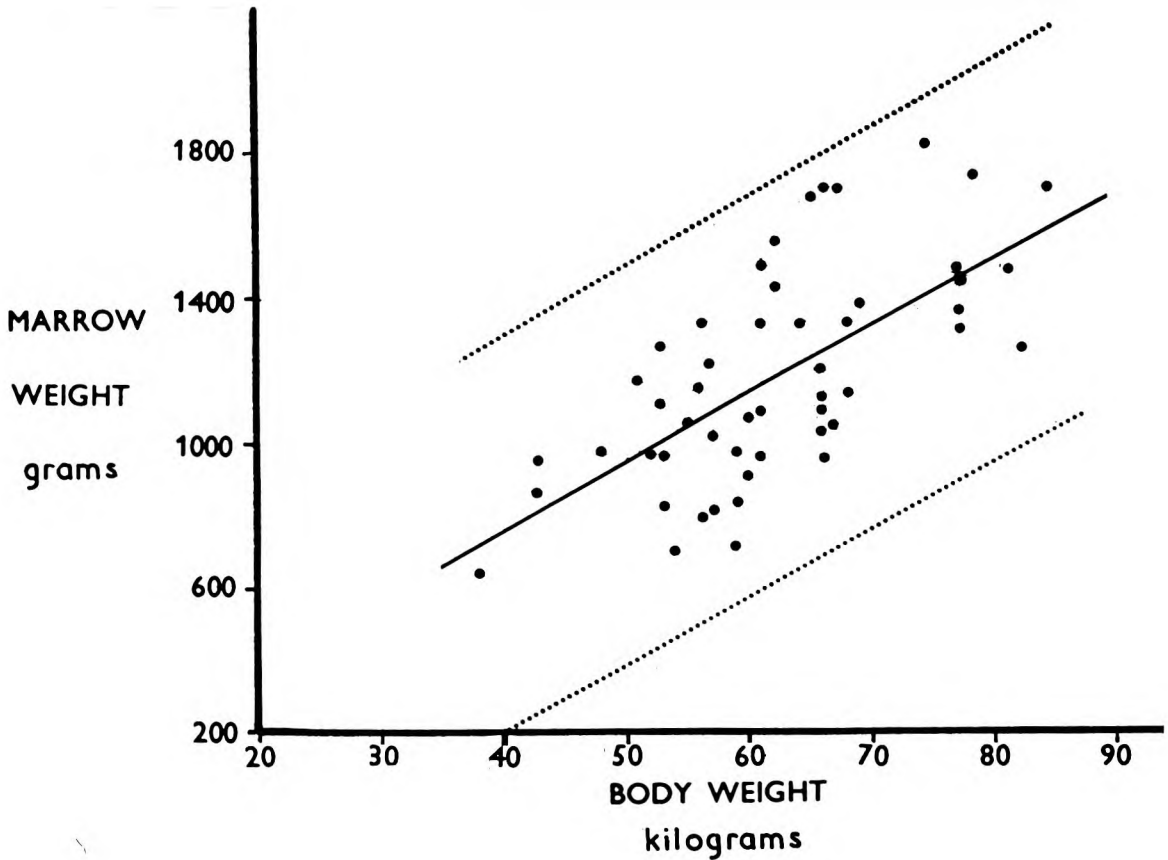


Fig. 8: The correlation between the body weights and marrow weights of 51 subjects. The regression line is shown ($y = 6.08 + 18.84 x$). The dotted lines represent its 95, 95 tolerance limits. $r = + 0.66$, $p < 0.01$.

unsuitable for predicting marrow weight from body weight in a single individual. Thus a subject weighing 70 kg. might have a marrow weight of any value between 780 and 1,860 g. It is suggested that a better correlation might have been obtained using lean body mass although it is felt that the possible advantage conferred by this parameter did not warrant its determination in each subject.

Iron Incorporation into Haem

The isotopic study presented the opportunity of determining what proportion of radioiron had been incorporated into haem in the marrow 24 hours after its intravenous injection. Because the significance of these results is not germane to the theme of this thesis, and yet is deemed worthy of record, the data are not included here but are presented and discussed in the Appendix III (page 134).

2. Studies on Necropsy Material

Results of histological assessments of stainable marrow iron and of chemical determinations of storage iron

concentrations in marrows and livers of 156 necropsy subjects are shown in Figs. (9) to (11).

Marrow Iron Concentrations

The mean for these values in all necropsy subjects was 957 $\mu\text{g. per g.}$ of wet tissue. Breakdown according to race and sex again showed the differences which were so obvious in the isotopic study. The mean value in 28 white females was 209 $\mu\text{g. per g.}$ (range 24 - 1,072 $\mu\text{g. per g.}$) and in 25 white males 318 $\mu\text{g. per g.}$ (range 20 - 1,066 $\mu\text{g. per g.}$). In the Bantu subjects the mean figure for 45 females was 479 $\mu\text{g. per g.}$ (range 28 - 4,032 $\mu\text{g. per g.}$) and for 58 males was 1,976 $\mu\text{g. per g.}$ (range 68 - 8,647 $\mu\text{g. per g.}$).

With the exception of the results for the white females group these results are very similar to those obtained for marrow iron concentration in the isotopic study on living subjects. White females of the necropsy series have approximately double the marrow iron concentration found in the corresponding group of the isotopic study. This may well

Assessments of Marrow Iron in 53 White Necropsy Subjects

<u>Sex</u>	<u>Age</u>	<u>Storage iron concentration</u> (μ g. per g. wet tissue)		<u>Marrow</u> <u>Histology</u>	<u>Diagnosis</u>
		<u>Liver</u>	<u>Marrow</u>		
M	51	235	186	+	Rheumatic heart disease
M	74	1297	555	3+	Laryngeal carcinoma
F	64	120	45	0	Pneumonia
F	47	85	54		Rheumatic heart disease
F	65	5	30	0	Bronchial carcinoma
F	67	150	131	+	Carcinoma of colon
M	87	48	186	2+	Carcinoma of maxillary antrum
M	78	0	50	0	Bronchopneumonia
M	54	210	476	3+	Bronchial carcinoma
M	64	253	183	2+	Diverticulitis
F	83	110	63	+	Ruptured aortic aneurysm
F	89	28	79	+	Ischaemic heart disease
F	66	240	313	2+	Myocardial infarction
F	74	25	37	0	Carcinoma of colon
F	53	23	82	+	Cerebral haemorrhage
F	83	260	228	2+	Acute pancreatitis
M	61	108	186	2+	Cor pulmonale
M	55	0	51	0	Bleeding gastric ulcer
M	54	385	305	2+	Dissecting aortic aneurysm
M	42	153	415		Pneumonia and empyema
F	44	255	243	2+	Carcinoma of breast
M	69	28	28	0	Bleeding gastric ulcer
M		565	1051	5+	Lymphoma
M	55	263	1066	5+	
F	74	20	98	+	Pyelonephritis
M	67	343	426	2+	Myocardial infarction
F	66	10	65	0	Acute tubular necrosis
F	51	20	48	0	Endomyocardial fibrosis
M	78	0	64	0	Lobar pneumonia
F	90	960	735	4+	Biliary peritonitis
F		70	166	2+	Hepatic cirrhosis
F	90	120	231	2+	Myocardial infarction
F	84	23	55	0	Myocardial infarction
F	71	248	151	+	Bronchopneumonia
F	50	420	490	3+	Carcinoma of breast
M	75	5	20	0	
M	67	108	270	2+	Hypertensive heart disease
F	74	513	462	3+	Pulmonary embolus
M	67	23	57	+	Calcific aortic stenosis
F	49	28	94	+	Rheumatic heart disease
M	47	1253	585	3+	Acute pancreatitis
F	67	18	24	0	Cerebral thrombosis
M	69	180	141	+	
M		185	140	+	Cerebral haemorrhage
M	47	333	223	2+	Myocardial infarction
F	81	225	139	2+	Bronchopneumonia
F	47	280	286	3+	Hepatic cirrhosis
F	74	83	226	2+	Carcinoma of colon
F		55	143	+	Hypokalaemia
M	70	300	835	4+	Ischaemic heart disease
F	47	573	1072	5+	Leukaemia
M	65	195	312	3+	Bronchial carcinoma
M	58	200	371	3+	Perforated duodenal ulcer

Fig. 9

Bantu Necropsy Subjects (continued)

<u>Sex</u>	<u>Age</u>	<u>Storage iron concentration</u> (µg. per g. wet tissue)		<u>Marrow</u> <u>Histology</u>	<u>Diagnosis</u>
		<u>Liver</u>	<u>Marrow</u>		
F	70	3940	1237	4+	
M	23	1078	1421	5+	Subacute bacterial endo- carditis
M	30	2254	5505	6+	Bronchopneumonia
F	70	135	172	2+	Oesophageal carcinoma
F	15	571	1011	4+	Psoas abscess
M	56	68	34	0	Gastric carcinoma
M	57	673	2292	5+	Bronchopneumonia
M	55	3895	2971	6+	Hepatic cirrhosis
M	52	2241	642	3+	Disseminated tuberculosis
M	49	1618	3969	6+	Oesophageal carcinoma
M	65	6362	2138	6+	Chronic pyelonephritis
M	30	488	1066	4+	Acute tubular necrosis
M	40	5655	3676	6+	Bronchopneumonia
M	36	1203	2256	6+	Oesophageal carcinoma
M	61	65	120	+	Cerebral thrombosis
F	42	155	482	3+	Chronic nephritis
M	40	190	678	3+	Chronic nephritis
M	65	6188	4346	6+	Hepatic cirrhosis
F	35	13	65	+	Renal sarcoma
M	40	11020	3729	6+	Haemothorax
M	60	2915	4219	6+	Hydronephrosis
M	61	5510	3034	6+	Pneumonia
M	50	2843	3225	6+	Bronchiectasis
M	32	210	804	4+	Bowel lymphoma
M	52	4730	3840	6+	Spinal tuberculosis
F	21	744	343		Acute leukaemia
F	40	180	148	+	Pulmonary tuberculosis
M	60	4483	953	4+	Hepatic cirrhosis
M	67	616	657	4+	Cerebral tuberculoma
M	34	295	269	2+	Chronic nephritis
F	24	49	380	2+	Amoebiasis
F	26	63	130	+	Septic endometritis
M	51	6775	4600	6+	Oesophageal carcinoma
F	34	808	270	2+	Cardiac aneurysm
F	27	350	540	4+	Faecal fistula
M	42	875	632	4+	Perforated colonic ulcer
M	51	2300	2543	6+	Pancreatic carcinoma
M	26	215	257	+	Chronic nephritis
M	46	13	68	0	Rheumatic heart disease
M	29	1485	1431	4+	Status epilepticus
F	21	45	186		Cerebral softening
M	64	1442	804	4+	Generalized tuberculosis
M	56	2344	2009	5+	Oesophageal carcinoma
F	35	266	336	3+	Puerperal sepsis
M	80	489	458	3+	Chronic pancreatitis
F	28	355	156		Chronic nephritis
M	40	10915	8647	6+	Hepatic cirrhosis
M	42	560	915	5+	Idiopathic cardiac failure
M	45	539	839	4+	Oesophageal carcinoma
F	28	125	83	+	Diabetic coma
M	53	237	472	4+	Oesophageal carcinoma
M	68	332	214	+	Perforated duodenal ulcer

Page
109

Fig. 10.

