IRON METABOLISM.

A series of publications on

various aspects of iron metabolism

by

T.H. BOTHWELL, M.D. (Witwatersrand),
M.R.C.P. (London).

Thomas Hamilton

Presented for the degree of Doctor of

Science of University of the Witwatersrand.

October, 1964.

Iron Metabolism

The
QP
535
.F4
BOT

WITWATERSRAND
18-5-1966
MEDICAL LIBRARY
42925

FOR ALIX.

DECLARATION.

My interest in iron metabolism was initially aroused in 1948 by a young patient with idiopathic haemochromatosis who was admitted to Professor Elliott's ward while I was serving my medical internship. With the support of the Council for Scientific and Industrial Research it was possible to carry out radioisotopic studies on this patient and over the next four years a number of other subjects with the same disease were investigated. As the study continued, attention was also directed to the siderosis which is so common in adult Bantu, and to the iron overload which results from the administration of repeated blood transfusions to subjects with refractory anaemias. The results of these various investigations were collected together in 1952 and were presented as a thesis, entitled "Radioiron Absorption Studies in Idiopathic Haemochromatosis, Malnutritional Cytosiderosis and Transfusional Haemosiderosis". This thesis was accepted by the University of the Witwatersrand in partial fulfilment of the requirements for the degree of Doctor of Medicine.

The present series of publications represent the results of subsequent studies carried out between 1952 and 1964. Over the first two years this work was done in the Department of Medicine of the University of the Witwatersrand. I then spent a period of 15 months in Professor L.J. Witts'

- department -

department at the University of Oxford and this was followed by a similar period with Professor C.A. Finch at the University of Washington in Seattle. On returning to South Africa in 1956 I joined the full-time staff of the Department of Medicine of the University of the Witwatersrand and was able, with support from the Council for Scientific and Industrial Research and from the United States Public Health Service, to continue investigations on a number of aspects of iron metabolism. Latterly this research support has been consolidated by the C.S.I.R. which has established an Iron and Red Cell Metabolism Unit in the Department of Medicine under my direction.

From the table of contents it is apparent that the studies I have done have been carried out in collaboration with a large number of workers. These have included senior and junior medical colleagues, biochemists, physicists and technical assistants. This type of collaboration is, I believe, not only highly profitable but also inevitable in this type of work since the studies which have been included, embrace a number of different disciplines. To dissect out in retrospect the contributions of individual collaborators is not possible and the only proof I can supply of my own contribution to these studies is the continuation and development of certain concepts through a number of papers. At the same time I can affirm that I have been intimately

concerned in all the investigations described in the individual papers and that I have made a major contribution to those in which I am the senior author. In this context, my work with Professor C.A. Finch deserves special mention as we not only carried out several studies together but also collaborated on the preparation of a book on iron metabolism. Since we were working in different departments at the time the book was being written it was necessary in the first instance to divide responsibility for the separate chapters. My own responsibilities were in the sections on technical methodology, serum iron, ferrokinetics, iron stores and iron overload. However, once the drafts of all the chapters had been prepared they were modified by each author so that the final product represents a synthesis of our viewpoints in each area. Further details on the nature of our collaboration are provided in a separate joint statement which follows on this opening declaration.

None of the papers has been submitted previously for any degree in any university. However No. 17 includes data presented by Dr. G.E. Gale for a Doctorate in Medicine at the University of the Witwatersrand, and No. 18 contains some material which Dr. S. Kramer included in one of the sections of his M.D. thesis. In addition, Dr. P. Jacobs intends to include data from papers No. 8 and 9 as part of an M.D. thesis. Similarly, data from paper No. 19 form part of

the work which Dr. S.S. Zail will submit for an M.D. thesis.

In conclusion, I should like to mention my indebtedness to the many workers with whom I have been associated over the course of these studies. My continuing interest in the field of iron metabolism is in large measure the result of the stimulation, ideas and advice which I have received from many quarters. In particular I must acknowledge the debts I owe to Professors J. and T. Gillman for their encouragement in the initial phases of this work, to Professor L.J. Witts for the facilities and support he provided in his department at Oxford, and to Dr. H. Seftel, Dr. C. Isaacson and Dr. K. Keeley at Baragwanath hospital for their many contributions. In addition, I have had the pleasure of being associated with a number of colleagues and technical assistants in the Department of Medicine of the University of the Witwatersrand who have been concerned in various aspects of the work. These include Dr. S. Kramer, Dr. P. Jacobs, Dr. G.E. Gale, Dr. S.S. Zail and, in particular, Dr. R.W. Charlton. I must make mention, too, of the warm cooperation of Professor H.B. Stein, and Dr. B.A. Bradlow of the Department of Pathology of the University of the Witwatersrand. Special mention must also be made of the rewarding and fruitful working partnership I enjoyed with Professor C.A. Finch. Finally it is a special pleasure to acknowledge the unfailing support and encouragement I have

received over a period of sixteen years from Professor G.A. Elliott, Head of the Department of Medicine of the University of the Witwatersrand.

LONDON.

August 27th., 1964.

Early in 1955 we collaborated together for the first time and over the next fifteen months embarked on several projects concerned with the absorption of iron and its transport through the body. When we began these studies we had both been working in the field of iron metabolism for several years and the studies we did together represented the natural development of these interests. Although it is impossible in retrospect to disentangle the individual contributions made to each study, the partnership was a full and equal one. Over the next four years we worked separately in our respective departments and although we were both still actively concerned with various aspects of iron metabolism the emphasis and direction of our investigations diverged. In 1959 it was decided that it might be profitable to pool our experience and collaborate on the preparation of an overall review on current concepts of the metabolism of iron in the body in health and disease. Since we were working in different parts of the world it was necessary in the first instance to divide responsibility for the various aspects of the subject but once initial drafts had been prepared it was possible to combine our individual viewpoints into an integrated whole. The end result of this four year project was the book "Iron Metabolism".

Clement A Finch

C.A. FINCH.

T H Bothwell

T.H. BOTHWELL.

TABLE OF CONTENTS.

VOLUME I

Part I - Technical Methods.

1. Bothwell, T.H. and Mallett, B.: The Determination of Iron in Plasma or Serum. Biochem. J. 59: 599-602, 1955.

2. Bothwell, T.H., Jacobs, P., and Kamener, R.: The Determination of the Unsaturated Iron-Binding Capacity of Serum using Radioactive Iron. S. Afr.. J. Med. Sci. 24: 93-98, 1959.

*3. Bothwell, T.H., Roos, N. and Lifschitz, M.L.: A Method for Measuring the Storage Iron Content of Formalinised Tissues. South Afr. J. Med. Sci. In Press.

Part 2 - Iron Absorption.

4. Bothwell, T.H., Mallett, B., Oliver, R. and Smith, M.D.: Inability to assess Absorption of Iron from Plasma Radioiron Curves. Brit. J. Haematol. 1: 352-357, 1955.

5. Bothwell, T.H., Pirzio-Biroli, G. and Finch, C.A.: Iron absorption. Factors influencing Absorption. J. Lab. and Clin. Med. 51: 24-36, 1958.

6. Pirzio-Biroli, G., Bothwell, T.H., and Finch, C.A.: Iron Absorption II. The Absorption of Radioiron administered with a Standard Meal in Man. J. Lab. and Clin. Med. 51: 37-48, 1958.

7. Charlton, R.W., Jacobs, P., Torrance, J.D. and Bothwell, T.H.: The Role of Ferritin in Iron Absorption. Lancet, 2: 3-8, 1963.

8. Jacobs, P., Bothwell, T.H. and Charlton, R.W.: Role of Hydrochloric Acid in Iron Absorption. J. Appl. Physiol. 19: 187-188, 1964.

*9. Charlton, R.W., Jacobs, P., Seftel, H. and Bothwell, T.H. The Effect of Alcohol on Iron Absorption. Brit. Med. J. In Press.

Part 3 - Ferrokinetic Studies.

10. Bothwell, T.H., Ellis, B.C., van W. Doorn-Wittkampf, H., and Abrahams, D.: Radioiron Studies in Hemochromatosis. The Effects of Repeated Phlebotomies. J. Lab. and Clin. Med., 45: 167-178, 1955.

11. Bothwell, T.H. and Mallett, B.: Diurnal Variation in the Turnover of Iron through the Plasma. Clin. Sci., 14: 235-239, 1955.

12. Bothwell, T.H., Callender, S., Mallett, B. and Witts, L.J.: The Study of Erythropoiesis using Tracer Quantities of Radioactive Iron. Brit. J. Haematol. 2: 1-16, 1956.

13. Bothwell, T.H., Hurtado, A.V., Donohue, D.M. and Finch, C.A.: Erythrokinetics, IV. The Plasma Iron Turnover as a Measure of Erythropoiesis. Blood: 12, 409-427, 1957.

14. Bothwell, T.H., Pribilla, W., Mehust, W. and Finch, C.A.: Iron Metabolism in the Pregnant Rabbit. Iron Transport across the Placenta. Amer. J. Physiol. 193: 615-622, 1958.

15. Noyes, W.D., Bothwell, T.H. and Finch, C.A.: The Role of the Reticulo-Endothelial Cell in Iron Metabolism. Brit. J. Haematol. 6: 43-55, 1960.

16. Bothwell, T.H., Kramer, S., Jacobs, P. and Barnes, H.D.: Iron Kinetics during Acute Episodes of South African Porphyria. South Afr. J. Med. Sci. 25: 141-147, 1960.

17. Gale, G.E., Torrance, J., and Bothwell, T.H.: The Quantitative Estimation of Total Iron Stores in Human Bone Marrow. J. Clin. Invest. 42 : 1076-1082, 1963.

18. Bothwell, T.H., Bradlow, B.A., Jacobs, P., Keeley, K., Kramer, S., Seftel, H. and Zail, S.: Iron Metabolism in Scurvy with Special Reference to Erythropoiesis. Brit. J. Haemat. 10 : 50-58, 1964.

19. Zail, S.S., Charlton, R.W., Torrance, J.D. and Bothwell, T.H.: Studies on the Formation of Ferritin in Red Cell Precursors. J. Clin. Invest. 43 : 670-680, 1964.

20. Bothwell, T.H. The Use of Radioisotopes in the Study of Iron Metabolism. Radioisotopes in Tropical Medicine. International Atomic Energy Agency. Vienna, 1962. pp. 87-102.

Part 4 - Iron Overload.

21. Bothwell, T.H., Cohen, I., Abrahams, G.L. and Perold, S.: A Familial Study in Idiopathic Hemochromatosis : Amer. J. Med. 27: 730-738, 1959.

22. Bothwell, T.H., Jacobs, P. and Torrance, J.D.: Studies on the Behaviour of Transferrin in Idiopathic Haemochromatosis. S. Afr. J. Med. Sci. 27: 35-39, 1962.

23. Bothwell, T.H. and Bradlow, B.A.: Siderosis in the Bantu. A Combined Histopathological and Chemical Study. Arch. Path. 70: 279-292, 1960.

24. Seftel, H., Isaacson, C. and Bothwell, T.H.: The Relationship between Siderosis and Diabetes in the Bantu. South Afr. J. Med. Sci. 25: 89-98, 1960.

25. Isaacson, C., Seftel, H.C., Keeley, K.H. and Bothwell, T.H.: Siderosis in the Bantu. The Relationship between Iron Overload and Cirrhosis. J. Lab. and Clin. Med. 58: 845-853, 1961.

26. Seftel, H.C., Keeley, K.J., Isaacson, C. and Bothwell, T.H.: Siderosis in the Bantu. The Clinical Incidence of Hemochromatosis in Diabetic Subjects. J. Lab. and Clin. Med. 58: 837-844, 1961.

27. Bothwell, T.H. and Isaacson, C.: Siderosis in the Bantu. A Comparison of the Incidence in Males and Females. Brit. Med. J. 1: 522-524, 1962.

28. Bothwell, T.H., Seftel, H., Jacobs, P., Torrance, J.D. and Baumslag, N.: Iron Overload in the Bantu. Studies on the Availability of Iron in Bantu Beer. Amer. J. Clin. Nutrit. 14: 47-51, 1964.

*29. Bothwell, T.H., Abrahams, C., Bradlow, B.A. and Charlton, R.W.: Idiopathic and Bantu Hemochromatosis: A Comparative Histological Study. Arch. Path. In Press.

*30. Mayet, F.G.H. and Bothwell, T.H.: Hepatic Iron Stores in Three Different Racial Groups. South Afr. J. Med. Sci. In Press.

31. Bothwell, T.H. Iron Overload in the Bantu. Ciba Symposium on Iron Metabolism. J.A. Churchill Ltd., London. 1964, pp. 362-373.

*32. Bothwell, T.H. Pathophysiological and Clinical Aspects of Iron Overload. Proceedings Xth Congress of the International Society of Haematology. In Press.

* Papers marked with an asterisk have been accepted for publication but are still in press.

VOLUME II.

Bothwell, T.H. and Finch, C.A.: IRON METABOLISM. Little, Brown and Co., Boston, and J.A. Churchill Ltd., London, 1962.
Introduction: (pp. 3-7).
Chapter I: Technical Methods employed in Studies on Iron Metabolism (pp. 8-44).

(i) Histological Methods.
(ii) Chemical Methods.
(iii) Isotopic Methods.

Chapter 2: Some General Applications of Chemical and Isotopic Methods (pp. 46-90).

(i) Measurements of Iron Absorption.
(ii) Measurements of Iron Excretion.
(iii) Ferrokinetic Measurements.

Chapter 3: Body Iron Turnover (pp. 92-137).

(i) Iron Absorption.
(ii) Iron Excretion.

Chapter 4: Plasma Transferrin and its Iron (pp. 138-178).

(i) Iron-binding Protein.
(ii) Plasma Iron.
(iii) Degree of Saturation of Transferrin with Iron.

Chapter 5: Ferrokinetics (pp. 180-219).

(i) Plasma Iron Turnover.
(ii) Utilization of Radioiron for Red Cell Production.
(iii) Measurement of Erythrocyte Life Span.
(iv) In Vivo Measurements.
(v) Clinical Measurement of Plasma Iron Turnover and Red Blood Cell Utilization of Radioiron.
(vi) Significance of Ferrokinetic Data in Clinical Studies.