Liver and Marrow Storage Iron Concentrations and Histological
Assessments of Marrow Iron in 103 Bantu Necropsy Subjects

Page
110

<u>Sex</u>	<u>Age</u>	<u>Storage iron concentration</u> (μ g. per g. wet tissue)		<u>Marrow</u> <u>Histology</u>	<u>Diagnosis</u>
		<u>Liver</u>	<u>Marrow</u>		
M	49	4440	4888	6+	Idiopathic cardiac failure
M	50	50	135	+	Cor pulmonale
M	55	842	1692	5+	Miliary tuberculosis
M	42	1055	475	3+	Hepatic cirrhosis
M	21	265	129	+	Cerebral infarcts
M	70	2605	1413	5+	Disseminated tuberculosis
F	60	157	139	2+	Idiopathic cardiac failure
M	40	1577	2084	5+	Oesophageal carcinoma
F	26	93	293	2+	Hepatoma
M	17	430	116	2+	Cerebral haemorrhage
F	20	33	35	0	Ventricular septal defect
M	45	640	292	2+	Hepatic cirrhosis
M	56	2923	2336	6+	Renal carcinoma
F	40	878	253	2+	Lymphadenoma
M	46	817	644	4+	Lung abscesses
F	66	153	288	2+	Subarachnoid haemorrhage
F	30	422	397	2+	Septic abortion
F	25	90	285	2+	Pyelonephritis
F	44	1260	253	3+	Volvulus
F	21	83	94	0	Pulmonary emboli
F	40	75	94	0	
F	22	83	223	2+	
F	32	28	22	0	Massive liver necrosis
F	51	3708	4032	6+	Miliary tuberculosis
F	47	13	142	+	Chronic pyelonephritis
F	45	3180	3600	6+	Hepatic cirrhosis
F	25	45	159	+	Acute tubular necrosis
F	60	120	282	2+	Chronic nephritis
F	42	1428	536	3+	Uterine sarcoma
F	30	28	28	0	Chronic nephritis
F	21	165	240	+	Cholelithiasis
F	69	23	72	0	Idiopathic cardiac failure
F	49	3	38	0	Uterine sarcoma
F	49	418	411	4+	Oesophageal carcinoma
F	89	160	84	0	Chronic pyelonephritis
F	75	302	788	4+	Chronic pyelonephritis
M	60	2412	6028	6+	Acute pyelonephritis
F	22	50	231	+	Pneumonia
M	73	3165	5031	6+	Acute pyelonephritis
M	46	295	243	2+	Gastric carcinoma
M	70	394	164	2+	Chronic nephritis
F	60	2455	2337	5+	Cholelithiasis
M	44	73	138	2+	Pulmonary infarction
F	45	215	495	3+	Pneumonia
M	40	13	152	2+	Gastric carcinoma
F	36	100	66	0	Ruptured uterus
F	69	73	117	+	Disseminated tuberculosis
M	44	4650	3714	6+	Disseminated tuberculosis
M	54	1800	2564	6+	Oesophageal carcinoma
M	54	4945	3572	6+	Oesophageal carcinoma
M	52	1485	3087	6+	Osteomyelitis

Fig. 11.

be related to the fact that their mean age is much higher - 67 years as against 35 years in the isotopic group. After maturity advancing age is not thought to influence iron stores (Finch and Finch, 1955), since in adulthood absorption and excretion of iron are normally balanced, but in the case of postmenopausal females it is possible that storage increments occur following the cessation of the menstrual source of iron loss.

Liver Iron Concentrations

Liver Iron concentrations also show marked race and sex differences. Mean values and ranges were as shown in Table IX.

The accepted limits of normal for liver iron concentration are 0.05 to 0.1 per cent dry weight (Sheldon, 1935 (b)), which corresponds approximately to 125 to 250 $\mu\text{g.}$ per g. of fresh tissue. Morgan and Walters (1963) found a mean value of 244 $\mu\text{g.}$ per g. in fresh analyses. The mean figure obtained for all white subjects in the present study was 219 $\mu\text{g.}$ per g. Most of the individual values in whites exceeding

T A B L E IX

Storage -Iron Concentrations in Livers of 156 Necropsy Subjects
(μ g. iron per g. of Wet Tissue)

	No. of Cases	Mean	Range
White females	28	177	5 - 960
White Males	25	267	0 - 1297
Bantu Females	45	525	3 - 3940
Bantu Males	58	2207	12 - 11020

the upper limit of normal occurred in subjects with neoplastic disease. Values below 125 $\mu\text{g. per g.}$ were, however, not uncommon, and could in only a few cases be explained on the basis of chronic haemorrhage.

As has been found in previous studies, the liver iron concentrations in Bantu subjects were a good deal higher. The mean concentration of 1,472 $\mu\text{g. per g.}$ was nearly 7 times greater than that obtained for white subjects and the highest value was 52 times greater. The wide range of values indicates considerable variability in degree of siderosis. In the very high concentration range there is a close correlation between the results of the present study, in which 20 per cent of Bantu subjects had liver iron concentrations exceeding 1,500 $\mu\text{g. per g.}$ and those of Bothwell and Bradlow (1960) who found equivalent concentrations in 19 per cent of their cases.

The Quantitative Relationship Between Storage Iron Concentrations in Bone Marrow and Liver

It was of considerable interest to find that the iron

concentrations in the marrows and livers of individual subjects were very similar when stores were depleted or normal and that a fair correlation existed in all degrees of iron overload.

Fig.(12) demonstrates this relationship in 156 white and Bantu necropsy subjects of both sexes having a range of tissue iron varying between 0 and 11,020 $\mu\text{g. per g.}$ The data do not permit calculation of the absolute amounts of storage iron in bone marrow and liver (since the total weight of each organ is not known), but some idea of their relative capacities can be obtained by applying results of the isotopic study. The latter gave a mean total marrow weight of only 18 per cent less than the predicted normal for mean liver weight in the same group. This implies that the total quantities of iron stored in each of these two organs is normally roughly equal. This relationship probably holds true also when stores are moderately increased, but heavy iron overload is usually associated with hepatomegaly and consequently an increase in the capacity of the liver.

In quantitative terms, the highest marrow iron

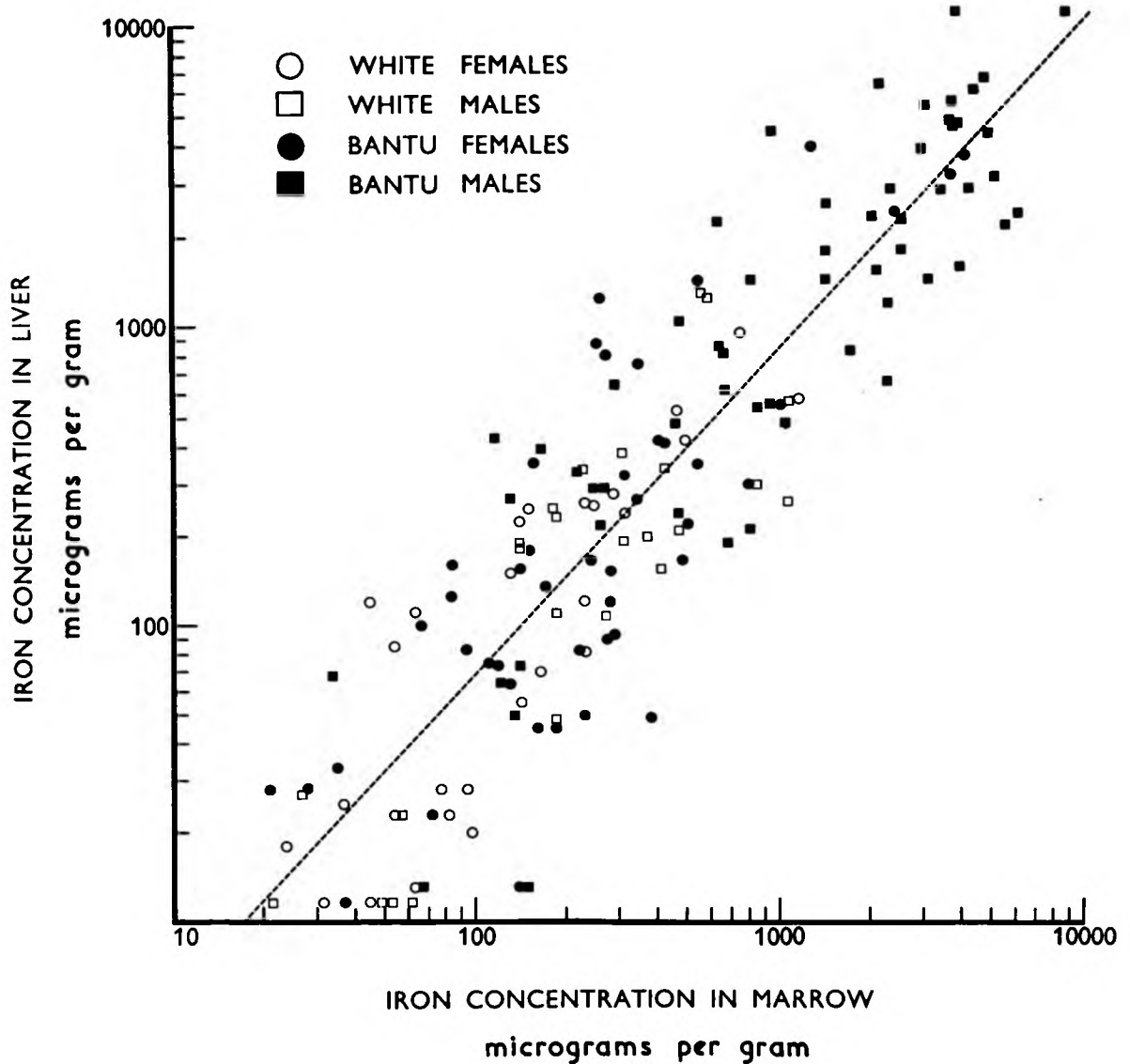


Fig. 12: The correlation between storage iron concentrations in the marrows and livers of 156 necropsy subjects. The regression line is indicated. $r = +0.88$, $p < .001$.

concentration encountered was 8,647 $\mu\text{g. per g.}$ If it is presumed that the total marrow weight in this individual was 1,182 g. then his total marrow iron stores must have been approximately 10 g. This is about half the amount usually present in the liver in idiopathic haemochromatosis (Sheldon, 1935 (a); Finch and Finch, 1955). The bone marrow thus possesses a remarkable capacity for expansion of its iron stores. This appears to be achieved without any derangement of its other functions. It may be concluded that in Bantu iron overload, where in the absence of complicating factors hepatomegaly is uncommon, the distribution of iron between bone marrow and liver is approximately equal.