Chapter 6: Erythrokinetics (pp. 220-267).

(i) The Single Cell.
(ii) Quantitative Aspects of Erythropoiesis.
(iii) Measurement of Red Cell Production and Destruction.
(iv) Diagnosis of Anemia.

Chapter 7: Tissue Iron Stores (pp. 268-298).

(i) Composition of Ferritin and Hemosiderin.
(ii) Distribution and Functional Relationships.
(iii) Possible Pharmacologic Effects of Ferritin.
(iv) Quantitative Aspects of Iron Storage.
(v) Alterations in Body Stores.
(vi) Clinical Evaluation of Iron Stores.

Chapter 8: Iron-Deficiency Anemia (pp. 300-363).

(i) Incidence.
(ii) Iron Requirements.
(iii) Etiology of Iron-Deficiency Anemia.
(iv) Pathogenesis of Iron Deficiency.
(v) Diagnosis of Iron Deficiency.
(vi) Treatment of Iron-Deficiency Anemia.
(vii) Iron Poisoning.

Chapter 9: Pathologic and Clinical Aspects of Iron Overload (pp. 364-434).

(i) Parenchymal Iron Overload.
(ii) Reticuloendothelial Iron Overload.
(iii) Summary of Association between Iron Overload and Secondary Pathologic Changes.
(iv) Focal Iron Overload.

/DH.

INTRODUCTION.

The publications are presented in two parts. The first section represents a series of investigations done during the period 1952 to 1964. The second section is the book 'Iron Metabolism' which provides a comprehensive coverage of current concepts on how the body handles iron in health and disease. As such, it represents a background to the papers in the first section since it is possible to appreciate how each study fits into the total scheme.

VOLUME I.

The work is divided into four parts. This division is, of necessity, somewhat arbitrary as there is a certain degree of overlap between the different parts. In spite of this, the division of the papers into 4 separate areas does provide a means of following the chronological development of certain ideas and conclusions over a period of time.

Technical Methods

The three papers included in this part describe the development and validation of certain techniques required in the investigation of iron metabolism. The serum iron method (paper I) was developed because of the technical difficulties we experienced with other methods in use at the time. Similarly, the isotopic technique for measuring the iron-binding capacity of serum (paper 2) was established as

a considerable amount of laboratory time was being taken up with more laborious and less accurate chemical methods. Latterly, interest has centred on simple ways and means of assessing the incidence of iron deficiency in different population groups. Although this can theoretically be done by estimating the storage iron concentrations in samples of liver obtained at necropsy the development of such an approach has been hindered by the technical difficulties involved in freeing formalinised tissues of haemoglobin iron. For this reason a method was devised (paper 3) whereby this can be achieved. (The method has now been extensively applied and some preliminary results utilising it are given in paper 29).

Iron Absorption

In this section a number of facets of iron absorption are discussed. The first paper (No. 4) describes an attempt to assess radioiron absorption from the levels of radioactivity appearing in the serum after due allowance had been made for the rate of removal of iron from the plasma. In the next paper (No. 5) a series of experiments were carried out to try to define the factors controlling the amount of iron absorbed at any one time. The results of these experiments established the important influence of the rate of erythropoiesis and of the level of iron stores on the absorptive process. In the following paper (No. 6) the principles established in the previous investigation were applied to a large number of subjects suffering from

a variety of haematological disorders. In more recent studies a systematic attempt has been made to define the effects of certain luminal factors and of mucosal cells on the absorptive process. Results obtained in these studies are presented in papers No. 7, 8 and 9.

Ferrokinetic Measurements

With the advent of radioactive isotopes of iron it became possible not only to measure the absorption of the metal but also to trace its passage within the body. In 1950 Huff and his co-workers reported studies on the rate of turnover of plasma and of red cell iron and the technique was subsequently used by a number of laboratories. Paper No. 10 gives an account of our initial experiences with the method in subjects with idiopathic haemochromatosis who were being treated by repeated venesection.

During this study it became apparent that there were a number of technical and theoretical difficulties associated with the interpretation of such data and in subsequent investigations attempts were made to define the significance of these factors. In one investigation (No. 12) tracer data were collected on a large number of subjects with various types of anaemia and the results were compared with other measurements. The discrepancies which were noted indicated the complexity of the factors involved in iron transport and further studies were carried

out with the purpose of studying each of the major variables in more detail. In one investigation (paper No. II) the effect of the diurnal variation in plasma level on plasma iron turnover was studied. In a further extension, the effects of changes in the rate of red cell destruction, of red cell production and of iron stores were examined and, in addition, a number of technical problems associated with the execution of such tests were investigated (paper No. 13). On the basis of these various results it was possible to define the significance of ferrokinetic data in a way that allowed for their clinical application to subjects suffering from a variety of haematological diseases. The results presented in two later studies illustrate the use of this approach in two specific problems. In the one paper (No. 16) a peculiar disorder in iron transport associated with acute episodes of South African porphyria was studied and in the second study (paper No. 18) several types of kinetic measurement were carried out in patients and experimental animals suffering from scurvy.

As a more general interest developed in the application of tracer techniques it seemed possible that useful information might be collected on how other cellular systems handled iron in health and disease. Specific attention was initially paid to the kinetics

of iron transport to the developing foetus (paper No. 14). and to the role of the reticulo-endothelial system in iron metabolism (paper No. 15). More recently an isotopic dilution technique was developed in order to define the capacity of the bone marrow as a storage organ for iron (paper No. 17). In another study both *in vivo* and *in vitro* experiments were carried out to find out the factors influencing the uptake and utilisation of iron by red cell precursors (paper No. 19). In this study special attention was paid to the possible role of ferritin as a precursor in the synthesis of haemoglobin.

The section closes with a short account of the value of radioisotopes in the investigation of iron metabolism (paper No. 20).

Iron Overload

South Africa offers a unique opportunity for studying the effects of iron overload, since it is possible to compare the findings in subjects suffering from idiopathic haemochromatosis with those which occur in the siderosis so commonly present in adult Bantu. In the first paper (No. 21) in this section a genetic study was performed to find out what proportion of the immediate relatives of patients with idiopathic haemochromatosis were affected. Although the results suggested a genetic defect they did nothing to elucidate the nature of this defect. In a subsequent study

(paper No. 22) the iron-carrying transferrin of subjects with the disease was examined to find out whether it differed from normal, but with negative results.

The iron overload which is found in the Bantu has been examined from several angles. In one group of histological and chemical studies the incidence and severity of the problem in the two sexes and in different age groups was systematically examined (papers No. 23 and 27) and the iron status of Bantu subjects was also compared with that of other population groups in South Africa (paper No. 29). In a second series of studies, the pathological sequelae of iron overload in the Bantu, especially in so far as they parallel those in idiopathic haemochromatosis, were defined (papers No. 24, 25, 26 and 30). In another recent investigation a combined chemical and isotopic approach was used to examine the amounts of iron present in the Bantu diet, and to find out the availability for absorption of the iron present in Bantu beer, which is the major source of the metal (paper No. 28).

The two final papers summarise our current concepts on the significance of siderosis in the Bantu (No. 31) and of its relationship to other forms of iron overload (No. 32).

VOLUME II.

During the past 25 years extensive information has accumulated on the various roles which iron fulfils in the body. The book "Iron Metabolism" represents an attempt to consolidate as much of this information as possible into a unified concept of iron metabolism and to relate it to clinical medicine. It is divided into several sections. The opening one is a critical analysis of the techniques which have been used in the study of iron metabolism and of the ways in which they can be applied. Although this section is directed primarily to those working in the field it has a more general relevance since it is felt that some of the widely accepted concepts concerning iron metabolism are based on experimental methods not acceptable by present day standards. The section which follows is divided into several chapters concerned with external and internal iron exchange and with the complexes involved in these processes. It includes a detailed consideration of the absorption and excretion of the metal, its transport through the plasma, its utilisation for haemoglobin production and its storage in various organs of the body. The relationship between the iron and haemoglobin cycles receives special attention and the subsequent discussion includes an overall assessment of quantitative aspects of red cell turnover and of the factors which modify it. In the final section iron deficiency anaemia

and iron overload are described in detail from the point of view of pathogenesis, pathological changes, clinical manifestations and therapy.

Throughout the book an attempt has been made to tie together the various facets of iron metabolism into a coherent whole. This has involved a certain amount of speculation and it is realised that a number of the views and interpretations expressed are controversial ones. It is hoped that such speculations will not only serve to underline the areas in which current knowledge is lacking but will act also as a stimulus to further work in the field of iron metabolism.

TECHNICAL METHODS

[From THE BIOCHEMICAL JOURNAL, Vol. 59, No. 4, pp. 599–602, 1955]
[*All rights reserved*] PRINTED IN GREAT BRITAIN

The Determination of Iron in Plasma or Serum

By T. H. BOTHWELL* and BARBARA MALLETT
Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford

(*Received* 10 *August* 1954)

Iron circulates in blood plasma as a complex with β_1-globulin (Schade & Caroline, 1946) which is neither dialysable nor ultrafiltrable (Barkan, 1927). Most methods for estimating this iron involve three stages, (i) incubation of the plasma or serum with dilute hydrochloric acid for periods of 15 min. to several hours to separate the iron from its protein, (ii) precipitation of plasma proteins with trichloroacetic acid, (iii) formation of a coloured complex with reagents such as *o*-phenanthroline or 2:2′-dipyridyl (Hemmeler, 1951). Recently, Ramsay (1953) has described a method in which the plasma proteins are coagulated in the presence of a buffered solution containing a reducing agent and 2:2′-dipyridyl. In this way a coloured complex is formed under conditions which render reversibility of the

* Nuffield Travelling Fellow in Medicine.

reaction unlikely. Ramsay's results, however, are 30–60 μg./100 ml. higher than those previously published, and he has suggested that the lower values found by other workers may be explained by a subsequent chemical or physical reaction between a proportion of the dissolved iron and the precipitated proteins.

In view of the discrepancies between Ramsay's findings and those of others, it seemed important to find out whether all the iron is liberated from its protein by hydrochloric acid, and also whether any of it is subsequently lost by entrainment with the protein precipitated by trichloroacetic acid. These two points have been investigated using radioactive iron. The results of these experiments made it possible to design a method for plasma-iron estimation which incorporates the basic features of other methods but which is more simple and rapid in use.

EXPERIMENTAL

Determination of iron in blood plasma

Glassware. All-glass syringes with stainless-steel needles were used for taking blood. The syringes and other glassware were thoroughly washed and then immersed in chromic acid for several hours. They were then rinsed with hot and cold tap water, distilled water and finally with glass-distilled water.

Reagents. The following reagents were used (all were AR grade and were made up in glass-distilled water): 2N-HCl, 20% (w/v) trichloroacetic acid (TCA), thioglycollic acid, 0·4% (w/v) 2:2′-dipyridyl (0·4 g. dissolved in 5 ml. glacial acetic acid and made up to 100 ml. with glass-distilled water), saturated soln. of sodium acetate, standard soln. of iron (100 mg. of pure iron wire dissolved in dilute H_2SO_4 and oxidized with $KMnO_4$; the volume made up to 100 ml. with glass-distilled water and diluted 1 in 100 to give a soln. for use containing 10 μg./ml.).

Procedure. 2 ml. 2N-HCl are added to 4 ml. plasma or serum and the mixture is stirred with a glass rod. TCA (2 ml.) is then added and the final mixture stirred vigorously for at least 45 sec. After centrifuging for 20 min. at 2500 rev./min. the supernatant fluid is decanted. The supernatant fluid (5 ml.) is then added to a tube containing 2 drops of thioglycollic acid, 0·5 ml. 0·4% 2:2′-dipyridyl and 2·5 ml. sat. soln. of sodium acetate and the mixture shaken thoroughly. The colour intensity is measured in a Hilger New Biochem. absorptiometer with a 520 mμ. filter. This is calibrated by means of solutions of iron containing from 2 to 10 μg. Fe together with 1·25 ml. of 2N-HCl and 1·25 ml. 20% TCA; the volume is made up to 5 ml. and the mixture treated as above. A reagent blank is also measured.

Comment on procedure

2:2′-Dipyridyl, first used in the estimation of iron in biological material by Hill (1930), forms a stable coloured complex with iron within the pH range 3–9 (Moss & Mellon, 1942). In the method described above, thioglycollic acid, which rapidly reduces iron from ferric to the ferrous state at pH between 4·5 and 4·9 (Laurell, 1952), was used as the reducing agent and the solution buffered with a saturated soln. of sodium acetate. Experiments were carried out to find out the importance of the various factors in the colour reaction and the accuracy of the method.

Accuracy of the method (Table 1). The error in measurement was relatively greater with low concentrations of iron but was small enough to allow for moderate accuracy over a wide range of iron concentrations.

Clarity of the centrifuged solution. Hemmeler (1951) has reported difficulty in obtaining a clear supernatant after centrifuging. However, in the present method it was found that vigorous stirring of the mixture before centrifuging yields a clear supernatant that gave readings in the colorimeter no higher than a blank made up with the same reagents and glass-distilled water.

Importance of pH. The mean pH of the final soln. was 4·7 (range 4·54–4·77) in forty-eight estimations carried out with a glass electrode. By varying the experimental conditions it was also shown that the final pH was not critical enough to necessitate the titration of each sample as is described in some methods (Table 2).

Efficiency of thioglycollic acid as a reducing agent. The present method was compared with procedures in which the colour was developed by different techniques. The supernatant fluid was treated in one of two ways. In the first, 0·1 ml. 0·1M-Na_2SO_3 was used as a reducing agent and the

Table 1. *Accuracy of 2:2′-dipyridyl in determining iron in standard solutions*

Ten estimations for each concentration.

Fe in standard solutions (μg./100 ml.)	Coefficient of variation
20	7·0
80	2·4
160	0·9

Table 2. *Effect of variations in* pH *on the recovery of iron from standard solutions*

Solutions contained 20–400μg. Fe/100 ml. In this and subsequent tables results are given as mean ±S.D.

Range of pH	No. of estimations	Fe recovered (%)
3·7–4·5	18	98·9±2·07
4·5–5·2	20	100·6±1·50

Table 3. *Efficiency of thioglycollic acid as a reducing agent*

Each estimation was carried out in duplicate on two samples, of which one was treated by the present method and the other either with Na_2SO_3 or with HNO_3 and KCNS (for details see text). The iron content of the samples varied from 30 to 224μg./100 ml. (mean 106μg./100 ml.).