3. Histological Studies

A comparison was made between histological gradings of stainable iron content of marrow preparations and the storage iron concentrations estimated chemically. Individual results in 206 marrows obtained from both the isotopic and the necropsy series of cases are shown in Fig. (13). Examples of each histological grade, assessed on the criteria indicated

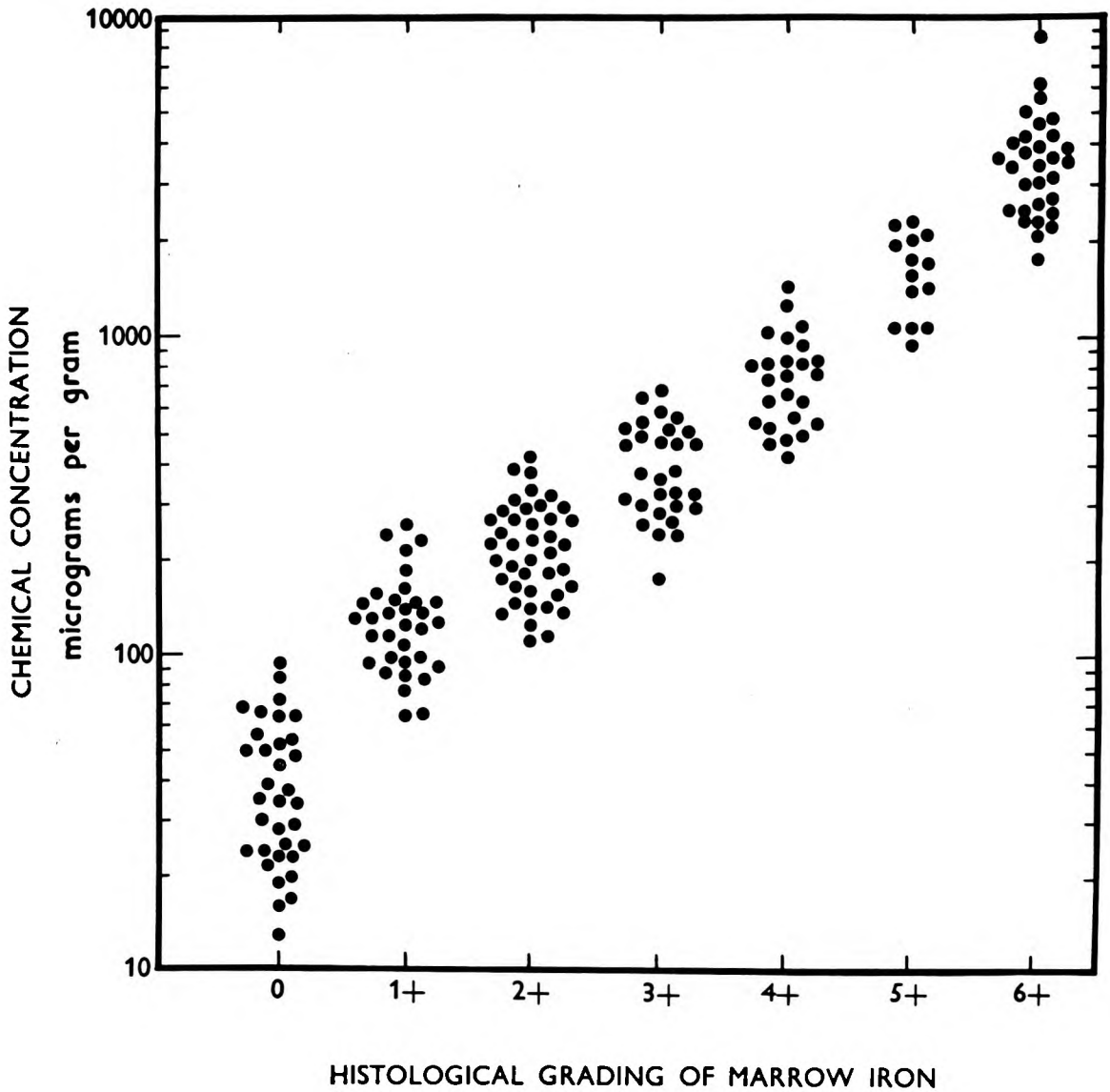


Fig. 13: The range of chemical concentrations of storage iron in the marrows of 206 subjects in whom the extent of reticulo-endothelial stores was assessed histologically.

on pages 79 and 80 , are illustrated by photomicrographs in Figs. (14) to (20).

It was found that the histological appearance correlated well with the chemical determination of iron in the marrow over a wide range of iron concentrations. The mean chemical values and ranges for each histological grading are shown in Table X. When visible iron was relatively scanty it was possible to predict with certainty to within 150 $\mu\text{g.}$ per g., and usually more accurately than this, the concentration of storage iron which would be found on chemical determination.

In grade 3+ marrows, which were regarded as the upper histological limit of normal, predictions were not quite so accurate but could usually be made to within 150 to 200 $\mu\text{g.}$ per g. and always to within 250 $\mu\text{g.}$ per g. With higher gradings the range of chemical concentrations become progressively wider, indicating that fine variations are not demonstrated or cannot be assessed histologically when

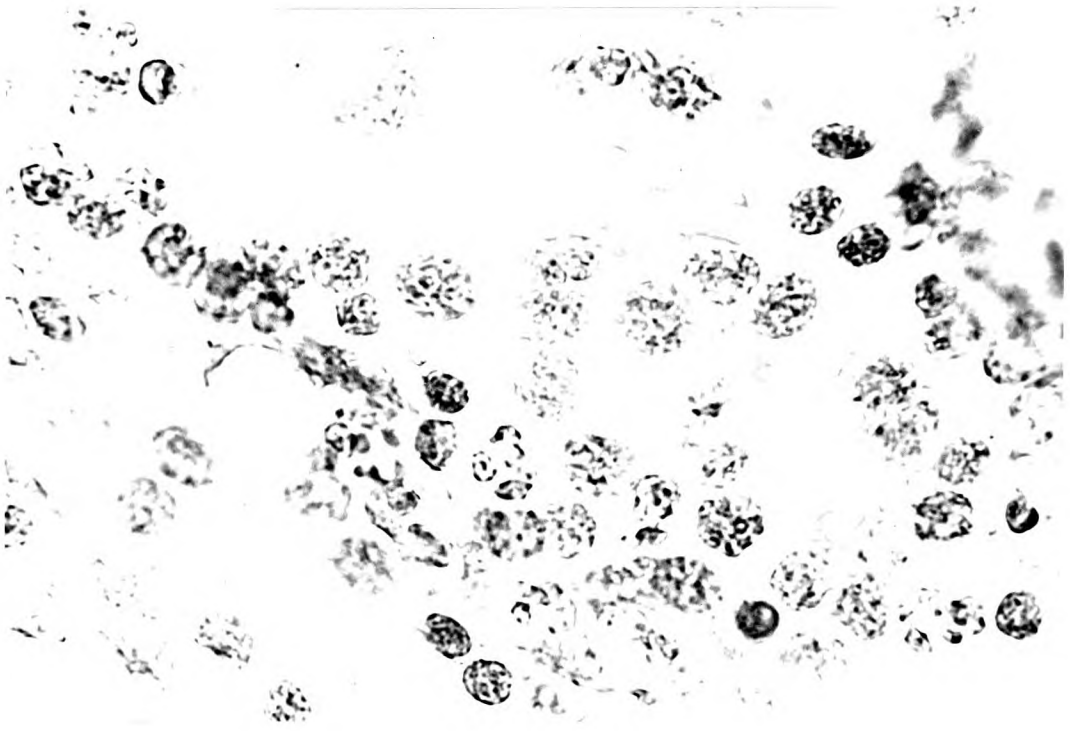


Fig. 14: Grade 0. Magnification X 1200

Figures 14 to 20 : Histological gradings of marrow iron stores.
Iron deposits are represented by black granules or aggregated clumps. Stain: potassium ferrocyanide.