Alternative treatment of iron in supernatant	No. of duplicate estimations	Mean difference between each pair of estimations (μg. Fe/100 ml.)
Reduced with 0·1M-Na_2SO_3	10	3·4±2·4
Oxidized by boiling with HNO_3 and converted into thiocyanate	10	5·6±3·2

solution buffered to pH 4·7 with saturated soln. of sodium acetate. In the second, the iron was oxidized by boiling for 30 min. with 0·1 ml. HNO_3. By the addition of KCNS the iron was converted into thiocyanate and extracted with amyl alcohol. In both sets of experiments there was good agreement with results obtained by the present method (Table 3).

Time taken for development of final colour. In thirty samples and the reagent blank the intensity of colour was read immediately after preparation, then the samples were covered with cellophan, incubated at 37° for 1 hr., and the reading repeated. The mean difference in plasma iron concentration after re-reading was only 0·9 μg./100 ml. (S.D. $\pm$1·2 μg./100 ml.).

Reproducibility and effect of anticoagulants. The method gave reproducible results unaffected by the use of anticoagulants (Table 4).

Recovery of added iron. It was shown that iron added to plasma either as a salt or bound to β_1-globulin (supplied by Cutter Laboratories, U.S.A.) could be recovered quantitatively (Table 5).

Effect of haemolysis. Red cells of known haemoglobin content were washed 3 times with 0·9 % NaCl and were then haemolysed with water. Their approximate iron content was calculated on the assumption that iron forms 0·34 % of the haemoglobin molecule by weight (Bernhart & Skeggs, 1943). Small volumes of haemolysed solution with iron content varying from approximately 60 to 600 μg./100 ml. were then added to twenty plasma samples and the mixtures were analysed for iron content. The mean difference in plasma iron concentration after the addition of haemolysed erythrocytes was only 3·5 μg./100 ml. (S.D. $\pm$4·5 μg./100 ml.).

Comparison with other methods. There was good agreement with the results obtained by two other methods for plasma iron determination, namely that of Ramsay (1954) and that of Barkan & Walker (1940), which is similar in principle to the present method but which has longer time intervals and involves the use of hydrazine sulphate as reducing agent and *o*-phenanthroline for the development of the colour (Table 6).

Table 4. *Reproducibility of results obtained on heparinized and oxalated plasma and serum*

The iron concentration of the samples varied from 18 to 234 μg./100 ml. (mean 106 μg./100 ml.).

	No. of estimations	Mean difference between each pair of estimations (μg. Fe/100 ml.)
Duplicate samples of heparinized plasma	30	2·6 $\pm$ 3·8
Comparison between heparinized plasma and oxalated plasma	15	3·2 $\pm$ 3·9
Comparison between heparinized plasma and serum	15	2·0 $\pm$ 1·9

Experiments with ^{59}Fe

Experiments were carried out to determine whether all the iron was detached from the metal-binding protein by precipitating the proteins with acids.

Administration of ^{59}Fe. $^{59}FeCl_3$ of high specific activity (1 μc/0·5 μg. Fe), obtained from an American source through A.E.R.E., Harwell, was given in tracer doses of 10–20 μc to a number of subjects in three ways:

(*a*) Injected intravenously after having been added *in vitro* to a solution of β_1-globulin as described by Huff *et al.* (1951).

(*b*) Injected intravenously after preliminary incubation with the patient's plasma as described by Huff *et al.* (1950).

(*c*) Fed by mouth together with approximately 5 mg. of unlabelled ferrous ascorbate.

By taking blood samples at periods of 10–60 min. after the administration of ^{59}Fe it was possible to obtain plasma labelled with ^{59}Fe.

Separation of iron from protein. The radioactivity in plasma was compared with that present in the supernatant fluid from samples which had been treated in various ways with HCl and TCA (Table 7). The counting was done in a scintillation counter (type 1186 on loan from A.E.R.E., Harwell) by the technique of Badenoch & Callender (1954). With the quantities of ^{59}Fe used in these studies the maximum counting error was $\pm$5 %.

RESULTS AND DISCUSSION

Table 7 shows that complete recoveries were obtained in 101 estimations. There was no apparent difference between the results obtained after allowing the plasma to stand for varying periods with HCl and trichloroacetic acid and those after

Table 6. *Comparison of results obtained by present method with those of two published methods*

The iron concentration of the plasma samples varied between 30 and 224 μg./100 ml. (mean 103 μg./100 ml.). Ten duplicate estimations were carried out for each comparison.

Method	Mean difference between each pair of estimations (μg. Fe/100 ml.)
Present method compared with Barkan & Walker's (1940) method	3·0 $\pm$ 2·6
Present method compared with Ramsay's (1954) modified technique	4·6 $\pm$ 2·0

Table 5. *Recovery of iron added to plasma samples*

Form in which iron was added to 3 ml. plasma samples	Quantity of added iron (μg./100 ml.)	No. of estimations	Recovery (%)
Standard ferric sulphate solutions	2–6	25	98·6 $\pm$ 4·2
Ferric sulphate solution buffered to pH 6·4 with sodium citrate and bound to β_1-globulin	2	12	99·0 $\pm$ 4·2

Table 7. *Recoveries of* ^{59}Fe *obtained from plasma treated with* 2 N-HCl *and* 20 % *trichloroacetic acid*

(*A*) Samples were mixed with HCl and allowed to stand for 10–60 min. Trichloroacetic acid was then added and the samples were mixed thoroughly and allowed to stand for a further 5–60 min. (*B*) Samples were treated in the same way as in (*A*) except that they were not left to stand after the addition of HCl and trichloroacetic acid.

Method of administration of ^{59}Fe	Treatment of plasma samples	No. of estimations	% recoveries of radioiron from supernatant fluid after centrifuging
^{59}Fe bound to β_1-globulin and injected intravenously (six subjects)	*A*	28	101·2±3·7
	B	16	100·8±5·2
^{59}Fe incubated with plasma and injected intravenously (three subjects)	*A*	16	97·8±6·2
	B	15	101·0±7·1
^{59}Fe fed by mouth (three subjects)	*A*	7	107·1±7·6
	B	19	104·7±6·6

immediate mixing, nor did the initial manner of binding the iron to the β_1-globulin affect the results. However, in four estimations in which the plasma proteins were precipitated with trichloroacetic acid without having been treated with HCl, recoveries of only about 65 % were obtained.

By giving tracer doses of ^{59}Fe of high specific activity parenterally or orally, it was possible to obtain plasma samples in which the transport iron attached to β_1-globulin was labelled with radioactivity. It was shown that this iron was rapidly and completely separated from its protein attachment after thorough mixing with dilute HCl and trichloroacetic acid. As there is no reason to believe that radioactive iron behaves differently from ordinary iron it seems valid to assume that the many methods which use such a technique for the separation of iron from protein should give reliable results.

The present technique for iron estimation in plasma or serum, although it involves no new principles, has the advantage of simplicity and speed. The results of duplicate analyses have shown good agreement, and the occurrence of haemolysis has not affected the results significantly. A comparison of the results obtained by this method with those obtained by Barkan & Walker (1940) showed satisfactory agreement. In addition, it was also found that the present method gave results no lower than those obtained with Ramsay's modified technique (1954).

SUMMARY

1. A simple and rapid modification of previous techniques for the estimation of iron in plasma is described.

2. Recovery experiments with ^{59}Fe showed that iron is effectively separated from β_1-globulin in plasma by treatment with dilute hydrochloric acid and trichloroacetic acid.

Thanks are due to Mr J. R. P. O'Brien, Dr Sheila T. Callender and Dr G. H. Spray for helpful criticisms and to Professor L. J. Witts for advice and encouragement.

REFERENCES

Badenoch, J. & Callender, S. T. (1954). *Blood*, **9**, 123.
Barkan, G. (1927). *Hoppe-Seyl. Z.* **171**, 194.
Barkan, G. & Walker, B. (1940). *J. biol. Chem.* **135**, 37.
Bernhart, F. W. & Skeggs, L. (1943). *J. biol. Chem.* **147**, 19.
Hemmeler, G. (1951). *Métabolisme du Fer.* Paris: Masson.
Hill, R. (1930). *Proc. Roy. Soc.* B, **107**, 205.
Huff, R. L., Elmlinger, P. J., Garcia, J. F., Oda, J. M., Cockrell, M. C. & Lawrence, J. H. (1951). *J. clin. Invest.* **30**, 1512.
Huff, R. L., Hennessy, T. G., Austin, R. E., Garcia, J. F., Roberts, B. M. & Lawrence, J. H. (1950). *J. clin. Invest.* **29**, 1041.
Laurell, C.-B. (1952). *Pharm. Rev., Baltimore*, **4**, 371.
Moss, M. L. & Mellon, M. G. (1942). *Industr. Engng Chem. (Anal.)*, **14**, 862.
Ramsay, W. N. M. (1953). *Biochem. J.* **53**, 227.
Ramsay, W. N. M. (1954). *Biochem. J.* **57**, xviii.
Schade, A. L. & Caroline, L. (1946). *Science*, **104**, 340.

S. Afr. J. med. Sci. (1959), **24**, 93-98.

THE DETERMINATION OF THE UNSATURATED IRON-BINDING CAPACITY OF SERUM USING RADIOACTIVE IRON

by

T. H. BOTHWELL, P. JACOBS and R. KAMENER

Department of Medicine, University of the Witwatersrand, Johannesburg

[RECEIVED 20th JULY, 1959]

Normally the plasma iron-binding protein is only about one-third saturated with iron. The level of plasma iron is therefore no reflection of the total amount of iron-binding protein available for combination with iron in the plasma, i.e. the total iron-binding capacity (T.I.B.C.). The T.I.B.C. can be measured in a variety of ways. In essence, the techniques are of three different types: (1) The first one is an in vivo method in which the plasma is saturated by injecting an iron salt intravenously [Gitlow and Beyers, 1952]. An iron estimation is then carried out on a sample of saturated serum. (2) In the second, an iron salt is added to serum in vitro and the red colour developed by the iron-protein complex is measured. This can either be done by adding small increments of iron until no further change in optical density occurs [Rath and Finch, 1949] or the iron can be added in a single step and the total change in optical density measured [Ventura, 1952]. When the second method is used it must be calibrated against a curve obtained from several samples of serum measured by the first method. With these techniques the unsaturated iron-binding capacity (U.I.B.C.) is obtained and the T.I.B.C. can then be calculated by adding this figure to the plasma iron level. (3) The last approach is one in which an excess of iron is added to the serum in vitro and the unbound iron is then separated in various ways from that taken up by the iron-binding protein. It is then possible to measure (*a*) the amount of unbound iron present in the serum by complexing it with some colour reagent, or (*b*) the excess iron can be removed and an iron estimation is then carried out on the saturated serum. Both of these latter methods have been extensively applied and both appear to give reasonably accurate results.

The method 3 (*a*) was initially introduced by Holmberg and Laurell [1945]. In their original technique a known excess of iron was added to serum. The unbound iron was then measured colorimetrically as a phenanthroline complex. In this way it was possible to calculate the amount taken up by the iron-binding protein and hence the U.I.B.C. The actual technique involved several steps and has subsequently been simplified in two recent modifications [Schade *et al.*, 1954; Ressler and Zak, 1958]. The one described by Ressler and Zak is extremely practical and simple since only one tube is used. In this method a known excess of ferrous ions is added to a buffered sample of serum and the absorbance is read. Buffered iron colour reagent (2 : 2′-dipyridyl) is then added and the colour produced by the complex formed with the free ferrous ions is measured and compared with a standard. The use of a single serum tube for both blank and sample has the advantage of requiring less serum and of eliminating aberrant absorbance effects. Since the proteins are not precipitated in this type of method it is essential that the serum be fresh and clear.

12-03)

The alternative approach 3 (*b*) is exemplified by the method described by Laurell [1947] in which an amount of iron in excess of the T.I.B.C. is also added to serum. The unbound iron is complexed with 1,10-phenanthroline and the serum proteins are subsequently precipitated with hydrochloric and trichloracetic acids. Under these conditions the iron-phenanthroline complex is entrained with the proteins while the protein-bound iron is released into the supernatant. An iron estimation on the supernatant therefore gives a measure of T.I.B.C. In new modifications of this approach the unbound iron has been removed without precipitating the proteins. In one method iron is added to serum as ferric chloride and the excess ferric ions are then adsorbed onto magnesium carbonate. An iron estimation is then carried out on the saturated serum [Ramsay, 1957]. In another ingenious technique the iron is added as ferric ammonium citrate and the excess citrate is then removed by a strongly basic anion exchange resin (IRA-410) [Peters *et al.*, 1956]. Under these conditions the free iron remains attached to the citrate and is therefore also removed by the resin.

This last method has proved an extremely useful one. However, it does involve a final colorimetric estimation to obtain the amount of iron present in the saturated serum. This step can be eliminated by using radioactive iron. Although the originators of the method did in fact employ ^{59}Fe to assess the validity of the technique, the method, as described, is not specifically set up to be carried out in this way. We therefore decided to investigate the practicality of such an approach. This modified method has proved a rapid and simple one which can be easily carried out in any laboratory equipped to do radioactive measurements.

METHOD

Reagents

(*a*) *Ferric Ammonium Citrate.* This is prepared from ferric chloride. About 3 mg. is dissolved in 10 ml. water. To this is added a small amount of high specific activity ^{59}Fe chloride. (Enough is added to ensure that about 15,000 counts/min. are equivalent to about 5 μg. iron.) The ferric chloride is then precipitated with ammonium hydroxide, centrifuged and the supernatant is discarded. The precipitate is dissolved with a few crystals of citric acid and adjusted to pH 6·5-7 with ammonium hydroxide using bromthymol blue as indicator. The solution is then diluted to a volume of 20 ml. so that about 5 μg. iron is contained in 0·2 ml. The exact iron content of the solution is then obtained by measuring colorimetrically against suitable iron standards. The radioactive standard solution is stable for at least a month when kept refrigerated.

(*b*) *Tris* (2-*Amino*-2-(*Hydroxy-Methyl*)-1 : 3-*Propanediol*) *Buffer.* This is made up in a molarity of 0·08 (0·97 g./100 ml.) and adjusted to pH 8·4 with 2N hydrochloric acid. This solution must be iron-free and each batch should be tested colorimetrically for iron prior to use.

(*c*) *Amberlite IRA* - 401 *resin* (*A.R. grade*).* The resin is suspended and mixed in 3 N HCl for 24 to 36 hours to free it of iron and to saturate it with chloride. It is then repeatedly washed with glass distilled water and is adjusted to a pH of approximately 7·5 (phenol-red indicator) by suspension in the Tris buffer and, if necessary, by adding small amounts of dilute sodium hydroxide. It is then dried at 90° C in an oven. (Although this treatment did not render the resin completely iron-free as judged by results of iron analyses on dry-ashed material, there was no evidence that this iron was made available under the conditions of the test since it was shown

*Manufactured by British Drug Houses Ltd.

that an iron-free supernatant was obtained when the resin was mixed with Tris buffer or with 2N hydrochloric acid for as long as 30 minutes.)

Apparatus

To ensure that they are iron-free all pipettes and tubes are thoroughly washed and then immersed in chromic acid for several hours. They should then be rinsed in hot and cold tap water, distilled water and glass-distilled water prior to drying in an oven.

Procedure

One ml. serum is pipetted into the bottom of a test-tube and to this is added 0·2 ml. of iron standard followed by 5 ml. of the Tris buffer. The contents are mixed and allowed to stand at room temperature for 15 minutes. Approximately 400 mg. of resin are then added (the exact amount is not critical). The tube is then covered with "parafilm",* corked and placed on a slowly revolving vertical turn-table for 10 minutes. On removal it is spun at 3,000 r.p.m. for 2-3 minutes and a 4 ml. aliquot of the supernatant is pipetted into a tube. The activity is then counted in a scintillation counter against a 4 ml. aliquot of a control made up of 0·2 ml. standard and 6 ml. water.

The unsaturated iron-binding capacity is then calculated from the following formula:

$$\text{U.I.B.C. } (\mu\text{g./100 ml. serum}) = \frac{\text{Counts per minute in Test}}{\text{Counts per minute in Standard}} \times \text{Iron Content of Standard} \times 100$$

VALIDITY OF METHOD

Removal of ^{59}Fe ammonium citrate by resin

The resin IRA-401 was added in the same amount as used in the test to a mixture of 1 ml. water, 5 ml. buffer, and 0·2 ml. iron standard. After mixing for 10 minutes the residual activity in the supernatant was measured. In 10 estimations between 99·4 and 100% of the activity was removed by this procedure. Similar results were obtained using IRA-410. The resin De-Acidite FF was also tested and it was found that 98% was removed. In all subsequent experiments IRA-401 was used.

Removal of ^{59}Fe ammonium citrate in the presence of serum

Two series of experiments were done.

In the first, 10 buffered samples of serum were allowed to stand for 15 minutes after adding non-active ferric ammonium citrate in the same amount as used in the tests. Immediately prior to resin treatment 0·2 ml. ^{59}Fe ammonium citrate containing 0·1 μg. iron was added. After resinating the samples aliquots of the supernatant were counted for activity against suitable standards. It was found that between 99·0 and 99·4% of the activity was removed by the resin.

In the second series of experiments the standard test was carried out on 10 samples of serum using non-active ferric ammonium citrate. After resin treatment a 4 ml. aliquot was taken from each tube and to this was added 0·2 ml. ^{59}Fe ammonium citrate containing approximately 5 μg. iron. Resin was then again added and the tubes were mixed for 10 minutes. Two ml. aliquots of the supernatant were finally

*Manufactured by Marathon Corporation, U.S.A.

measured for activity against standards. It was found that between 98·0 and 98·7% of the activity was removed by the procedure. In 6 further experiments the saturated serum was allowed to stand in contact with the ^{59}Fe ammonium citrate for 10-30 minutes prior to the second resin treatment. When this was done 96·0-98·6% of the activity was removed.

Effect of resin treatment on physiologically bound iron

Eight samples of serum were made radioactive by adding ^{59}Fe ammonium citrate. The amount of iron added was always less than 0·1 μg. The standard test was then carried out using non-active ferric ammonium citrate. The recovery of activity in in the final supernantant varied between 101·7 and 105·0%. In addition the test was done on 12 samples of pooled serum. Four ml. aliquots of the saturated serum were then taken and to each was added 0·2 ml. of non-active ferric ammonium citrate standard containing 5 μg. iron. Resin was added to 10 tubes which were then mixed for periods varying from 5-30 minutes. Two ml. aliquots of the supernatant were withdrawn and were compared with similar aliquots from the two tubes which had not been treated with resin. It was found that recoveries varied between 97·5 and 104·0%. Similar results were obtained in 6 experiments in which the saturated radioactive serum was left in contact with non-active ferric ammonium citrate for as long as 30 minutes prior to the second resin treatment.

It was concluded that very little exchange occurs between physiologically bound iron and free iron under these various conditions.

Effects of heparin and oxalate

A series of duplicate analyses were carried out in which the results obtained with serum were compared with those obtained when either heparin or oxalate were used as anticoagulants. Heparin was used in a concentration of 10 units/ml. blood and potassium oxalate in a concentration of 2 mg./ml. The mean difference in U.I.B.C. between serum and heparinised plasma was found to be 4·2 μg./100 ml. (range 0 to 7) in 7 duplicate estimations. With oxalated plasma, however, the mean results in 10 duplicate estimations were 21 μg./100 ml. (range 0-33) lower than with serum.

It was concluded that oxalate interferes in some way with complete saturation of the iron-binding protein and is therefore not suitable for routine estimations using this method.

Reproducibility of results

Ten duplicate analyses were carried out on samples of serum. The mean difference in U.I.B.C. was 4·1 μg./100 ml. (range 0 to 9). In addition 7 analyses on one sample of serum gave a mean result of 154·7 μg./100 ml. (range 152 to 159).

Effect of storage

Repeated analyses were carried out on two samples of serum which were kept refrigerated at 4° C. for a period of 7 days and on one sample which was kept at room temperature over the same period. The results are shown in Table 1. It can be seen that results were reproducible over the period of observation.

Normal values

The U.I.B.C. was estimated in 10 healthy male students and the results were added to the figures obtained for serum iron as obtained by a modification of the method of Bothwell and Mallett [1958]. In this way a measure of T.I.B.C. was obtained in each case. The mean value was 331 μg./100 ml. (range 282 to 395).

TABLE I

Effects of storage on unsaturated iron-binding capacity (μg./100 *ml. serum*)

Days	1	2	3	4	5	6	7
Sample 1.. ..	264	260	265	260		263	264
Sample 2.. ..	246				254	250	254
Sample 3.. ..	246				254	250	256

Samples 1 and 2 were kept refrigerated at 4° C. over the period of observation and sample 3 was kept at room temperature.

Comparison with other methods

The technique was compared with the colorimetric methods described by Ramsay [1957], by Ressler and Zak [1958] and by Peters *et al.* [1956]. (Using Ramsay's method it was found necessary to centrifuge longer than recommended in order to obtain a clear supernatant after treatment with magnesium carbonate.) The results of the comparison are shown in Table II. It can be seen that agreement was good between all four methods.

TABLE II

Results for unsaturated Iron-binding capacity (μg./100 *ml. serum*) *by different methods*

Sample No.	Present Method	Ramsay [1957]	Ressler and Zak [1958]	Peters *et al.* [1956]
1	194		197	
2	207		203	
3	225		200	
4	200		229	
5	211		214	
6	356		361	
7	126		128	118
8	200		192	183
9	203		200	195
10	80			89
11	142			146
12	8			0
13	283	270		
14	212	207		
15	165	167		
16	245	239		
17	172	186		
18	205	211		
19	242	254		
20	152	161		

In the method of Ramsay [1957] and of Peters *et al.* [1956] the U.I.B.C. was calculated by subtracting the figure for serum iron level from the T.I.B.C. In both instances the colorimetric estimations were carried out by a modification of the method of Bothwell and Mallett [1955] in which sulphonated bathophenanthroline [Trinder, 1956] was used instead of 2,2′-dipyridyl as the colour reagent.

12-03)

COMMENT

The present radioactive technique for estimating the unsaturated iron-binding capacity of serum or heparinised plasma, which is based on the colorimetric method of Peters *et al.* [1956], appears to be a reliable one. Results obtained using it are in close agreement with those obtained by three other methods. Its major advantage lies in the fact that it can be rapidly carried out and no colorimetric estimation is required. It gives reproducible results on serum stored for as long as a week and on serum which is icteric or haemolysed. For accurate results one precaution is necessary. The buffer solution should be checked to make sure that it is iron-free since the presence of extraneous iron will tend to give falsely low results.

SUMMARY

A method for measuring the unsaturated iron-binding capacity of serum using radioactive iron is described. The results with this method are in close agreement with those obtained using three other techniques.

REFERENCES

BOTHWELL, T. H. and MALLETT, B. 1955). The determination of iron in plasma or serum. *Biochem. J.*, **59**, 599-602.

GITLOW, S. E. and BEYERS, M. R. (1952). Intravenous iron tolerance tests in normal subjects and patients with hemochromatosis. *J. Lab. Clin. Med.*, **40**, 541-549.

HOLMBERG, C. G. and LAURELL, C-B. (1945). Studies on the capacity of serum to bind iron. The regulation mechanism of serum iron. *Acta Physiol. Scand.*, **10**, 307-319.

LAURELL, C-B. (1947). Studies on the transportation and metabolism of iron in the body. *Acta Physiol. Scand.*, (Suppl. No. 46), **14**, 1-129.

PETERS, T., GIOVANNIELLO, T. J., APT, L. and ROSS, J. F. (1956). A new method for the determination of serum iron-binding capacity. *J. Lab. Clin. Med.*, **48**, 274-279.

RAMSAY, W. N. M. (1957). The determination of the total iron-binding capacity of serum. *Clin. Chim. Acta*, **2**, 221-226.

RATH, C. E. and FINCH, C. A. (1949). Chemical, clinical and immunological studies on the products of human plasma fractionation. XXXVIII. Serum iron transport. Measurement of the iron-binding capacity of serum in man. *J. Clin. Invest.*, **28**, 79-88.

RESSLER, N. and ZAK, B. (1958). Serum unsaturated iron-binding capacity. *Amer. J. Clin. Path.*, **30**, 87-90.

SCHADE, A. L., OYAMA, J., REINHART, R. W. and MILLER, J. R. (1954). Bound iron and unsaturated iron-binding capacity of serum: rapid and reliable quantitative determination. *Proc. Soc. Exp. Biol. & Med.*, **87**, 443-448.

TRINDER, P. (1956). The improved determination of serum iron. *J. Clin. Path.*, **9**, 170-172.

VENTURA, S. (1952). Determination of the unsaturated iron-binding capacity of serum. *J. Clin. Path.*, **5**, 271-274.

A METHOD FOR MEASURING THE STORAGE IRON CONTENT OF FORMALINISED TISSUES.

by

T.H. BOTHWELL*, N. ROOS and M.L. LIFSCHITZ

Department of Medicine, University of the Witwatersrand, Johannesburg, and the Council of Scientific and Industrial Research Iron and Red Cell Metabolism Unit.

This work was supported in part by grants from the United States Public Health Service (AM04912-03) and the World Health Organisation, Geneva.

Under optimal conditions about one quarter of the body iron content is present in storage depots such as the liver, bone marrow and spleen. From a functional standpoint iron stores represent a buffer which can be drawn upon when the need arises. They therefore tend to be less than normal when there are added losses from the body or when dietary deficiency is present. In contrast, increased iron stores are a feature when the iron content of the diet is high.

From a practical point of view an assessment of the level of iron stores has been found to be of major importance in defining the iron status of individual patients. This is done most conveniently by examining particles of bone marrow histologically in order to gauge the amounts of stainable iron present in reticulum cells (Rath and Finch, 1948). In necropsy studies more quantitative information has been obtained by estimating the concentration of iron present in liver specimens (Bothwell and Bradlow, 1960). There are, however, certain technical difficulties associated with such chemical measurements since different livers contain varying amounts of haemoglobin iron. To obtain valid figures for storage iron concentrations it is therefore necessary either to remove the iron present as haemoglobin or to estimate its concentration.

The present study was undertaken as a preliminary to a proposed collaborative investigation into the incidence of

iron deficiency in different population groups. It was felt that a fair reflection of the iron nutrition in different areas of the world might be obtained if the concentration of storage iron were estimated in samples of liver obtained from subjects dying acute traumatic deaths. However, before this could be attempted it was necessary to develop a simple method for carrying out large numbers of these analyses on formalinised liver specimens. This paper records the development of such a method.

METHODS

The estimation of total iron concentrations in liver samples presented no major problems since reproducible results could be obtained using conventional wet and dry ashing procedures. However some difficulty was experienced in measuring the haemoglobin content of the samples and several different approaches were tried.

Estimation of Haemoglobin Concentrations in Formalinised Samples:

An initial attempt was made to convert the haemoglobin to acid haematin prior to extracting it with an alcohol-ether mixture. Although this approach has been reported as satisfactory (Koeyman, 1949) recoveries were found to be incomplete with formalinised tissues and it was therefore abandoned. These results suggested that it might be necessary to digest samples in order to release the haemoglobin completely. This was initially done by

- incubating -

incubating samples in 1.0 N hydrochloric acid for 24 hours at 56°C. Since this did not effect complete dissolution of the tissue a similar procedure was carried out in which approximately 1g. tissue was incubated in 50 ml. 1.0 N sodium hydroxide at 56°C. After 24 hours the liver was completely dissolved and the solution was coloured brown due to the presence of alkaline haematin and other pigments. In livers with high concentrations of storage iron a brown deposit of ferric hydroxide was macroscopically visible. Although the solutions obtained after cooling and filtration were usually clear, soapy solutions were formed with fatty livers. This could be prevented by the addition of 0.5 ml. of the detergent sodium alkyl sulphate*.

Attempts were then made to estimate the haematin content of filtered solutions in three different ways.