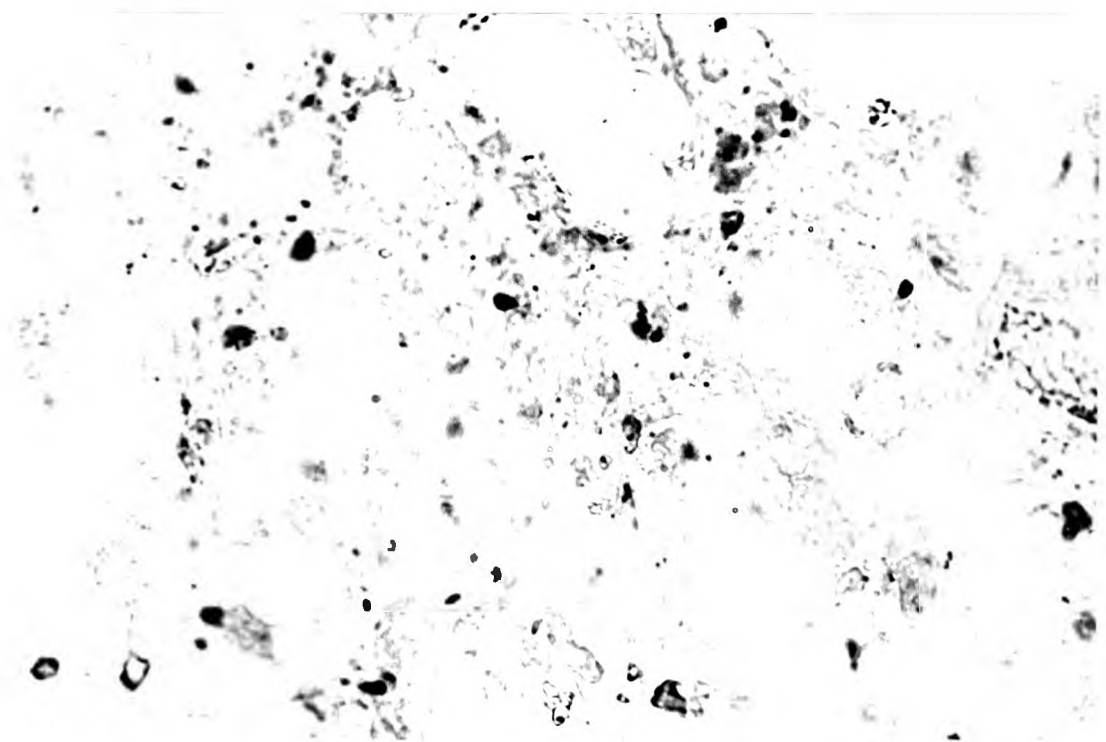


Fig. 15: Grade 1+. Magnification X 1200

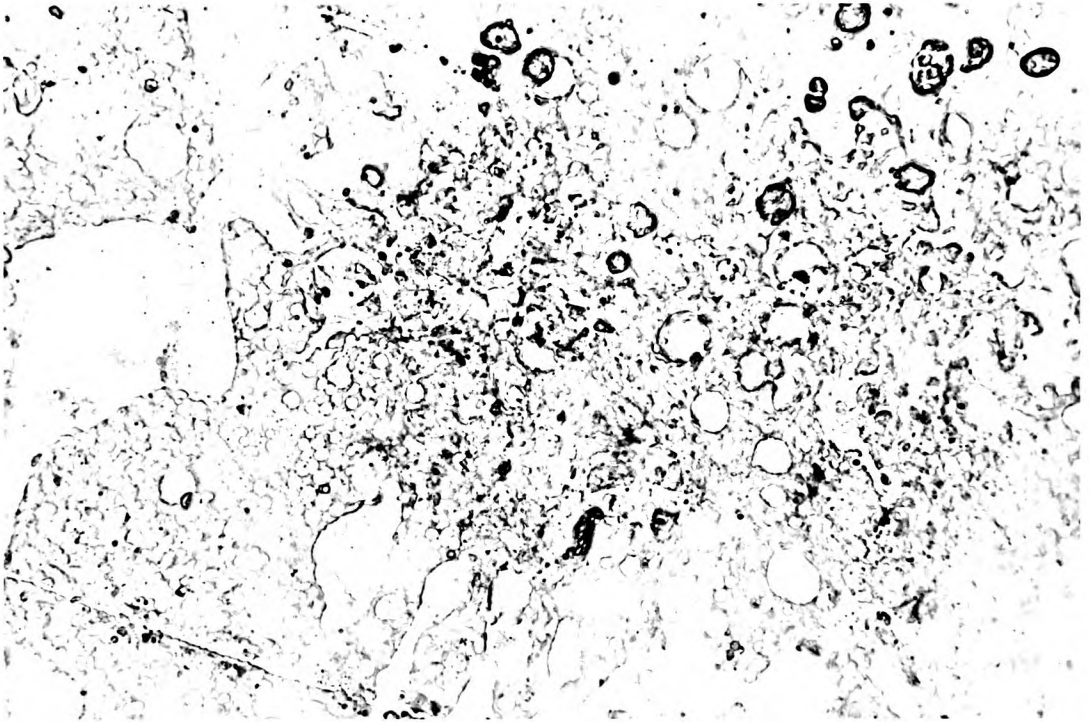


Fig. 16: Grade 2+. Magnification X 120.

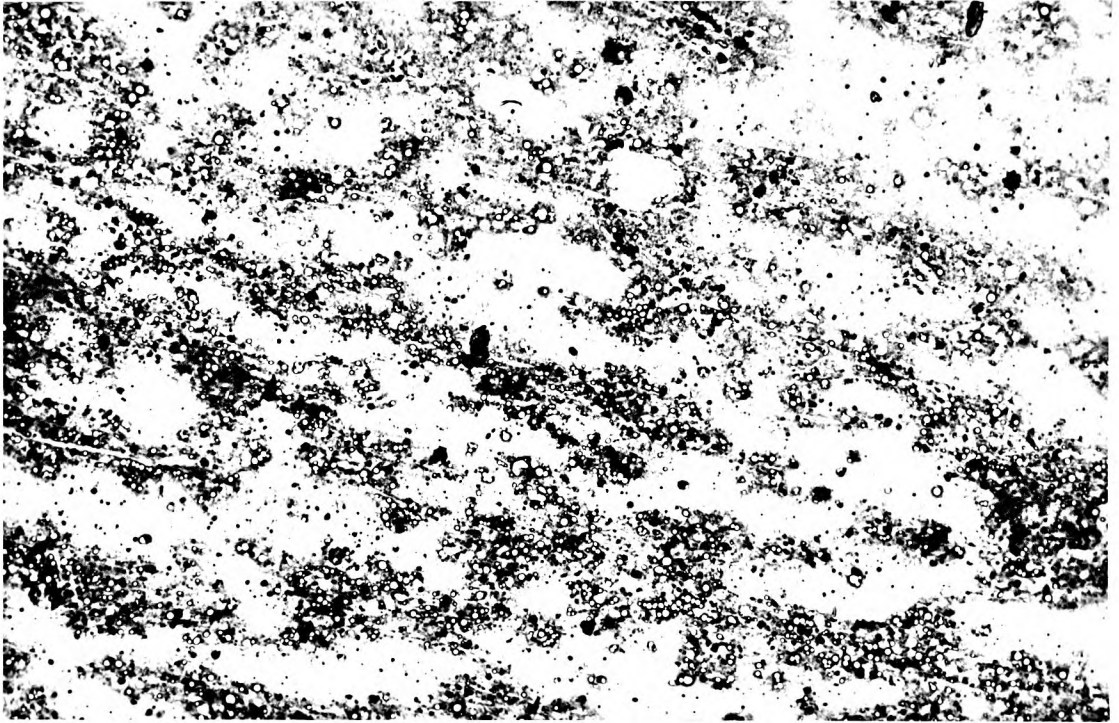


Fig. 17: Grade 3+. Magnification X 120

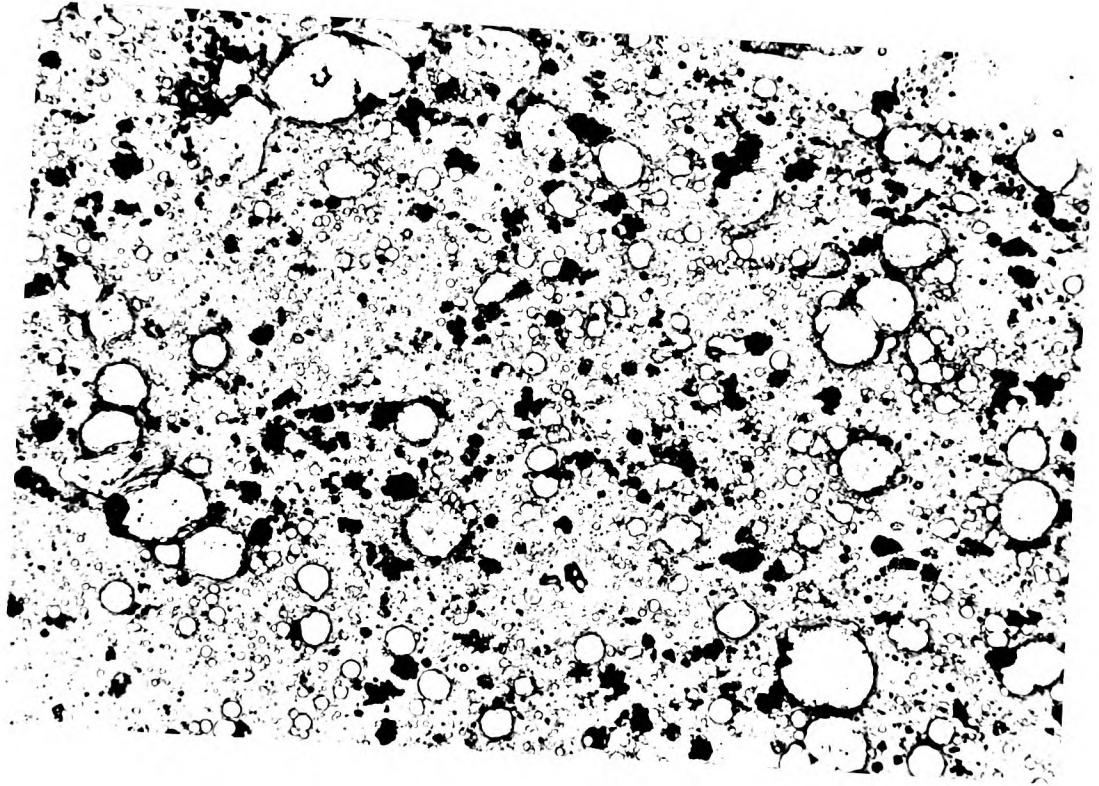


Fig. 18: Grade 4+. Magnification X 120.