(a) Alkaline Haematin: Since it seemed likely that most of the colour present in digests was due to alkaline haematin a number of estimations were carried out in which direct measurements were made of the optical density at 540 mμ of filtered digest solutions. The concentration of haemoglobin iron present was then read from a calibration curve constructed from haemoglobin solutions of known iron content which had been treated in the same way. (With this technique reproducible calibration curves were

- obtained -

*Marketed as 'Teepol' by the Shell Chemical Company, London, England.

obtained on solutions of haemoglobin that had been stored in formalin for as long as 3 months).

Although it was realised that the method was not a completely specific one it was felt it might prove adequate in epidemiological studies. However when batches of liver were tested from an area in which iron deficiency was known to be common a proportion of the values obtained for haematin iron were greater than those obtained for total iron. It was therefore apparent that the alkaline haematin method tended to give too high values, this being presumably due to other pigments, such as bile, present in the liver. In further experiments the percentage absorption of alkaline haematin and an alkanised solution of bile were compared at different wave lengths but the spectra were too close for adequate separation to be achieved.

(b) Benzidine Reaction: Since haematin gives a positive reaction with benzidine in both the bound and free forms, aliquots of the alkaline haematin solution were tested using a modification of the standard benzidine test (Bing and Baker, 1931). The procedure was as follows: 1 ml. of benzidine reagent* and 0.5 ml. 0.6% hydrogen peroxide were pipetted into a test-tube.

- The -

*Half a gram of benzidine hydrochloride was dissolved in 15 ml. water at 80°C. Twenty five ml. 95% (v/v) ethanol were then immediately added, followed by 10 ml. glacial acetic acid (AR grade). The solution was then stirred with a glass rod to ensure final clarity. (It was necessary to make up this reagent freshly each day).

The mixture was allowed to stand for 5 minutes to ensure that the tubes were free from contamination. An aliquot (0.02 ml.) of the alkalinised liver solution was then added and after thorough mixing the tube was allowed to stand for exactly 1 hour. At the end of this period 10 ml. of 20% (v/v) acetic acid was added and the optical density was read immediately in an Evelyn colorimeter using a 515 mμ filter. A blank solution in which 0.02 ml. 1.0 N sodium hydroxide was substituted for the test solution was read at the same time. The iron concentration was then read directly off a standard curve prepared from alkaline haematin solutions of known iron content which had been treated in the same way as the liver samples. (Since the benzidine reagent had to be made up freshly each day a solution of alkaline haematin of known iron content was set up with each batch of livers to check the validity of the standard curve).

Although the technique gave reproducible results this was only achieved when rigid precautions were taken to ensure cleanliness of glassware and standardisation of the various steps. On this basis the technique was not rugged or simple enough for the routine analysis of large numbers of samples.

(c) Pyridine haemochromogen: Since the major disadvantage of the alkaline haematin method was the interference

produced by other pigments, attempts were made to convert the haematin to a substance with a more distinctive absorption spectrum. This was done by adding pyridine and sodium dithionite to produce the pyridine haemochromagen (Flink and Watson, 1942). The absorption spectrum of the pyridine haemochromagen was then compared with the absorption spectrum of solutions prepared from 12 rat livers which had been exhaustively perfused to rid them of haemoglobin. It was found that these spectra were very dissimilar. The mean ratio of the optical density of the perfused specimens at 470 mμ and 540 mμ was 2.7 : 1 (range 2.2 to 3.2) while that of the pyridine haemochromagen was 1 : 1.07. These findings suggested that the differences could be utilised to obtain an absolute measure of the haematin concentrations present in specimens of liver. This was checked further by selecting a number of formalinised specimens of human liver which appeared very pale. After the usual treatment with sodium hydroxide, 5 ml samples were taken and 1 ml pyridine added to each. The optical density was read at 540 mμ. Sodium dithionite was then added and the optical density again measured. If there was no change (i.e. no pyridine haemochromagen present) the specimen was then used for determining the ratio of the optical density at 470 and 540 mμ. The mean ratio in 20 specimens was 2.7 (range 1.9 to 3.3).

Since this figure was identical to that obtained from perfused rat livers it was used as a correction factor in the final method. (The error that might be introduced by using an incorrect ratio in an individual specimen was tested by comparing figures for haematin iron in a series of 16 unselected livers, using ratios of 2.0 and 3.0 respectively. The mean difference (expressed as µg. haematin iron/g. (wet weight) was 7.7 (range 1.0 to 14.0)).

Validation of Pyridine Haemochromagen Method: Although there was no absolute standard of reference, an attempt was made to validate the method by comparing results on a series of livers with those obtained using the benzidine technique. In this comparison corrected figures for haematin iron using the pyridine haemochromagen method were calculated from the following formula:

$$\text{Corrected Haematin Iron} = \frac{(2.7 \times \text{Optical Density}_{540\ m\mu}) - (\text{Optical Density}_{470\ m\mu})}{1.7}$$

The degree of agreement was good, with a coefficient of correlation of + 0.96 ($p < 0.01$)(Fig. 1).

Estimation of Total Iron Concentrations in Formalinised Samples:

One gram aliquots of formalinised liver were digested and the iron concentrations were then measured

using the thioglycollic acid method (McCance et al., 1936). Storage iron concentrations were then obtained by subtracting figures obtained for haematin iron on a second aliquot. Although the method was adequate it involved the use of two samples from each liver. The alternative possibility was to measure the iron content of an aliquot of the alkalinised digest. Since the iron was present as a ferric hydroxide precipitate the 50 ml. suspensions were shaken vigorously prior to removing two samples of 10 ml. each. These were then wet digested and the iron contents measured. Agreement in 21 such estimations was good; the average difference from the mean of each pair of observations was 3.7% (range 0 to 12%). In a further comparison, results obtained from an aliquot were compared with those from a separately wet-digested 1g. sample. There was a good correlation ($r = + 0.986$; $p < 0.01$)(Fig. 2). These results indicated that an aliquot of alkalinised liver was adequate for the estimation of the total iron concentration.

RECOMMENDED METHOD.

Approximately 1g. formalinised liver is accurately weighed out after excess formalin has been removed by blotting gently with tissue paper. The sample is then transferred to a 50 ml. standard flask and 0.5 ml. Shell 'Teepol' is added. The volume is then made up to 50 ml. with 1.0 N sodium hydroxide and after corking is incubated at 56°C for 24 hours. At the end of this period the flask is shaken thoroughly and is cooled at 4°C for

1 hour. After further shaking a 10 ml. aliquot is immediately pipetted off for total iron estimation (see below).

(a) Estimation of Haematin Iron: The alkalinised suspension is filtered through No. 5 Whatman paper and a 5 ml. aliquot of the filtrate is pipetted into a test-tube containing 1 ml. pyridine (Analar grade). Approximately 30 mg. sodium dithionite (enough to cover the end of a small spatula) is added and the solution is allowed to stand for 10 minutes after thorough mixing. The solution is then read at 540 mμ and at 470 mμ against a blank containing 5 ml. 1.0 N sodium hydroxide, 1 ml. pyridine and about 30 mg. sodium dithionite. (It should be noted that the pyridine haemochromagen colour is stable for at least 1 hour).

The absolute value for pyridine haemochromagen is calculated as follows:

$$\text{Corrected value} = \frac{(2.7 \text{ Optical Density}_{540\ m\mu}) - (\text{Optical Density}_{470\ m\mu})}{1.7}$$

This reading is then read off a calibration curve constructed from haemoglobin solutions of known iron contents. Results are expressed as μg iron/gm. wet weight.

(b) Estimation of Total Iron: A 10 ml. aliquot of the well-shaken alkalinised liver solution is placed in a micro-Kjeldahl flask. To this is added 2.5 ml. of a mixture containing concentrated sulphuric acid (2 parts) and

concentrated nitric acid (1 part). Two glass beads are also put into the flask to prevent bumping and the mixture is digested until black and oily. This takes approximately 30 minutes. The flask is then allowed to cool for a short period and 0.25 ml. of 30% hydrogen peroxide is then added and digestion is then continued for a further 20 minutes. The colourless solution is again cooled and about 5 ml. distilled water are added. This is followed by about 0.2 ml. thioglycollic acid. Ammonium hydroxide (25%) is finally added carefully until a purple colour has fully developed. The solution is then transferred to a measuring cylinder and made up to 50 ml. with water. (In livers with high iron concentrations further dilution is necessary). The optical density of the final solution is read at 540 mμ against a water blank. The reading obtained is then read off a standard curve calibrated to a volume of 50 ml. and the concentration of iron is calculated from the formula:

$$\text{Iron concentration (μg./g. wet weight)} = \text{Reading on graph} \times \frac{\text{final Volume}}{50} \times \frac{1.0}{\text{Sample wt. (g.)}} \times 5$$

The figure for storage iron concentration is then obtained by subtracting haematin iron from this value.

C O M M E N T

Experience with the method has shown the importance of making allowance for the haemoglobin content in livers

when estimating storage iron concentrations. This is due to the fact that the amount of haemoglobin present varies considerably, depending as it does not only on the haematological status prior to death but also on the mode of death. With the present technique the median concentration of haemoglobin iron in 124 subjects was 42 µg./g. wet weight with a range from 0 to 214.

S U M M A R Y

A method is described for the estimation of storage iron concentration in formalinised tissue samples. It is especially suited for epidemiological studies in which large numbers of samples require to be analysed.

/DH.

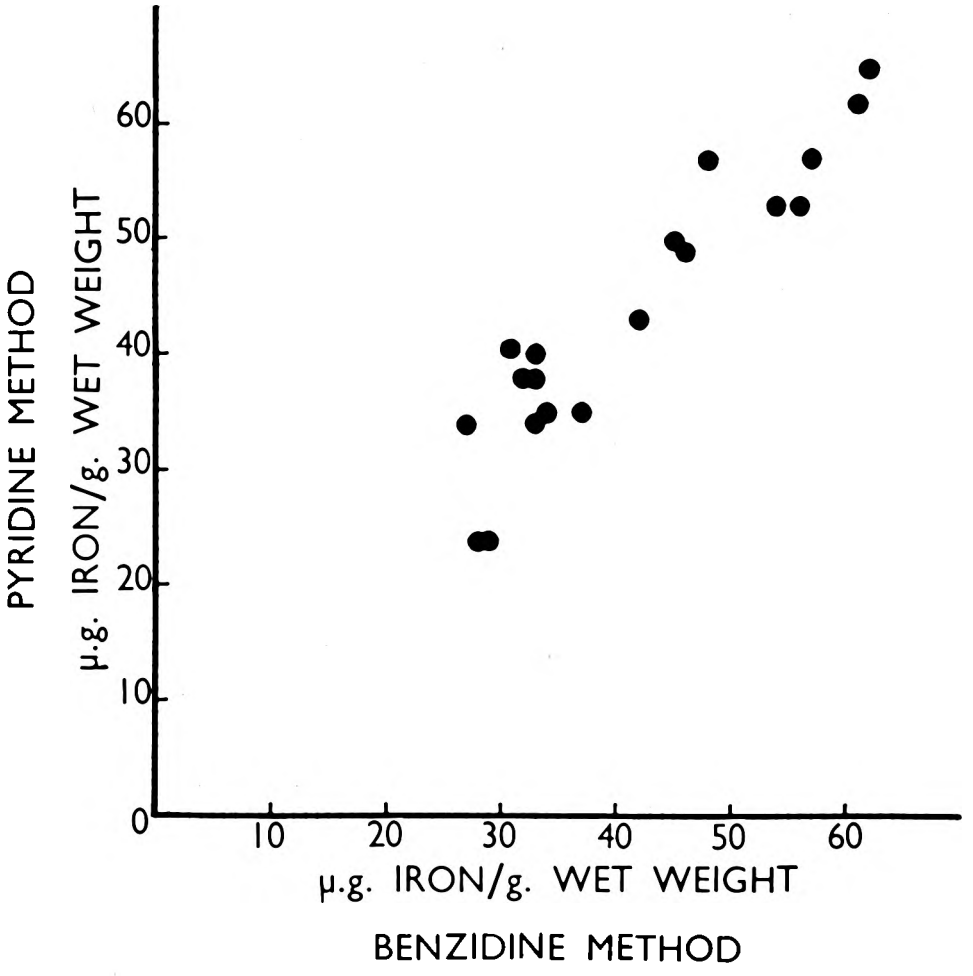


FIGURE I. A comparison of haematin iron values on formalinised liver samples as estimated by the benzidine and pyridine haemochromogen methods.

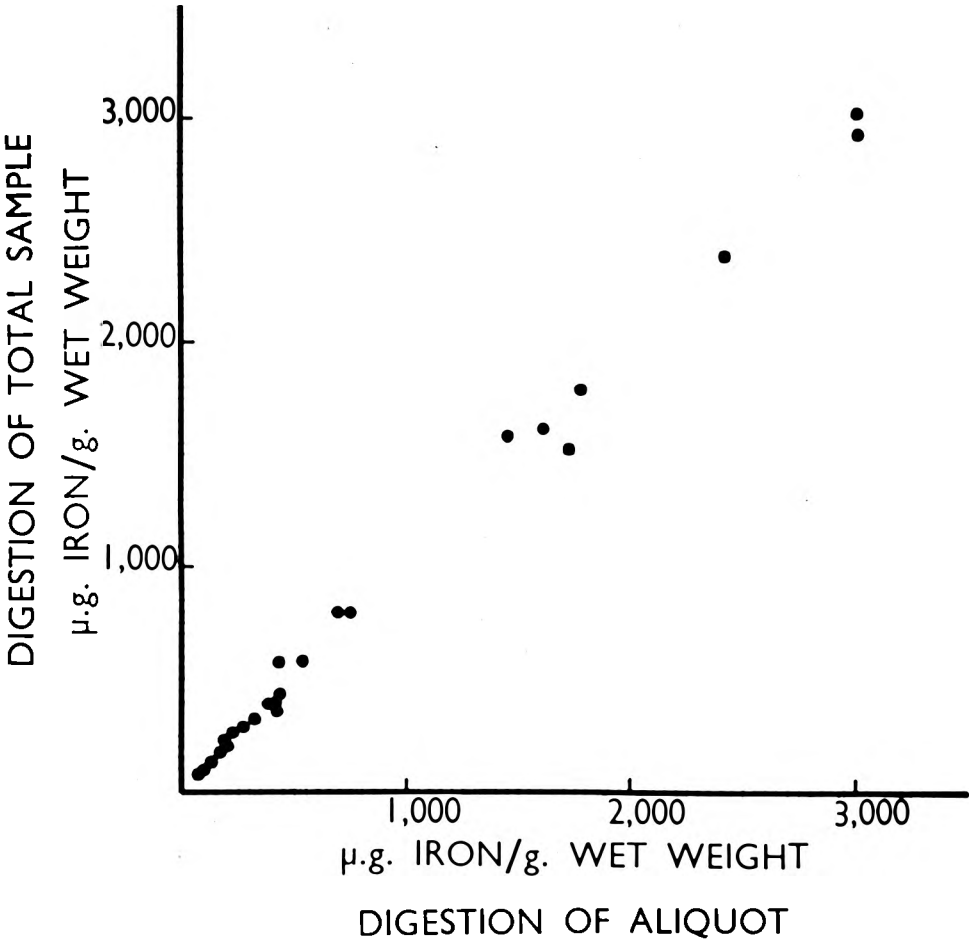


FIGURE 2. A comparison of liver iron concentrations as estimated by (a) wet acid digestion of 1 g. samples and (b) wet acid digestion of 10 ml. aliquots obtained after 1 g. samples had been hydrolysed for 24 hours in 1.0 N Sodium hydroxide.

REFERENCES

BING, F.C. and BAKER, R.W. (1931). The determination of hemoglobin in minute amounts of blood by Wu's method. J. biol. Chem., 92: 589-600.

BOTHWELL, T.H. and BRADLOW, B.A. (1960). Siderosis in the Bantu. A combined histopathological and chemical study. Arch.Path., 70: 279-292.

FLINK, E.B. and WATSON, C.J. (1942). A method for the quantitative determination of hemoglobin and related heme pigments in feces, urine and blood plasma. J. biol. Chem., 146: 171-178.

KOOYMAN, J.C. (1949). Investigations of serum and tissue iron. Acta Med. Scandinav., 134: 205-220.

McCANCE, R.A, WIDDOWSON, E.M. and SHACKLETON, L.R.B. (1938). The nutritional value of fruits, vegetables and nuts. Med. Research Council (Brit.) Spec. Rept. Ser. No. 213 p. 21.

RATH, C.E. and FINCH, C.A. (1948). Sternal marrow hemosiderin. A method for the determination of available iron stores in man. J. Lab. and clin. Med., 33: 81-86.

IRON ABSORPTION

INABILITY TO ASSESS ABSORPTION OF IRON FROM PLASMA RADIOIRON CURVES

BY

T. H. BOTHWELL, BARBARA MALLETT, R. OLIVER
and MARY D. SMITH

Reprinted from British Journal of Haematology, Vol. 1, No. 4, October 1955

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD

Inability to assess Absorption of Iron from Plasma Radioiron Curves

T. H. Bothwell,* Barbara Mallett, R. Oliver, and Mary D. Smith†

Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford and Department of Radiotherapy, Churchill Hospital, Oxford

Following the administration of a large dose of iron by mouth there is a prompt increase in the concentration of iron in the plasma. This fact has been used by several workers in studying iron absorption (Jasinski, 1949; Thedering, 1949; Hein and Keibl, 1950; Jasinski and Ott, 1951; Crawley, 1952; Smith, 1952; Begemann, Keiderling and Walter, 1953; Thedering and Beck, 1953; Grace, Doig and Wolff, 1954). In most investigations it has been assumed that there is a relation between the level to which the plasma iron is raised and the actual quantity absorbed. However, Yuile and his colleagues (1950) gave small doses of labelled iron to dogs and found that neither the height nor shape of the plasma radioiron curves correlated well with the amount absorbed. This is not surprising since the character of tolerance curves depends not only on the amount absorbed but on the rate of absorption and the rate of removal of iron from the plasma (Cartwright *et al.*, 1946; Smith, 1952). In the present study radioactive iron was used to find out whether a more accurate index of iron absorption could be obtained from the amount appearing in the plasma if corrections were made for the rate of removal of iron. The corrected figures for absorption were compared with those obtained by estimating the quantity of unabsorbed radioactivity in the faeces. The results of the investigation form the substance of this paper.

METHODS

All the subjects were patients with peptic ulcers who showed no signs clinically or radiologically of pyloric retention and who were ambulant at the time of study. None was anaemic and there was no evidence of recent gastro-intestinal bleeding. Haematological data on each case can be seen in Table I.

The rate of disappearance of radioiron from the plasma was measured on the morning of the day prior to the oral absorption studies. ^{59}Fe of high specific activity (1 to 2 μc per μg. Fe) was obtained from an American source through A.E.R.E., Harwell, in the form of a sterile solution of ferric chloride. 0.1 ml. (5 μc) of the radioiron solution was added to 2 ml. of a 1 per cent sterile solution of β_1 globulin‡ buffered with 10 to 20 ml. of the patient's plasma. After incubation for 30 minutes at 37° C. the mixture was injected intravenously from a calibrated syringe, a small quantity being kept for the preparation of the standard solutions. 15-ml. blood samples were taken at intervals following the injection, the samples being usually withdrawn after 10, 20, 40, 60 and 90 minutes. Radioactivity was measured using a scintillation counting technique as described by Badenoch and Callender (1954). 5 ml. of plasma were counted from each sample and the results plotted on semi-logarithmic

* Nuffield Travelling Fellow.
† Medical Research Council Clinical Research Fellow.
‡ Generously supplied by Cutter Laboratories, U.S.A.

paper to give a straight line for the exponential fall in activity. This line was extrapolated to zero time to find the level of maximum plasma activity. With a knowledge of the total administered dose it was then possible to estimate the plasma volume. The fraction of iron removed per hour was calculated by dividing the natural logarithm of 2 by the time taken for the circulating radioiron to reach one-half concentration (Huff *et al.*, 1950).

The oral absorption studies were started at 9 a.m. in the morning, the patient having fasted overnight. 10 to 15 μc of ^{59}Fe were added to a mixture containing 5 mg. of iron in the form of ferrous sulphate and 50 mg. of ascorbic acid. This solution was made up to 100 ml. with distilled water. 1 ml. was set aside for the preparation of standards and the remainder given to the patient. Before the test, blood samples were taken in order to measure the plasma iron, using an α : α′-dipyridyl, thioglycollic acid technique (Bothwell and Mallett, 1955a), and to estimate any residual radioactivity from the previous day's test. Seven to eight 15-ml. blood samples were withdrawn over the next 3 to 5 hours and the activity of 5 ml. of plasma from each specimen was counted as before. Using the figures for plasma volume obtained in the intravenous study it was possible to calculate the percentage of the oral dose in the plasma and to plot a plasma absorption curve. In calculating the results the graph showing the observed uptake of radioiron in the plasma in each case was divided into 20-minute intervals and the average level of radioactivity for each period worked out. In order to estimate the total radioactivity reaching the plasma from the gut it was assumed that the fraction of iron leaving the plasma per unit time on the day of the oral study approximated closely to the figure obtained at the same time on the previous day. The loss for each period, calculated from the average activity and the fraction of iron leaving the plasma per unit time, was then added to the observed rise in radioactivity to give the true value for the amount arriving in the plasma during each period. A curve was then constructed to show the rise in the level of radioiron in the plasma in the absence of any loss process. An example of such a graph can be seen in Fig. 1.

All faeces passed in the 7 days following the oral test were collected, and blended aliquots were counted for radioactivity as described by Badenoch and Callender (1954). From the quantity of unabsorbed radioactivity it was then possible to calculate the amount of radioiron which had been absorbed into the body. The maximum error in measurement by this method is approximately ±5 per cent.

RESULTS

The reconstructed graphs of radioiron absorption into the plasma in the 10 subjects investigated are shown in Fig. 2. There was a wide variation in total absorption as calculated by this method, the range being 5.4 to 44.0 per cent of the orally administered dose. The same initial pattern was seen in all cases, the absorption rate being highest in the first hour. After the level of circulating radioactivity had reached its maximum level different patterns occurred. In seven cases (Nos. 1, 3, 4, 6, 7, 8 and 10) the level declined in an exponential manner at a rate which approximated closely to the known exponential rate factor for the loss process. This suggested that no further significant absorption was occurring over this period. However, in two cases (Nos. 5 and 9) there was evidence that further absorption was continuing at a slow rate, while in another (No. 2) there was a second peak of absorption.

The results for the absorption of iron as calculated from plasma radioiron curves were compared with those obtained from the quantity of unabsorbed radioactivity present in the

faeces (Table I). Good or moderate agreement between the two results was found in five cases (Nos. 2, 4, 5, 9 and 10). In the other five, however, large discrepancies occurred, with the plasma radioiron data giving lower figures for absorption in each case. The appearance of the curves in these cases did not suggest that absorption was still going on at the end of the oral test. On the other hand, there was no reason to suppose that the faecal collections had been incomplete.

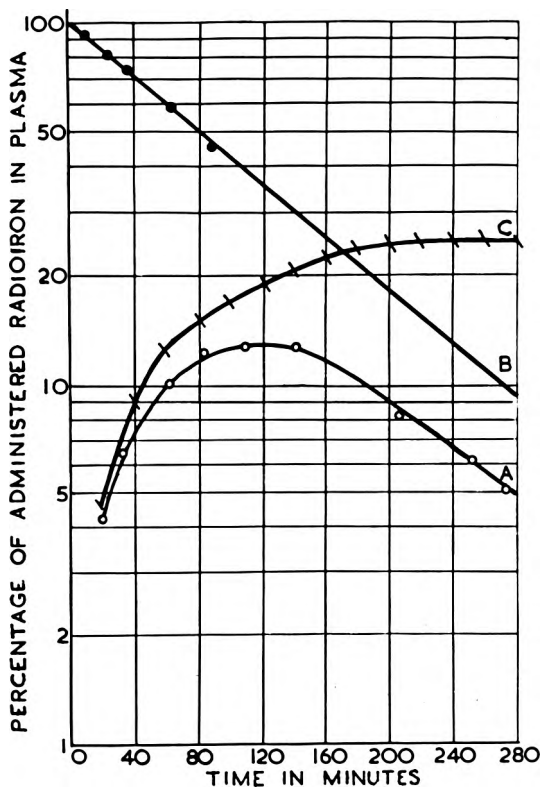


FIG. 1. Data on Case 4.
(A) Plasma curve after oral dose of radioiron. From this, the average level of circulating radioactivity in each 20-minute period is calculated.
(B) Line showing disappearance from the plasma of a tracer quantity of radioiron administered intravenously. Time taken to reach one-half concentration = 80 minutes; hence fraction of iron removed per 20 minutes $= \frac{\log_e 2 \times 20}{80}$ = 0.173.
Calculated loss for each 20-minute period (average activity × 0.173) was added to the observed rise in activity in the plasma over same period.
(C) Corrected curve showing rise in level of plasma radioiron in the absence of any loss process.

DISCUSSION

From the results of this study it seems apparent that it is difficult to get an accurate quantitative measure of radioiron absorption from the amount appearing in the plasma. In some cases there was good agreement between the figures obtained by this method and those

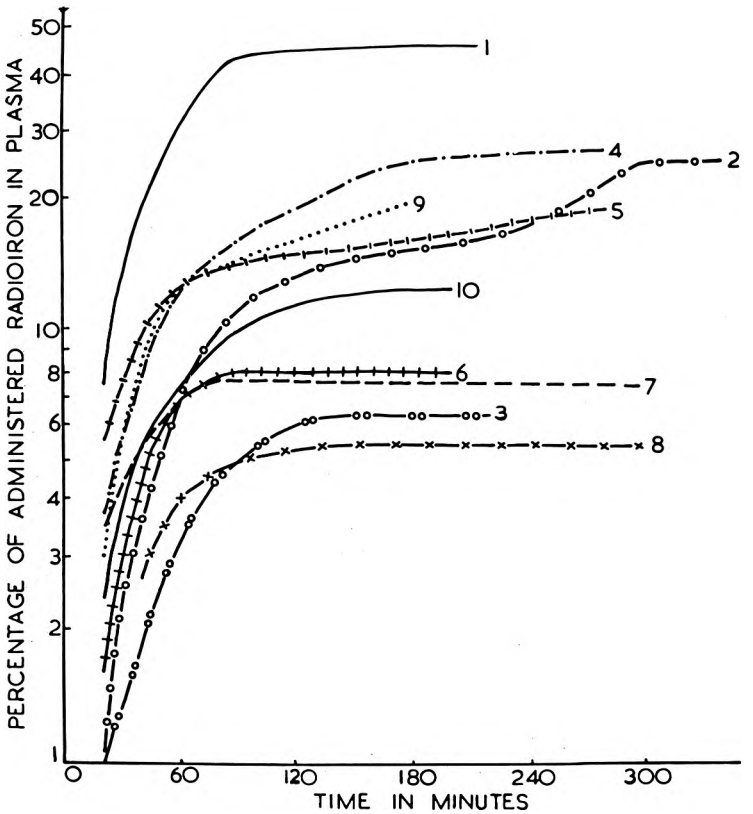


FIG. 2. Corrected graphs of plasma radioiron absorption to show the levels which would have been reached in each case if no radioiron was being removed from the plasma.

TABLE I

HAEMATOLOGICAL DATA AND RESULTS OF IRON ABSORPTION TESTS IN 10 SUBJECTS

No.	*Sex*	*Age*	*Haemo-globin (g./100 ml.)*	*Packed cell volume (%)*	*Red cells (mill./c.mm.)*	*Plasma iron (µg./100 ml.)*	*Fraction of iron removed per hour*	*Radioiron absorption calculated from amount appearing in plasma (%)*	*Radioiron absorption calculated from amount recovered from faeces (%)*
1	M	45	15.9	48	5.28	124	0.338	44.0	69.0
2	M	41	18.7	54	5.60	174	0.473	24.0	33.0
3	M	41	16.5	50	5.82	100	0.462	6.4	29.0
4	F	30	15.6	45	4.60	84	0.520	25.0	28.0
5	M	49	14.6	46	...	108	0.338	17.9	25.6
6	M	44	15.6	45	4.76	126	0.260	8.0	22.2
7	M	45	15.6	47	4.55	114	0.327	7.5	20.6
8	M	60	16.2	51	4.78	128	0.396	5.4	18.3
9	F	65	12.7	39	4.97	62	0.378	19.5	18.0
10	M	38	15.3	44	4.80	44	0.784	13.0	17.0

calculated from the quantity of unabsorbed radioactivity appearing in the faeces, but in others the discrepancies were large enough to invalidate the technique.