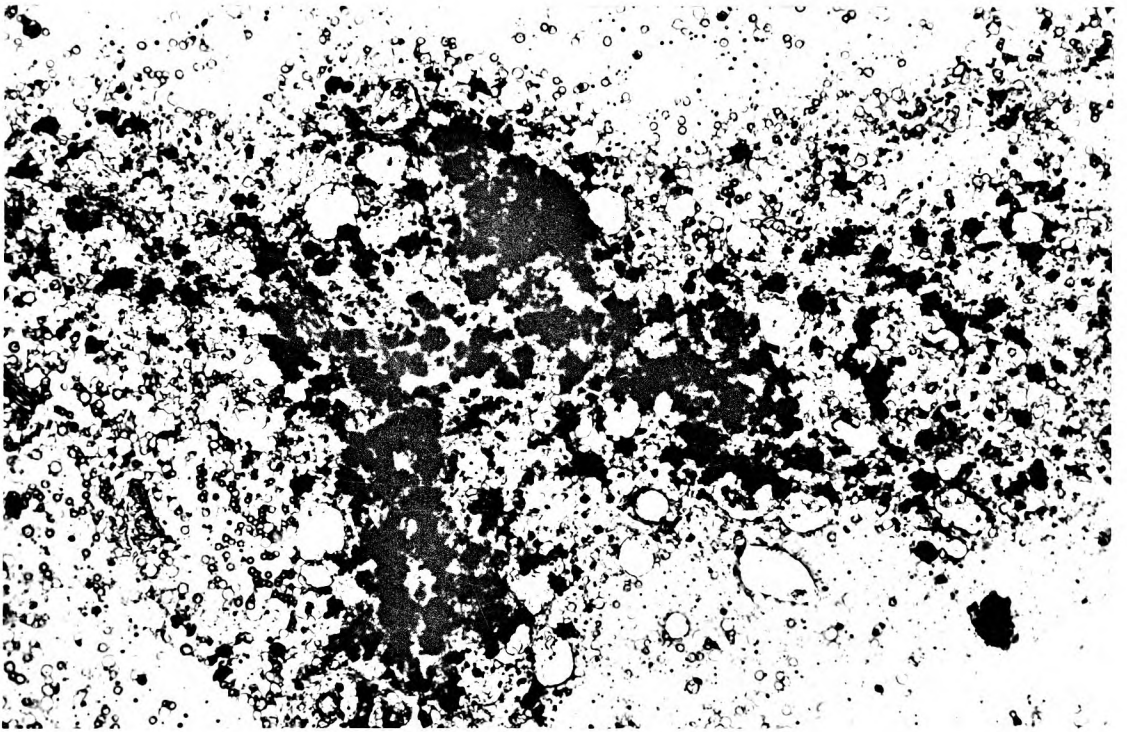


Fig. 19: Grade 5+. Magnification X 120

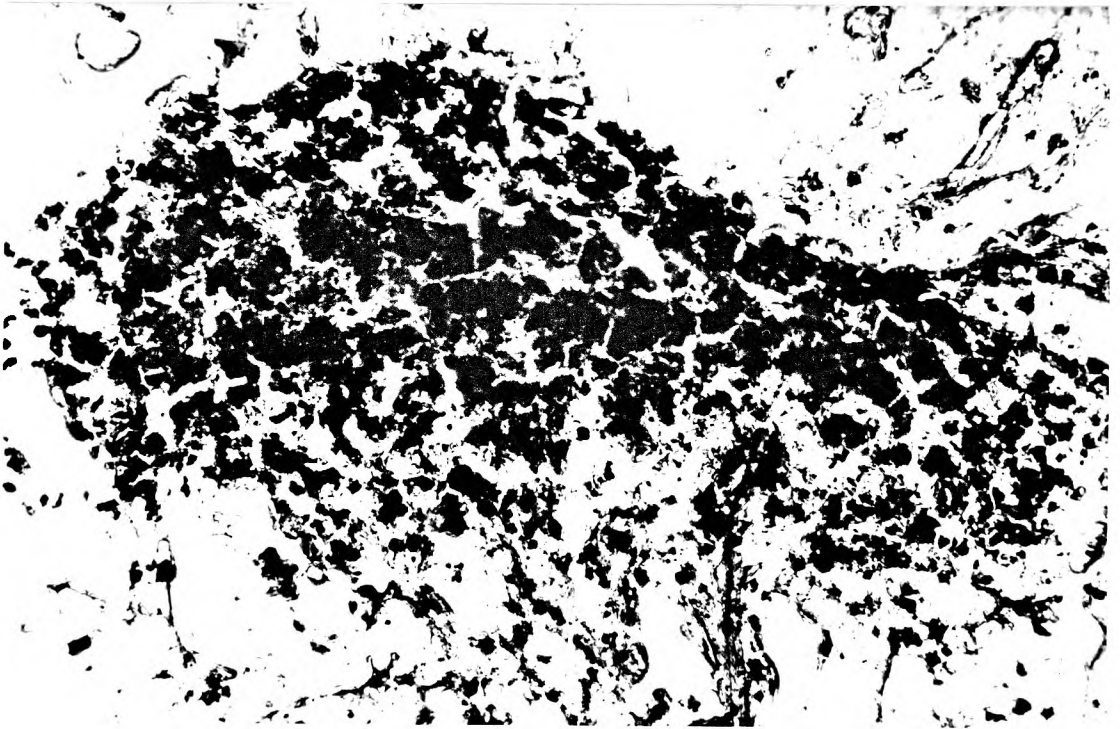


Fig. 20: Grade 6+. Magnification X 120.

T A B L E X

Storage Iron Concentrations According to Histological Grading
of Stainable Iron in 206 Marrows

Histological grading	Number of subjects	Chemical concentration (μ g. per g.)		
		Mean	Range	Standard Deviation
0	34	43	43 - 94	23
1+	33	130	63 - 257	50
2+	41	223	110 - 426	75
3+	29	406	176 - 678	131
4+	25	762	411 - 1431	247
5+	14	1618	915 - 2337	464
6+	30	3681	1778 - 8647	1400

staining is heavy. Correlations were fairly good, however, and there was no more overlap between the high grades than among the low. This is indicated by Fig. (13). Concentrations of iron above 2,000 $\mu\text{g. per g.}$ were almost always graded as 6+.

These results add further confirmation to the validity of the histological assessment of haemosiderin in marrow particles as a useful means of gauging the size of reticulo-endothelial iron stores in the marrow.

Since marrow stores correlate well with liver stores particularly when they are low or normal, and these two organs are the major ones for iron storage, it follows that histological assessment of marrow iron is a valuable gauge of total body iron stores especially in the lower ranges. When stores are abundant the quantitative significance of marrow histology is less precise.

CHAPTER VII

SUMMARY

It has been estimated that iron stores in man, which are deposited chiefly in the liver, bone marrow, spleen and possibly skeletal muscle, normally amount to about 1 g. or one quarter of the total body content. The liver content is well documented, but review of the current literature reveals that although the histological assessment of haemosiderin in the reticulum cells of bone marrow has been widely used to gauge the individual status of iron stores, there is no information on what amount these visible marrow reserves represent. The present study was therefore undertaken to measure the iron stores of human bone marrow in normal and abnormal conditions of iron metabolism.

A review of present knowledge of iron metabolism and iron stores in the body was made, with particular emphasis on various aspects of storage in marrow. The methods and clinical importance of the evaluation of these stores were discussed. An appraisal of known techniques for the quantitative differentiation of haem and non-haem or storage iron in tissues was made, and validation experiments

were conducted confirming the accuracy of the strontium chloride and the pyrophosphate methods.

Total bone marrow iron stores were then estimated quantitatively in 61 white and Bantu hospital patients by means of an isotopic technique using Fe^{59} . Results showed well-defined race and sex differences, but on the whole related poorly to haematological parameters such as plasma iron and transferrin concentrations. The mean value in 24 white males was 288 mg. as compared with 99 mg. in 17 white females. The high incidence of iron overload in Bantu adults was reflected in the finding of a mean value of 1,629 mg. in 15 Bantu males. The significance of these differences was discussed. The overall effects upon marrow iron reserves of the diseases from which the subjects were suffering were investigated, and, contrary to expectation, were found not to have been significant, but the total number of cases in each group was small. Other haematological data were also considered in relation to the diagnostic groups.

Data permitted the direct calculation of the total amount of haematologically active marrow in these subjects. The mean value was 1,182 g. and the individual variation was wide. A good positive correlation was shown to exist between body weight and marrow weight.

Storage iron concentrations in marrows and livers of 156 necropsy subjects were then determined. Results for marrows closely paralleled those of the isotopic study with the exception of the white female group, in which values were higher. A possible reason for the latter discrepancy was indicated. Results for livers showed similar race and sex differences. A comparison of results for the two organs indicated that the iron concentrations in the marrows and livers of individual subjects were very similar when stores were depleted or normal and that a fair correlation existed in all degrees of iron overload.

Histological gradings of stainable iron contents of preparations from 206 marrows were found to correlate well

with their chemically determined storage iron concentrations.

This confirmed the value of marrow histology in the assessment of iron stores.

APPENDIX I

Method for the Preparation of Radioactive Ferritin

Rats are loaded with iron by giving each animal an intramuscular injection of iron-sorbitol-citrate containing 1 mg. of iron on alternate days for 3 doses. On the day following the last dose approximately 100 μ c. of Fe^{59} as ferric chloride are administered intravenously to each animal in order to label the stored iron. The animals are sacrificed on the following day and the labelled ferritin is isolated from their livers by the method of Mazur and Shorr (1948).

APPENDIX II

Method for the Preparation of Radioactive Haemoglobin

Erythropoiesis is stimulated in rats by bleeding each animal 4 ml. on alternate days until 12 ml. have been removed. On the day following the last bleeding approximately 100 μ c. of Fe⁵⁹ as ferric chloride are administered intravenously to each animal. On the fifth day thereafter the animals are exsanguinated. The erythrocytes are separated by centrifugation and haemolysed with water to provide a solution of labelled haemoglobin.

APPENDIX III

An Aspect of Iron Exchange Between Plasma and Bone

Marrow

The understanding of the complexities of intermediary iron metabolism may be resolved into the delineation of the nature of the exchange of iron between the transport protein of the plasma and three major cell types - erythroid precursors in the marrow, reticulo-endothelial cells and parenchymal cells of various organs which may store iron, particularly those of the liver. Data obtained from the present study contribute towards the illumination of one facet of iron exchange between transferrin and the erythroid marrow. It is felt that these results are worthy of record since there appears to be some discordance of opinion on this aspect of iron metabolism between notable workers in the field.

Pollycove and Mortimer (1961) have used the disappearance curve of intravenously injected transferrin-

bound radioiron from the plasma of human subjects together with measurements of the subsequent appearance of radioactivity in marrow, liver, spleen and erythrocytes as a basis for the formulation of "mathematical models" of intermediary iron exchange. Their analysis of the plasma disappearance curve showed three exponential components and this was interpreted as being due to a single rapid exponential disappearance rate being superimposed upon by re-entry of radioiron into the plasma from two separate sources. Kinetic studies suggested that these sources were

- a) an "erythropoietic labile pool" in the bone marrow which took up approximately one third of the radioiron initially going to the bone marrow, and
- b) A storage pool from which the "feedback" into plasma was slower and which was quantitatively significant only when stores were abundant.

Similar ideas had been earlier expressed by Greenberg and Wintrobe (1946), who postulated a labile iron

pool comprising recently absorbed iron and iron recently liberated from destroyed red blood cells, from which the daily requirements of iron are drawn. On the basis of their own experiments, Dubach et al. (1946), accepted this concept, but regarded the "pool" as a form of storage from which the iron is more readily mobilized.

The concept of marrow pool of iron which is freely exchangeable with plasma iron is fundamental to this interpretation of intermediary iron metabolism. The hypothesis admirably fits the data of the study but it remains a theoretical possibility and not a proven reality.

It is, however, difficult to reconcile the observation that the red cell precursor maturation time is about 3 or 4 days (Osgood, 1954; Astaldi, 1960) and that the maximal incorporation of radioiron into circulating red cells occurs in 7 to 10 days with the view that initial marrow accumulation of radioiron represents its irreversible fixation in red cell precursors. This favours indirectly the labile erythropoietic pool hypothesis. Further support is cited by

Pollycove (1961) who found in later experiments that haem radioiron (which is irreversibly fixed in the red cell) accumulates relatively slowly in human marrow after the tracer injection and that at 36 hours only half of the marrow radioiron was in the haem form.

This result is at variance with the studies of Noyes and Finch (1961), who found that 20 minutes after the intravenous injection of radioiron in rabbits about 80 per cent of marrow radioiron was in the haem form. Similar findings were obtained by Hosain et al. (1962) in dog and man. The rapid fixing in the haem form of most of the iron taken up by erythroid precursors is incompatible with the presence of a labile marrow pool of the size originally postulated.

The present study provided an opportunity to determine what proportion of radioiron in the bone marrow was in haem form between 18 and 24 hours after its original injection. This would seem to be an important determination in view of the

conflicting evidence cited above. The method used has been described on page 77.

Details of individual results appear in Fig. (I) (page 82). In 53 observations a mean of 79 per cent of marrow iron was found to be in haem form; the range was 55 - 100 per cent and the standard deviation 10.7. This is in close agreement with the estimate of 80 per cent made by Hosain et al. (1962).

These data therefore indicate that there is rapid fixing of transferrin-derived marrow iron as haem in erythroid precursors. Since the fate of the remaining 21 per cent of iron could not be followed, it remains quite possible on the present evidence alone that this fraction could represent a labile pool. Jandl and Katz (1963) have, however, shown that at least a portion of this iron is irreversibly fixed on the cell membrane of erythroid precursors.

The limited scrutiny of the "labile erythropoietic iron pool" hypothesis permitted by this study would thus appear to

cast doubt upon its reality as an entity of any appreciable size.

BIBLIOGRAPHY

- Astaldi, G. (1960). Differentiation, proliferation and maturation of haemopoietic cells studied in tissue culture. In Ciba Foundation Symposium on Haemopoiesis (Wolstenholme, G.E.W., and O'Connor, M., eds.) J.A. Churchill Ltd., London. p. 99.
- Austoni, M.E., Rabinovitch, A., and Greenberg, D.M. (1940). The iron content of the tissues of normal, anemic, and iron-enriched rats freed from blood by viviperfusion. J. biol. Chem. 134, 17.
- Bannerman, R.M., Callender, S.T., and Williams, D.L. (1962). Effect of desferrioxamine and D.T.P.A. in iron overload. Brit. med. J. 2, 1573.
- Bessis, M.C., and Breton-Gorius, J. (1957). Iron particles in normal erythroblasts and normal and pathological erythrocytes. J. biophys. biochem. Cytol. 3, 503.
- Best, C.H., and Taylor, N.B. (1961). The physiological basis of medical practice. The Williams and Wilkins Co., Baltimore, p. 104.
- Beutler, E. (1957). Clinical evaluation of iron stores. New Engl. J. Med. 256, 692.
- Beutler, E., and Bittenwieser, E. (1960). The regulation of iron absorption. 1. A search for humoral factors. J. Lab. clin. Med. 55, 274.
- Beutler, E., Drennan, W., and Block, M. (1954). The bone marrow and liver in iron deficiency anaemia. J. Lab. clin. Med. 43, 427.
- Beutler, E., Robson, M.J., and Bittenwieser, E.A. (1958). A comparison of the plasma iron, iron-binding capacity, sternal marrow iron and other methods in the clinical evaluation of iron stores. Ann. intern. Med. 48, 60.

- Biorck, G. (1949). On myoglobin and its occurrence in man. *Acta med. scand. Supp. No. 226.*
- Bielig, H.J., and Bayer, E. (1955). Synthetisches Ferritin, ein Eisen (III) - Komplex des Apoferritins. *Naturwissenschaften* 42, 125. Cited by Bothwell, T.H., and Finch, C.A. (1962). *Iron Metabolism.* Little, Brown and Co., Boston. p. 275.
- Bognaird, R.P., and Whipple, G.H. (1932). The iron content of blood free tissues and viscera. Variations due to diet, anemia and hemoglobin injections. *J. exp. Med.* 55, 653.
- Bothwell, T.H., and Bradlow, B.A. (1960). Siderosis in the Bantu. A combined histopathological and chemical study. *Arch. Path.* 70, 279.
- Bothwell, T.H., Callender, S., Mallett, B., and Witts, L.J. (1956). The study of erythropoiesis using tracer quantities of radioactive iron. *Brit. J. Haemat.* 2, 1.
- Bothwell, T.H., and Finch, C.A. (1962). *Iron metabolism.* Little, Brown and Co., Boston. (a) p. 144. (b). p. 320. (c) p. 284. (d) p. 35.
- Bothwell, T.H., Hurtado, A.V., Donohue, D.M., and Finch, C.A. (1957). Erythrokinetics. IV. The plasma iron turnover as a measure of erythropoiesis. *Blood*, 12, 409.
- Bothwell, T.H., and Isaacson, C. (1962). Siderosis in the Bantu. *Brit. med. J.* 1, 522.
- Bothwell, T.H., Jacobs, P., and Kamener, R. (1959). The determination of the unsaturated iron binding capacity of serum using radioactive iron. *S. Afr. J. med. Sci.* 24, 93.

- Bothwell, T.H., and Mallett, B. (1955). The determination of iron in plasma or serum. *Biochem. J.* 59, 599.
- Bothwell, T.H., Pirzio-Biroli, G., and Finch, C.A. (1958). Iron absorption. I. Factors influencing absorption. *J. Lab. clin. Med.* 51, 24.
- Bothwell, T.H., Seftel, H., Jacobs, P., Torrance, J.D., and Baumslag, N. (in press). Iron overload in the Bantu. Studies on the availability of iron in Bantu beer. *Am. J. clin. Nutr.*
- Brendstrup, P. (1953). Serum iron, total iron-binding capacity of serum and serum copper in acute hepatitis. *Acta med. scand.* 146, 107.
- Brown, E.B., Jr., Dubach, R., Smith, D.E., Reynafarje, C., and Moore, C.V. (1957). Studies in iron transportation and metabolism. X. Long-term iron overload in dogs. *J. Lab. clin. Med.* 50, 862.
- Bruckmann, G., and Zondek, S.G. (1939). Iron, copper and manganese in human organs at various ages. *Biochem. J.* 33, 1845.
- Bruckmann, G., and Zondek, S.G. (1940). An improved method for the determination of non-hemin iron. *J. biol. Chem.* 135, 23.
- Bush, J.A., Ashenbrucker, H., Cartwright, G.E., and Wintrobe, M.M. (1956). The anemia of infection. XX. The kinetics of iron metabolism in the anemia associated with chronic infection. *J. clin. Invest.* 35, 89.
- Cappell, D.F. (1930). The late results of intravenous injection of colloidal iron. *J. Path. Bact.* 33, 175.

- Cappell, D.F., Hutchison, H.E., and Jowett, M. (1957).
Transfusional siderosis: the effects of excessive
iron deposits on the tissues. *J. Path. Bact.* 74, 245.
- Cartwright, G.E., and Wintrobe, M.M. (1949). Chemical,
clinical and immunological studies on the products
of human plasma fractionation. XXXIX. The anemia
of infection. Studies on the iron-binding capacity of
serum. *J. clin. Invest.* 28, 86.
- Case Records of the Mass. General Hospital. Case 38512.
(1952). *New Engl. J. Med.* 247, 992.
- Cavaye Hazen, E. (1949). Contribucion al estudio mineral de
la medula osea humana. *R. esp. Fisiol.* 5, 199.
- Charlton, R.W., Jacobs, P., Torrance, J.D., and
Bothwell, T.H. (1963). The role of ferritin in iron
absorption. *Lancet* 2, 762.
- Cleton, F.J., and Finch, C.A. (1961). Cited in Bothwell, T.H.,
and Finch, C.A. (1962). *Iron metabolism*. Little,
Brown and Co., Boston, p. 391.
- Conrad, M.E., and Crosby, W.H. (1963). Intestinal mechanisms
controlling iron absorption. *Blood* 22, 406.
- Copp, D.H., and Greenberg, D.M. (1946). A tracer study of
iron metabolism with radioactive iron. I. Methods:
absorption and excretion of iron. *J. biol. Chem.*
164, 377.
- Crawley, J. (1952). Iron absorption tests in anaemia: the use
of intravenous iron preparations. *Edinb. med. J.* 59,
478.
- Crosby, W.H. (1963). The control of iron balance by the
intestinal mucosa. *Blood* 22, 441.

- Davidson, W.M., and Jennison, R.F. (1952). The relationship between iron storage and anaemia. *J. clin. Path.* 5, 281.
- Donohue, D.M., Gabrio, B.W., and Finch, C.A. (1958). Quantitative measurement of hemopoietic cells of the marrow. *J. clin. Invest.* 37, 1564.
- Douglas, A.S., and Dacie, J.V. (1953). The incidence and significance of iron-containing granules in human erythrocytes and their precursors. *J. clin. Path.* 6, 307.
- Drabkin, D.L. (1951). Metabolism of the hemin chromoproteins. *Physiol. Rev.* 31, 345.
- Dry, D.S. (1945). Improved methods for the demonstration of mitochondria, glycogen, fat and iron in animal cells. *S. Afr. J. Sci.* 41, 298.
- Dubach, R., Callender, S.T., and Moore, C.V. (1948). Studies in iron transportation and metabolism. VI. Absorption of radioactive iron in patients with fever and with anemias of varied etiology. *Blood* 3, 526.
- Dubach, R., Moore, C.V., and Callender, S. (1955). Studies in iron transportation and metabolism. IX. The excretion of iron as measured by the isotope technique. *J. Lab. clin. Med.* 45, 599.
- Dubach, R., Moore, C.V., and Minnich, V. (1946). Studies in iron transportation and metabolism. V. Utilization of intravenously injected radioactive iron for hemoglobin synthesis, and an evaluation of the radioactive iron method for studying iron absorption. *J. Lab. clin. Med.* 31, 1201.

- Ellis, J.T., Schulman, I., and Smith, C.H. (1954).
Generalized siderosis with fibrosis of liver and
pancreas in Cooley's (Mediterranean) anemia.
Amer. J. Path. 30, 287.
- Elmlinger, P.J., Huff, R.L., Tobias, C.A. and Lawrence,
J.A. (1953). Iron turnover abnormalities in patients
having anemia: serial blood and in vivo tissue
studies with Fe⁵⁹. Acta haemat. 9, 73.
- Erlandson, M.E., Schulman, I., Stern, G., and Smith, C.H.
(1958). Studies of congenital hemolytic syndromes.
I. Rates of destruction and production of erythrocytes
in thalassemia. Pediatrics 22, 910.
- Fahey, J.L., Rath, C.E., Princiotto, J.V., Brick, I.B., and
Rubin, M. (1961). Evaluation of trisodium calcium
diethylenetriaminepentaacetate in iron storage
disease. J. Lab. clin. Med. 57, 436.
- Fiala, S., and Burk, D. (1949). On the mode of iron binding
by siderophilin, conalbumin, hydroxylamine,
aspergillic acid, and other hydroxamic acids.
Arch. Biochem. 20, 172.
- Finch, C.A. (1959). Body iron exchange in man. J. clin.
Invest. 38, 392.
- Finch, C.A., Gibson, J.G., Peacock, W.C., and Fluharty,
R.G. (1949). Iron metabolism. Utilization of
intravenous radioactive iron. Blood 4, 905.
- Finch, C.A., Hegsted, M., Kinney, T.D., Thomas, E.D.,
Rath, C.E., Haskins, D., Finch, S., and
Fluharty, R.G. (1950)(a). Iron metabolism. The
pathophysiology of iron storage. Blood 5, 983.