There are a number of ways in which the low figures for plasma radioiron absorption may be explained. Some of the radioiron may have been retained in the stomach; this seems unlikely as the small volume of solution was given to fasting patients and observations were carried on for several hours. It is possible that a proportion of the absorbed radioiron was removed by the liver. However, there is experimental evidence (Finch, *et al.*, 1950) that this could only account for a small percentage of the amount absorbed. An increase in the rate of disappearance of iron from the plasma on the second day of the study might explain some of the low plasma absorption figures. However, repeated intravenous tests at the same time of day have suggested that there is little change from day to day (Bothwell, Ellis, van Doorn-Wittkampf and Abrams, 1955) and even the diurnal variations which have been shown to occur (Bothwell and Mallett, 1955b) were not large enough to account satisfactorily for the low absorption results. It is also possible that a proportion of the radiorion was absorbed by a channel other than the plasma, e.g., through the lymphatic system, but experimental evidence does not support such a contention (Everett, Garrett and Simmons, 1954). According to Granick's hypothesis (1946) iron may be absorbed and temporarily stored as ferritin in the epithelial cells of the gut. Such a mechanism seems the most likely explanation of the discrepancies which were found. In addition, if iron were still being delivered into the plasma at the end of the observational period it may well have been at a rate not detectable by the techniques used in this study.

The inconsistent results which were found seem of some importance, as increasing use is being made of plasma-iron curves in measuring alimentary iron absorption. Although some authors have reported a wide scatter in results obtained with this technique (Smith, 1952), others have advocated oral absorption curves as a useful diagnostic procedure (Jasinski, 1949; Crawley, 1952; Thedering and Beck, 1953) and the method has been employed in assessing the relative merits of different iron salts (Hein and Keibl, 1950; Grace, Doig and Wolff, 1954). It is true that the results of the present study are not strictly comparable with previous ones. Using the radioiron technique it is possible to give a dose of iron which was within the physiological range while with chemical methods a massive dose is required in order to obtain significant changes in the plasma-iron level. In spite of this it is felt that the same inherent errors are present in the other studies, and although patterns obtained with plasma-iron curves may be useful in showing group differences, it is doubtful whether there is any value in attempting to assess the significance of individual results.

SUMMARY

An attempt has been made to assess quantitatively the absorption of radioiron from the gut in ten patients by estimating the total quantity of radioactivity appearing in the plasma.

The results have been compared with those obtained by estimating the percentage of unabsorbed radioactivity in the faeces.

In five cases there was moderately good agreement between the two methods; however, in the other five patients the estimates of absorption based on the plasma radioiron curves were lower.

It is concluded that estimation of the amount of radioactivity appearing in the plasma does not provide a reliable quantitative measure of iron absorption.

ACKNOWLEDGMENTS

We wish to thank Professor L. J. Witts and Dr. Sheila Callender for helpful criticisms. This work was supported by a grant to Professor L. J. Witts from the Medical Research Council

REFERENCES

BEGEMANN, H., KEIDERLING, W. and WALTER, F. (1953). 'Der Einfluss der Folsäure auf die Eisenresorption.' *Klin. Wschr., 31*, 881.

BADENOCH, J. and CALLENDER, S. T. (1954). 'Iron metabolism in steatorrhea.' *Blood, 9*, 123.

BOTHWELL, T. H. and MALLETT, B. (1955a). 'Determination of iron in plasma or serum.' *Biochem. J., 59*, 599.

BOTHWELL, T. H. and MALLETT, B. (1955b). 'Diurnal variations in the turnover of iron through the plasma.' *Clin. Sci., 14*, 235.

BOTHWELL, T. H., ELLIS, B. C., VAN DOORN-WITTKAMPF, H. VAN W. and ABRAMS, O. L. (1955). 'Radioiron studies in haemochromatosis. The effects of repeated phlebotomies.' *J. Lab. clin. Med., 45*, 167.

CARTWRIGHT, G. E., LAURITSEN, M. A., JONES, P. J., MERRILL, I. M. and WINTROBE, M. M. (1946). 'The anemia of infection. I. Hypoferremia, hypercupremia and alterations in porphyrin metabolism in patients.' *J. clin. Invest., 25*, 65.

CRAWLEY, J. (1952). 'Iron absorption tests in anaemia; use of intravenous iron preparations.' *Edinb. med. J., 59*, 478.

EVERETT, N. B., GARRETT, W. E. and SIMMONS, B. S. (1954). 'Lymphatics in iron absorption and transport.' *Amer. J. Physiol., 178*, 45.

FINCH, C. A., HEGSTED, M., KINNEY, T. D., THOMAS, E. D., RATH, C. E., HASKINS, D., FINCH, S. and FLUHARTY, R. G. (1950). 'Iron metabolism. The pathophysiology of iron storage.' *Blood, 5*, 983.

GRACE, W. J., DOIG, R. K. and WOLFF, H. G. (1954). 'Absorption of iron from the gastro-intestinal tract.' *J. clin. Nutr., 2*, 162.

GRANICK, S. (1946). 'Ferritin; increase of protein apoferritin in gastrointestinal mucosa as direct response to iron feeding. Function of ferritin in iron absorption.' *J. biol. Chem., 164*, 737.

HEIN, M. and KEIBI, E. (1950). 'Klinische Beobachtungen über die Wirksamkeit von Ferronicum.' *Klin. med. (Wien), 6*, 244.

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.

JASINSKI, B. (1949). 'Ueber Pathogenese und Therapie der nicht-anämischen und leicht-anämischen Eisenmangelzustände.' *Schweiz. med. Wschr., 79*, 1255.

JASINSKI, B. and OTT, W. (1951). 'Larvierter Eisenmangel, ein wesentlicher Teilfaktor des Dumping-Syndroms bei Magenresezierten.' *Schweiz. med. Wschr., 81*, 1141.

SMITH, M. D. (1952). 'Oral iron absorption and intravenous iron disappearance tests in the investigation of anaemia.' *Glasgow med. J., 33*, 425.

THEDERING, F. (1949). 'Wird die Eisenresorption durch Vitamin C messbar beeinflusst?' *Dtsch. med. Wschr., 74*, 921.

THEDERING, F. and BECK, R. (1953). 'Störungen der Eisenverdauung und Eisenresorption als pathogenetische Faktoren der essentiellen hypochromen Anämie.' *Klin. Wschr., 31*, 127.

YUILE, C. L., HAYDEN, J. W., BUSH, J. A., FESLUK, H. and STEWART, W. B. (1950). 'Plasma iron and saturation of plasma iron-binding protein in dogs as related to the gastrointestinal absorption of radioiron.' *J. exp. Med., 92*, 367.

IRON ABSORPTION

I. Factors Influencing Absorption

T. H. BOTHWELL, M.D.
G. PIRZIO-BIROLI, M.D.
and
C. A. FINCH, M.D.
Seattle, Wash.
With the Technical Assistance of
B. LODEN
and
A. MELLY

From the Department of Medicine, School of Medicine, University of Washington

Reprinted from

THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE
St. Louis

Vol. 51, No. 1, Pages 24-36, January, 1958

(Printed in the U. S. A.)

IRON ABSORPTION

I. Factors Influencing Absorption

T. H. Bothwell, M.D.,* G. Pirzio-Biroli, M.D., and C. A. Finch, M.D.
Seattle, Wash.

With the Technical Assistance of B. Loden and A. Melly

When the iron content of the body is altered by some cause, such as blood loss, balance is re-established mainly by changes in the amount of iron absorbed from the gut.[1] Because of its capacity to respond to the needs of the body, the gastrointestinal mucosa has been regarded as the chief regulator of iron balance.[2] The mechanisms by which this control is exerted are, however, still in doubt. One problem has been the technical difficulty in accurately measuring iron absorption. Recently, precise methods employing radioiron have been developed, but only limited observations have been made concerning the influence of anemia, anoxia, plasma iron level, iron stores, and altered erythropoiesis on iron absorption.

The present paper reports the results of studies involving several aspects of iron absorption. Experiments in animals were carried out in a variety of situations and the hypotheses which emerged were tested in man. The investigation was broadened to include observations on absorption of iron salts by normal subjects, by individuals after acute blood loss, and by patients with iron deficiency anemia. Information was obtained on the relative importance of certain internal factors in the regulation of iron absorption and, in addition, a rational approach to the problem of iron therapy was developed.

MATERIALS AND METHODS

Sprague Dawley rats weighing 250 to 350 grams, New Zealand white rabbits weighing 2.5 to 3.5 kilograms, and a strain of adult field mice with hereditary spherocytosis[3] were employed in various studies. In each experiment control animals of the same sex, age, weight, and dietary background were employed. The purpose was to indicate whether a specific factor altered absorption in these animals as compared to control animals. Variations, particularly in the amount of iron administered, made it impractical to interrelate individual studies.

Radioiron was administered directly by gastric intubation. Rats were rendered anemic by removing 5 ml. of blood at 5 and 3 days prior to study, or by the parenteral administration of an ethanolic solution of phenylhydrazine (30 mg./Kg.). Hemolytic anemia was also induced by the prior injection of methyl cellulose intraperitoneally twice

From the Department of Medicine, School of Medicine, University of Washington, Seattle, Wash.

This investigation was supported by a research grant from the United States Atomic Energy Commission (Contract AT-[45-1]-218); and a grant-in-aid from Smith, Kline & French Laboratories, Philadelphia, Pa.

Received for publication May 6, 1957.

*In receipt of Lederle Traveling Fellowship for South Africa. Present address: Department of Medicine, University of Witwatersrand Medical School, Hospital Street, Johannesburg, South Africa.

weekly as described elsewhere.[4] Excess body iron stores were produced in rats, rabbits, and mice by the intramuscular injection of iron-dextran (Imferon*). Exchange transfusion was performed in rats by the insertion of plastic cannulae into the femoral artery and vein with the simultaneous removal of circulating blood and the injection of blood previously obtained from donor animals.[5]

Normal human subjects studied had plasma iron concentrations between 80 and 160 μg/100 ml. plasma and hematocrits between 42 and 48 per cent. In patients the criteria of iron deficiency were plasma iron levels below 50 μg, the absence of marrow hemosiderin, and a response to iron therapy.

Absorption studies were performed on normal volunteer subjects, fasting or after meals, as specified under results in individual experiments. Phlebotomy was also employed in normal subjects to enhance iron absorption. Unless otherwise specified, such subjects were bled 400 ml. on the ninth and seventh days before the absorption study was performed.

Absolute iron absorption was measured by the double isotope method described by Saylor and Finch.[6] In this technique a weighed amount of Fe^{59} either as iron citrate or as iron bound to fresh plasma† was injected intravenously and at the same time a solution of iron (Fe^{55}) was given orally. Standards were then made from both solutions given. Fourteen days after administration of the two isotopes, a blood sample was collected and wet-ashed with sulfuric and perchloric acids. Samples were precipitated with the addition of carrier iron to a total of 10 mg. and electroplated as described previously.[7] Counting for Fe^{55} was done with an argon-filled geiger tube having a beryllium filter, whereas Fe^{59} was determined with a helium-filled tube. The cross-counting for which appropriate correction was made was less than 4 per cent in either direction. Calculation of results:

$$\text{Percentage absorbed} = \text{yl} \frac{\frac{(\text{x})}{(\text{xl})} \times 100}{\text{y}}$$

When yl = activity of Fe^{55} in blood sample (c.p.m.)
y = activity of Fe^{55} given by mouth (c.p.m.)
xl = activity of Fe^{59} in blood sample (c.p.m.)
x = activity of Fe^{59} injected intravenously (c.p.m.)

In several studies iron absorption was computed by measuring stool radioiron content. To determine stool activity, a 10-day stool collection was transferred from waxed cartons to a large weighed beaker. The bulked specimen was then covered with concentrated sulfuric acid for 10 to 14 days with occasinal stirring and addition of acid to a total of about 5,000 ml. Gentle heating was applied when necessary to produce a homogenous mixture. Ten gram aliquots were electroplated in the usual manner prior to counting.[7] Since the validity of methods such as these must ultimately rest on direct comparisons, results of absorption by the stool and double isotope methods in 4 normal subjects are summarized in Table I.

TABLE I. COMPARISON OF STOOL RECOVERY AND DOUBLE ISOTOPE METHODS FOR MEASURING IRON ABSORPTION IN 4 HUMAN SUBJECTS

	STOOL METHOD (PER CENT ABSORPTION)	DOUBLE ISOTOPE METHOD (PER CENT ABSORPTION)
1	5.3	4.8
2	15.2	12.5
3	5.5	2.6
4	20.9	16.6

*Obtained through the courtesy of the Benger Company, England, and Lakeside Laboratories, Milwaukee, Wis.

†It is essential that there be either adequate capacity of the recipient's plasma to bind the injected iron citrate, or that iron be bound to a plasma with adequate binding capacity before its injection.

In certain studies on human volunteers the major interest was centered not so much on the absolute amount of iron absorbed as on the relative amounts absorbed under two different sets of conditions. One isotope of iron in one form was given by mouth on the first morning, while the second was given by the same route on the following morning. The order of administration of the two test doses was alternated in different subjects. Analysis of activity in the blood drawn two to three weeks later permitted a comparison of absorption between the two test doses in the same subject. In order to estimate the absolute absorption, the per cent of red cell utilization of absorbed iron must be predictable. This was assumed to be 90 per cent in normal and iron deficient subjects and the blood volume was estimated at 68 ml./Kg. Estimated per cent absorption then equaled:

$$\frac{\dfrac{\text{activity per ml. blood} \times \text{blood volume (ml.)} \times 100}{0.9}}{\text{total radioactivity administered}}$$

The actual amount of radioactivity given varied in different studies depending upon the anticipated absorption and on the total amount of administered elemental iron. In man, the amount of Fe^{55} varied between 50 and 100 μc when carrier iron of between 100 and 500 mg. was added. $Iron^{59}$, in doses of approximately 1 μc, was given intravenously as ferrous citrate or was bound in vitro to the normal plasma when the patient's plasma iron binding capacity was inadequate.[8] The specific activity of the two isotopes used varied between 1 and 3 $\mu c/\mu g$. With the double isotope method of measuring absorption, the dosage of each isotope was designed to provide nearly equal counts in the circulating blood.