- Finch, S., Haskins, D., and Finch, C.A. (1950) (b). Iron metabolism. Hematopoiesis following phlebotomy. Iron as a limiting factor. J. clin. Invest. 29, 1078.
- Finch, S.C., and Finch, C.A. (1955). Idiopathic hemochromatosis, an iron storage disease. Medicine, 34, 381.
- Freireich, E.J., Miller, A., Emerson, C.P., and Ross, J.F. (1957). The effect of inflammation on the utilization of erythrocyte and transferrin bound radioiron for red cell production. Blood 12, 972.
- Gabrio, B.W., Shoden, A., and Finch, C.A. (1953). A quantitative fractionation of tissue ferritin and hemosiderin. J. biol. Chem. 204, 815.
- Gale, E., Torrance, J., and Bothwell, T. (1963). The quantitative estimation of total iron stores in human bone marrow. J. clin. Invest. 42, 1076.
- Gillman, J., Mandelstam, J., and Gillman, T. (1945). A comparison of chemical and histological estimations of the iron and copper content of the livers of Africans in relation to the pathogenesis of cytosiderosis and cirrhosis (haemochromatosis). S. Afr. J. med. Sci. 10, 109.
- Gillman, T., Hathorn, M., and Lamont, N.M. (1958). Liver disease in Durban Africans. Histopathological findings in biopsies. S. Afr. J. med. Sci. 23, 187.
- Goldberg, A., Lockhead, A.C., and Dagg, J.H. (1963). Histamine fast achlorhydria and iron absorption. Lancet 1, 848.
- Granick, S. (1958). Iron metabolism in animals and plants. In Trace Elements. Academic Press, New York. p. 367.

- Green, S., and Mazur, A. (1957). Relation of uric acid metabolism to release of iron from hepatic ferritin. *J. biol. Chem.* 227, 653.
- Greenberg, G.R., and Wintrobe, M.M. (1946). A labile iron pool. *J. biol. Chem.* 165, 397.
- Gubler, C.J., Cartwright, G.E., and Wintrobe, M.M. (1950). The anemia of infection. X. The effect of infection on the absorption and storage of iron by the rat. *J. biol. Chem.* 184, 563.
- Hahn, P.F. (1937). The metabolism of iron. *Medicine* 16, 249.
- Hahn, P.F., and Whipple, G.H. (1936). II. Iron metabolism. Its absorption, storage and utilization in experimental anemia. *Amer. J. med. Sci.* 191, 24.
- Hallgren, B. (1954). Haemoglobin formation and storage iron in protein deficiency. *Acta Soc. med. upsal.* 59, 79.
- Hamilton, L.D., Gubler, C.J., Cartwright, G.E., and Wintrobe, M.M. (1950). Diurnal variation in the plasma iron level of man. *Proc. Soc. exp. Biol., N.Y.* 75, 65.
- Hampton, J.K., Jr. (1954). Uptake of radioiron in tissue storage compounds in normal and hemosiderotic mice and its utilization for erythropoiesis. *Amer. J. Physiol.* 176, 20.
- Haskins, D., Stevens, A.R., Finch, S., and Finch, C.A. (1952). Iron Metabolism. Iron stores in man as measured by phlebotomy. *J. clin. Invest.* 31, 543.
- Hathorn, M., Gillman, T., Canham, P.A.S., and Lamont, N.M. (1960). Plasma iron and iron-binding capacity in African males with siderosis. *Clin. Sci.* 19, 35.

- Hawkins, C.F. (1955). Value of serum iron levels in assessing effect of haematinics in the macrocytic anaemias. *Brit. med. J.* 1, 383.
- Higginson, J., Gerritsen, T., and Walker, A.R.P. (1953). Siderosis in the Bantu of Southern Africa. *Amer. J. Path.* 29, 779.
- Higginson, J., Keeley, K.J., Andersson, M., and Walker, A.R.P. (1957). Serum iron levels in siderosis due to habitually excessive iron intake. *J. clin. Invest.* 36, 1723.
- Hosain, F., Marsaglia, G., Noyes, W., and Finch, C.A. (1962). The nature of internal iron exchange in man. *Trans. Ass. Amer. Physns.* 75, 59.
- Huff, R.L., Elmlinger, P.J., Garcia, J.F., Oda, J.M., Cockrell, M.C., and Lawrence, J.H. (1951). Ferrokinetics in normal persons and in patients having various erythropoietic disorders. *J. clin. Invest.* 30, 1512.
- Huff, R.L., Hennessy, T.G., Austin, R.E., Garcia, J.F., Roberts, B.M., and Lawrence, J.H. (1950). Plasma and red cell iron turnover in normal subjects and in patients having various hematopoietic disorders. *J. clin. Invest.* 29, 1041.
- Hutchison, H.E. (1953). The significance of stainable iron in sternal marrow sections. *Blood* 8, 236.
- Hynes, M. (1949). The iron reserve of a normal man. *J. clin. Path.* 2, 99.
- International Commission on Radiological Protection (1955). *Brit. J. Radiol. N.S. Supp. No. 6.* p.49.

- International Commission on Radiological Protection and on Radiological Units (1950). Cited by Levi, H. (1959). In Documenta Geigy Scientific tables. J.R. Geigy, Basle, p. 101.
- Jacobs, P., Bothwell, T.H., and Charlton, R.W. (1963). In Press.
- Jandl, J.H., and Katz, J.H. (1963). The plasma-to-cell cycle of transferrin. J. clin. Invest. 42, 314.
- Jasinski, B. (1949). Die Bedeutung der Eisenresorptionsversuche für die Diagnose und Differentialdiagnose der Eisenmangelanämien, insbesondere für die Erkennung der Eisenmangelzustände ohne Anämie. Schweiz. med. Wschr. 79, 291.
- Jasinski, B. (1950). Resorptionstypen nach peroraler Eisenbelastung mit Ferronikum. Schweiz. med. Wschr. 80, 59.
- Johnston, F.A. (1947). Serum iron levels in adolescent girls. Amer. J. Dis. Child. 74, 716.
- Kaldor, I. (1953). Studies on intermediary iron metabolism. II. Variations of the serum iron value in blood donors and control subjects. Austral. J. exp. Biol. med. Sci. 31, 49.
- Kaldor, I. (1954). Studies on intermediary iron metabolism. V. The measurement of non-haemoglobin tissue iron. Austral. J. exp. Biol. med. Sci. 32, 795.
- Kaldor, I. (1958). Studies on intermediary iron metabolism. XII. Measurement of the iron derived from water soluble and water insoluble non-haem compounds (ferritin and haemosiderin iron) in liver and spleen. Austral. J. exp. Biol. 36, 173.

- Kaplan, E., Zuelzer, W.W., and Mouriquand, C. (1954). Sideroblasts. A study of stainable nonhemoglobin iron in marrow normoblasts. *Blood* 9, 203.
- Keiderling, W. (1958). Über die Ferrokinetik beim Infekt. *Schweiz. med. Wschr.* 88, 965. Cited in Bothwell, T.H., and Finch, C.A. (1962). *Iron metabolism*. Little, Brown and Co., Boston. p. 210.
- Kerr, L.M.H. (1957). A method for the determination of non-haem iron in bone marrow. *Biochem. J.* 67, 627.
- Kleckner, M.S., Baggenstoss, A.H., and Weir, J.F. (1955). Iron-storage diseases. *Amer. J. clin. Path.* 25, 915.
- Kooyman, J.C. (1949). Investigations on serum- and tissue-iron. *Acta med. scand.* 134, 205.
- Korman, S. (1960). The differentiation of anemia in man with an iron chelate. In *Metal binding in medicine*. (Seven, M.J., and Johnson, L.A., eds.) J.B. Lippincott Co., Philadelphia. p. 230.
- Labbe, R.F., and Nishida, G. (1957). A new method of hemin isolation. *Biochim. biophys. Acta* 26, 437.
- Lahey, M.E., Behar, M., Viteri, F., and Scrimshaw, N.S. (1958). Values for copper, iron and iron-binding capacity in the serum in kwashiorkor. *Pediatrics*, N.Y. 22, 72.
- Lancet. (1962). A new treatment for haemochromatosis? 1, 1172.
- Laufberger, V. (1937). Sur la cristallisation de la ferritine. *Bull. soc. chim. biol.* 19, 1575.

- Laurell, C.B. (1947). Studies on the transportation and metabolism of iron in the body. *Acta physiol. scand.* 14. Supp. 46, 1.
- Laurell, C.B. (1952). Plasma iron and the transport of iron in the organism. *Pharmacol. Rev.* 4, 371.
- Laurell, C.B. (1953). The diurnal variation of the serum iron concentration. *Scand. J. clin. Lab. Invest.* 5, 118.
- Little, A.G., Power, M.H., and Wakefield, E.G. (1945). Absorption and excretion of iron. *Ann. intern. Med.* 23, 627.
- Loeffler, R.K., Rappoport, D.A., and Collins, V.P. (1955). Radioiron citrate as tracer to determine disappearance rate of plasma iron in normal subjects. *Proc. Soc. exp. Biol., N.Y.*, 88, 441.
- Mazur, A., Baez, S., and Shorr, E. (1955). The mechanism of iron release from ferritin as related to its biological properties. *J. biol. Chem.* 213, 147.
- Mazur, A., Carleton, A., and Carlsen, A. (1961). Relation of oxidative metabolism to the incorporation of plasma iron into ferritin in vivo. *J. biol. Chem.* 236, 1109.
- Mazur, A., Green, S., and Carleton, A. (1960). Mechanism of plasma iron incorporation into hepatic ferritin. *J. biol. Chem.* 235, 595.
- Mazur, A., Green, S., Saha, A., and Carleton, A. (1958). Mechanism of release of ferritin iron in vivo by xanthine oxidase. *J. clin. Invest.* 37, 1809.

- Mazur, A., and Shorr E. (1948). Hepatorenal factors in circulatory homeostasis. IX. The identification of the hepatic vasodepressor substance, VDM, with ferritin. J. biol. Chem. 176, 771.
- Mazur, A., and Shorr, E. (1950). A quantitative immunochemical study of ferritin and its relation to the hepatic vasodepressor material. J. biol. Chem. 182, 607.
- McCance, R.A., and Widdowson, E.M. (1937). Absorption and excretion of iron. Lancet 2, 680.
- McCance, R.A., Widdowson, E.M., and Shackleton, L.R.B. (1936). The nutritive value of fruits, vegetables and nuts. Medical Research Council Special Report. His Majesty's Stationery Office, London. p. 21.
- McFadzean, A.J.S., and Davis, L.J. (1947). Iron staining erythrocytic inclusions with especial reference to acquired haemolytic anaemia. Glasg. med. J. 28, 237.
- McFarlane, W.D. (1932). The determination of iron in organic substances. I. Micro-colorimetry as ferric thiocyanate. II. Potentiometric titration with titanous sulphate. Biochem. J. 26, 1034.
- McMahon, F.G. (1956). A comparison of effect of Fe-3-specific, versenol, and calcium disodium versenate on urinary iron excretion in patient with hemochromatosis. J. Lab. clin. Med. 48, 589.
- Mendel, G.A. (1961). Studies on iron absorption. I. The relationships between the rate of erythropoiesis, hypoxia and iron absorption. Blood 18, 727.

- Mendel, G.A., Weiler, R.J., and Mangalik, A. (1963).
Studies on iron absorption. II. The absorption of
iron in experimental anemias of diverse etiology.
Blood 22, 450.
- Metz, J., Levin, N.W., and Hart, D. (1962). Effect of
altitude on the body/venous haematocrit ratio.
Nature 194, 483.
- Michaelis, L., Coryell, C.D., and Granick, S. (1943).
Ferritin. III. The magnetic properties of ferritin
and some other colloidal ferric compounds. J. biol.
Chem. 148, 463.
- Millis, J. (1951). The iron losses of healthy women during
consecutive menstrual cycles. Med. J. Aust. 2, 874.
- Moore, C.V. (1961). Iron metabolism and nutrition. Harvey
lectures 55, 67.
- Moore, C.V., Arrowsmith, W.R., Welch, J., and Minnich, V.
(1939). Studies in iron transportation and metabolism.
IV. Observations on the absorption of iron from the
gastro-intestinal tract. J. clin. Invest. 18, 553.
- Moore, C.V., and Dubach, R. (1962). Iron. In Mineral
Metabolism. Vol. 2B. (Comar, C.L., and Bronner,
F., eds.). Academic Press, New York. p. 287.
- Moore, C.V., Dubach, R., Minnich, V., and Roberts, H.K.
(1944). Absorption of ferrous and ferric radioactive
iron by human subjects and by dogs. J. clin. Invest.
23, 755.
- Morgan, E.H., and Walters, M.N.I. (1963). Iron storage in
human disease. J. clin. Path. 16, 101.

- Morse, W.I. (1955). Stainable ferric iron particles in erythroid marrow cells and erythrocytes. *Canad. med. Ass. J.* 72, 418.
- Noyes, W.D., Bothwell, T.H., and Finch, C.A. (1960). The role of the reticulo-endothelial cell in iron metabolism. *Brit. J. Haemat.* 6, 43.
- Noyes, W.D., and Finch, C.A. (1961). In vivo heme synthesis in rabbits. *Fed. Proc.* 20, 67.
- Osgood, E.E. (1954). Number and distribution of human hemic cells. *Blood* 9, 1141.
- Pirzio-Biroli, G., and Finch, C.A. (1960). Iron absorption. III. The influence of iron stores on iron absorption in the normal subject. *J. Lab. clin. Med.* 55, 216.
- Pollycove, M. (1958). Iron kinetics. In *Iron in clinical medicine.* (Wallerstein, R.O., and Mettier, S.R., eds.). Univ. of California Press, Berkeley, California. p. 43.
- Pollycove, M. (1961). Presence of an erythropoietic labile iron pool. *J. clin. Invest.* 40, 1071.
- Pollycove, M., and Mortimer, R. (1961). The quantitative determination of iron kinetics and hemoglobin synthesis in human subjects. *J. clin. Invest.* 40, 753.
- Polson, C.J. (1929). The storage of iron following its oral and subcutaneous administration. *Quart. J. Med.* 23, 77.
- Pribilla, W., Bothwell, T.H., and Finch, C.A. (1958). Iron transport to the fetus in man. In *Iron in clinical medicine.* (Wallerstein, R.O., and Mettier, S.R., eds.). Univ. of California Press. Berkeley, California. p. 58.

- Quastel, M.R., Ross, J.F., and Dieter, B. (1961). Effect of inflammation on iron from transfused non-viable erythrocytes. *Fed. Proc.* 20, 64.
- Ramsay, W.N.M. (1958). In *Advances in clinical chemistry*. Vol. I. (Sobotka, H., and Stewart, C.P., eds.) Academic Press, New York. p. 1.
- Ramsay, W.N.M., and Campbell, E.A. (1954). Iron metabolism in the laying hen. *Biochem. J.* 58, 313.
- Rath, C.E., and Finch, C.A. (1948). Sternal marrow hemosiderin. A method for the determination of available iron stores in man. *J. Lab. clin. Med.* 33, 81.
- Reynafarje, C., Lozano, R., and Valdivieso, J. (1959). The polycythemia of high altitudes: iron metabolism and related aspects. *Blood* 14, 433.
- Roth, O., Jasinski, B., and v. Bidder, H. (1951). Das Gewebeeisen beim Menschen bei normalen und pathologischen Zuständen. *Helv. med. acta* 18, 159.
- Rothen, A. (1944). Ferritin and apoferritin in the ultracentrifuge. Studies on the relationship of ferritin and apoferritin; precision measurements of the rates of sedimentation of apoferritin. *J. biol. Chem.* 152, 679.
- Rumball, J.M., Stone, C.M., Jr., and Hassett, C. (1959). The behavior of serum iron in acute hepatitis. *Gastroenterology* 36, 219.
- Saylor, L., and Finch, C.A. (1953). Determination of iron absorption using two isotopes of iron. *Amer. J. Physiol.* 172, 372.
- Scott, E.M. (1945). The determination of iron in tissues. *Arch. Biochem.* 6, 27.

- Seftel, H.C., Keeley, K.J., Isaacson, C., and Bothwell, T.H. (1961). Siderosis in the Bantu: the clinical incidence of hemochromatosis in diabetic subjects. *J. Lab. clin. Med.* 58, 837.
- Seven, M.J. (1960). Observations on the toxicity of intravenous chelating agents. In *Metal binding in medicine.* (Seven, M.J., and Johnson, L.A., eds.). J.B. Lippincott Co., Philadelphia. p. 95.
- Sharpe, L.M., Peacock, W.C., Cooke, R., and Harris, R.S. (1950). The effect of phytate and other food factors on iron absorption. *J. Nutr.* 41, 433.
- Sheldon, J.H. (1935). *Haemochromatosis.* Oxford Univ. Press, London. (a) p. 206. (b) p. 205.
- Sherman, W.C., Elvehjem, C.A., and Hart, E.B. (1934). Factors influencing the utilization of the iron and copper of egg yolk for hemoglobin formation. *J. biol. Chem.* 107, 289.
- Shoden, A., Gabrio, B.W., and Finch, C.A. (1953). The relationship between ferritin and hemosiderin in rabbits and man. *J. biol. Chem.* 204, 823.
- Shoden, A., and Sturgeon, P. (1960). Hemosiderin. I. A physico-chemical study. *Acta haemat.* 23, 376.
- Shoden, A., and Sturgeon, P. (1961). Formation of hemosiderin and its relation to ferritin. *Nature* 189, 846.
- Smith, R.S. (1962). Iron excretion in thalassaemia major after administration of chelating agents. *Brit. med. J.* 2, 1577.
- Starkenstein, E., and Weden, H. (1928). Weitere Beitrage zur Pharmakologie und Physiologie des Eisens. *Klin. Wschr.* 7, 1220.

- Stevens, A.R., Coleman, D.H., and Finch, C.A. (1953).
Iron metabolism: clinical evaluation of iron stores.
Ann. intern. Med. 38, 199.
- Strachan, A.S. (1929). Haemosiderosis and haemochromatosis in South African natives, with a comment on the etiology of haemochromatosis. M.D. Thesis, Glasgow. Cited in Sheldon, J.H. (1935).
Haemochromatosis. Oxford Univ. Press. London, p. 275.
- Suit, H.D. (1957). A technique for estimating the bone marrow cellularity in vivo using ^{59}Fe . *J. clin. Path.* 10, 267.
- Surgenor, D.M., Koechlin, B.A., and Strong, L.E. (1949).
Chemical, clinical and immunological studies on the products of human plasma fractionation.
XXXVII. The metal-combining globulin of human plasma. *J. clin. Invest.* 28, 73.
- Thederling, F., Jr. (1953). *Über die Steuerung der Eisenresorption*. *Medizinische* 38, 1224.
- Tingey, A.H. (1937). The iron, copper and manganese content of the human brain. *J. Ment. Sc.* 83, 452.
- Tompsett, S.L. (1935). The copper and 'inorganic' iron contents of human tissues. *Biochem. J.* 29, 480.
- Turnbull, A., and Giblett, E.R. (1961). The binding and transport of iron by transferrin variants. *J. Lab. clin. Med.* 57, 450.
- Valquist, B.C. (1941). *Das Serumeisen. Eine padiatrisch-klinische und experimentelle Studie*. *Acta paediat.* 28 (Supp. 5), 1. Quoted by Bothwell, T.H., and Finch, C.A. (1962). *Iron metabolism*. Little, Brown and Co., Boston. p. 153.

- Ventura, S., and Klopper, A. (1951). Iron metabolism in pregnancy. The behaviour of haemoglobin, serum iron, iron-binding capacity of serum proteins, serum copper and free erythrocyte protoporphyrin in normal pregnancy. *J. Obstet. Gynaec., Brit. Emp.* 58, 173.
- Wainwright, J. (1957). Siderosis in the African. *S. Afr. J. lab. clin. Med.* 3, 1.
- Wakeham, G., and Halenz, H.F. (1936). The distribution of iron in certain tissues of normal and anemic albino rats. *J. biol. Chem.* 115, 429.
- Waldenstrom, J. (1946). The incidence of "iron deficiency" (sideropenia) in some rural and urban populations. *Acta med. Scand. Supp.* 170, 252.
- Walker, A.R.P., and Arvidsson, U.B. (1953). Iron 'overload' in the South African Bantu. *Trans. R. Soc. trop. Med. Hyg.* 47, 536.
- Wallerstein, R.O., and Robbins, S.L. (1953). Hemochromatosis after prolonged oral iron therapy in a patient with chronic hemolytic anaemia. *Amer. J. Med.* 14, 256.
- Whitby, L. (1954). The dynamics of haemopoiesis. *Brit. med. J.* 1, 1279.
- Widdowson, E.M., McCance, R.A., and Spray, C.M. (1951). The chemical composition of the human body. *Clin. Sci.* 10, 113.
- Wintrobe, M.M. (1961). *Clinical hematology*. Lea and Febiger, Philadelphia. p. 41.
- Wyatt, J.P. (1956). Patterns of pathological iron storage. II. Exogenous siderosis in chronic anemia due to prolonged oral iron medications. *Arch. Path.* 61, 56.

Yuile, C.L., Bly, C.G., Stewart, W.B., Izzo, A.J.,
Wells, J.C., and Whipple, G.H. (1949). Plasma
and red cell radioiron following intravenous
injection. Turpentine abscesses in normal and
anemic dogs. J. exp. Med. 90, 273.