Plasma iron was estimated by the method of Bothwell and Mallet,[9] and of Kitzes, Elvehjem, and Schuette.[10] Bone marrow iron was estimated from sternal aspiration as described by Rath and Finch.[11] Hemoglobin was determined as oxyhemoglobin in an Evelyn colorimeter and hematocrits were spun at 1,800 g for 30 minutes. Reticulocytes were stained with cresyl blue and the number in 1,000 erythrocytes counted.

RESULTS

A. Internal Factors Regulating Iron Absorption.—The object of these studies was to delineate the effect of alterations within the body on iron absorption. In order to minimize the possible interference which local gut factors might have on such absorption, initial studies were done with animals and experimental subjects in a fasting state. Iron was given as a freshly prepared solution of ferrous sulfate. The usual dosage of iron was standardized at 0.25 mg./Kg. of elemental iron in experimental animals. Data obtained may be summarized under four headings.

1. Effect of the rate of erythropoiesis on iron absorption: Increased erythropoiesis was produced in several different ways. One injection of phenylhydrazine was given to rats and absorption was measured one week later when the reticulocyte count was high and the hematocrit was rising rapidly. In rabbits, multiple injections were given and absorption was measured at variable times thereafter. Methyl cellulose injected rats which had increased red cell production and destruction, but little anemia, and mice with increased erythropoiesis due to hereditary spherocytosis were also studied. These results are summarized in Table II and consistently indicated an increase in iron absorption in animals with increased erythropoiesis as compared to normal animals of

the same species given the same dose of iron. Additional studies in rabbits given multiple injections of phenylhydrazine, while again indicating increased absorption with increased erythropoiesis, showed no correlation between a rising or falling hematocrit or with the level of plasma iron.

TABLE II. EFFECTS OF INCREASED ERYTHROPOIESIS ON IRON ABSORPTION

DIAGNOSIS	NUMBER OF ANIMALS	DOSAGE OF IRON (μG)	ABSORPTION (± S.D.) (PER CENT)
Phenylhydrazine in rats	5	50	59 ± 19
Controls	6	50	21 ± 8
Phenylhydrazine in rabbits	8	1,000	30 ± 19
Controls	8	1,000	12 ± 11
"Methyl Cellulose" in rats	6	100	16.2 ± 6.2
Controls	4	100	10.3 ± 1.9
Congenital spherocytosis in mice	7	3.5	7.2 ± 2.2
Controls	7	3.5	4.8 ± 1.9

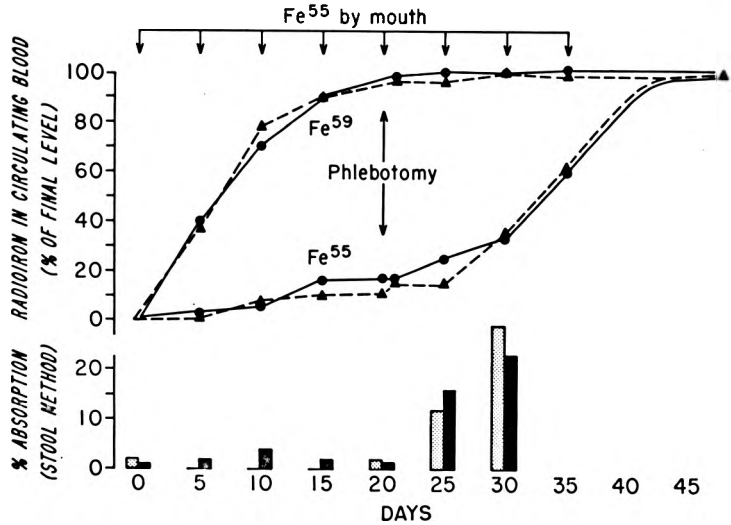


Fig. 1.—Effects of phlebotomy on iron absorption in 2 human subjects as measured by stool recoveries and by circulating radioactivity in the blood. During the control period there is almost complete recovery of orally administered Fe^{59} in stool and little radioactivity in circulation. After phlebotomy both measurements indicate an increased absorption of iron.

In an attempt to clarify further the time relationship between increased absorption and erythropoiesis, studies were carried out in experimental subjects before and after phlebotomy. As shown by stool measurements as well as by blood activities in two subjects, increased absorption occurred within 5 days of phlebotomy (Fig. 1). Absorption was also measured in normal fasting subjects given 25 mg. of iron (Fe^{59} sulfate) and phlebotomized 8 ml./Kg. on the following day. Repeat absorptions were then done with Fe^{55} under identical conditions through the following week and the pre- and postphlebotomy ratio was determined (Table III). In the first four days values were variable, but thereafter absorption showed a moderate but consistent increase.

The effect of *decreased erythropoiesis* was also studied. To retard red cell production, rats were made polycythemic by exchange transfusion with packed erythrocytes. The hemoglobin level after exchange in these animals was above 20 Gm. per cent. As a control, another group of rats was submitted to the same procedure except that they were exchanged-transfused with normal whole blood. Reticulocyte counts were depressed in the polycythemic animals, although the time lag in marrow response made this less conspicuous at two days. Absorption studies (Table IV) showed a reduction in iron absorption by polycythemic animals as compared to controls.

TABLE III. THE ABSORPTION OF A DOSE OF 25 MG. ELEMENTAL IRON BEFORE AND AFTER PHLEBOTOMY OF 8 ML. PER KILOGRAM IN 12 HUMAN VOLUNTEERS

SUBJECT	PLASMA IRON	BASAL ABSORPTION (PER CENT)	DAYS AFTER PHLEBOTOMY	PLASMA IRON	SECOND ABSORPTION (PER CENT)	$\frac{S.A.}{B.A.} \times 100$*
1	113	12.4	2	140	8.2	66
2	135	12.5	2	128	11.4	91
3	81	9.9	3	72	14.6	147
4	111	13.6	3	92	21.7	160
5	142	7.8	4	64	5.6	72
6	123	8.8	4	96	7.1	81
7	133	13.4	5	50	18.3	137
8	215	9.5	5	94	15.5	163
9	118	17.4	7	71	27.0	155
10	113	12.6	7	83	15.0	119
11	193	5.0	11	103	11.5	230
12	112	9.0	11	76	12.0	133

*$\frac{\text{Second absorption}}{\text{Basal absorption}} \times 100$.

TABLE IV. EFFECT OF DECREASED ERYTHROPOIESIS ON IRON ABSORPTION IN RATS

ANIMALS	DAYS FOLLOWING TRANSFUSION	DOSAGE (μG)	HEMOGLOBIN (GM.)	RETICULOCYTE COUNT (PER CENT)	ABSORPTION (PER CENT)
Control	2	20	14.8	2.8	19.4
			15.5	2.1	25.0
Polycythemic	2	20	20.2	1.1	0.6
			20.6	1.4	2.4
Control	5	100	19.3	1.4	8.0
			16.3	1.5	7.4
			17.8	1.5	6.8
			16.7	0.8	15.8
			16.9	1.8	14.3
Polycythemic	5	100	21.0	0.3	4.7
			19.4	0.1	2.0
			19.6	0.3	4.6

2. Effect of change in body iron stores on absorption of iron: Iron deficiency in rats was produced by feeding a low-iron diet from the time of weaning, and excessive iron stores were created by the injection of 100 mg. of iron 3 months before the study. Absorption in these animals was compared to that in normal animals (Fig. 2). In the group of 6 animals with excessive iron, absorption was decreased to 3.9 ± 0.9 per cent as compared to the normal group of animals with 20 ± 7 per cent absorption. The 4 iron deficient animals showed an increase to 47 ± 13 per cent. A comparable increase in absorption of iron deficient patients as compared to that in normal subjects is shown later.

3. Combined effect of iron stores and erythropoiesis on absorption: The studies previously described indicated that increased erythropoiesis was associated with increased absorption of iron and decreased erythropoiesis with decreased absorption. The interplay of two opposing factors was illustrated by the following experiment. The erythroid marrow was activated by bleeding two groups of animals, one having previously had normal iron stores and the other having been loaded with 100 mg. of iron by parenteral injection one month previously. As compared to the normal absorption of 20 per cent, 5 phlebotomized animals with hematocrits of 40 ± 2 per cent absorbed 40 ± 17 per cent. The

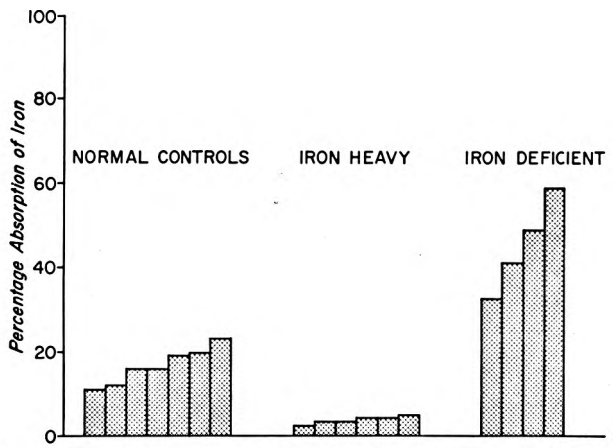


Fig. 2.—Effects of changes in body stores on iron absorption in rats.

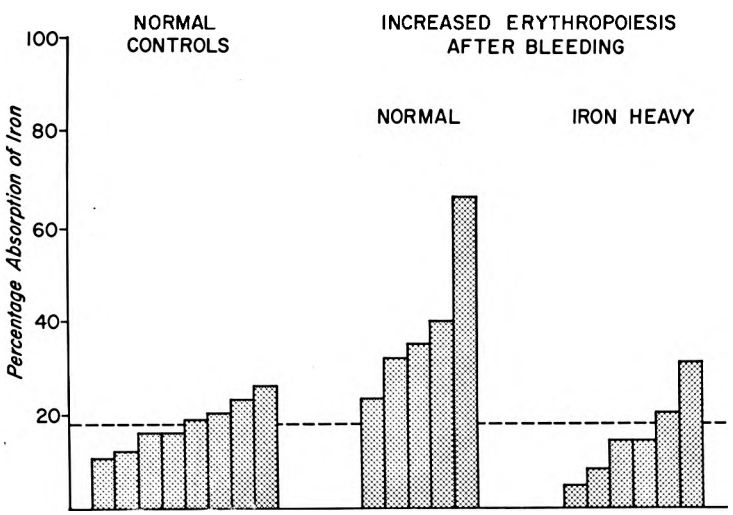


Fig. 3.—The combined effects of increased erythropoiesis and increased stores on iron absorption in rats. As compared with the group of normal animals, increased erythropoiesis increased iron absorption. However, the composite effect of a similar increase of erythropoiesis in animals with excess body iron was a normal absorption.

absorption by the 6 animals who were both iron loaded and phlebotomized was within the normal range (15 ± 9 per cent) (Fig. 3). Thus, with one factor tending to increase iron absorption and another to decrease it, the results fell within the normal range.

4. Effect of the plasma iron level on absorption from the gut: In the studies on normal and phlebotomized subjects there was no evident correlation between plasma iron and the amount of iron absorbed. To obtain more definite information, absorption studies were performed before and after the plasma iron had been raised to saturation of the iron-binding protein in 3 rabbits and in 2 normal subjects who had been previously phlebotomized. This elevation of plasma iron was accomplished by the infusion of erythrocytes rendered nonviable through in vitro storage in sodium citrate for 3 weeks. The marked increase in plasma iron which occurred between 2 and 8 hours after injection of the cells, due to the catabolism of their hemoglobin, did not significantly affect absorption (Table V).

TABLE V. EFFECT OF PLASMA IRON LEVEL ON IRON ABSORPTION

RABBIT STUDIES	Fe DOSAGE	PLASMA IRON (μG/100 ML.)		ABSORPTION (PER CENT)	
		BEFORE	AFTER	BEFORE	AFTER
R-1	500 μg	55	328	53	43
R-2	500 μg	162	314	87	81
R-3	500 μg	114	271	12	5
CLINICAL STUDIES					
S-1	5 mg.	92	315	40	53
S-2	50 mg.	87	267	8	7

B. Effects of Some Local Factors on Iron Absorption.—Many questions which fall outside the scope of this paper arise concerning local factors within the gastrointestinal lumen. However, certain observations relating to the form and amount of administered iron and the effects of food and ascorbic acid are presented because of their practical importance in iron therapy.

TABLE VI. EFFECT OF FORM OF IRON ON ABSORPTION IN PHLEBOTOMIZED NORMAL SUBJECTS

SUBJECT	DOSE OF IRON (MG.)	ABSORPTION (PER CENT)	
		FERROUS SULFATE SOLUTION	"QUICK" RELEASE SPANSULES
1	16	18.8	23.8
2	16	36.8	21.0
3	16	15.0	8.0
		FERROUS SULFATE SOLUTION	"SLOW" RELEASE SPANSULES
4	48	24.0	4.0
5	48	17.4	4.6
6	48	38.0	7.0
		FERROUS SULFATE SOLUTION	CALCIUM IRON CITRATE
7	50	9.9	3.0
8	50	10.7	8.7
9	50	20.8	22.0
10	100	4.7	5.5
11	100	4.5	2.4

1. Form of ingested iron: The absorption of a solution of ferrous sulfate was compared with that of ferrous sulfate in the form of quickly disintegrating

and slowly disintegrating spansules.* The spansule and ferrous sulfate preparations were fed to fasting phlebotomized subjects on alternate mornings. Results (Table VI) indicated little difference between the iron solution and the rapid release spansule, but did show a marked reduction in the amount of iron absorbed from the preparation with a slower release through the lower intestinal tract. Studies comparing ferrous sulfate with iron in the form of a calcium citrate chelate† showed little difference between these two compounds.

2. Relation between dosage of iron and amount absorbed: Data on 6 normal male volunteers, 13 other normal volunteers who had previously been phlebotomized, and on 8 iron deficient patients are summarized in Fig. 4. All tests were done with subjects in a fasting state and all were given freshly prepared solutions of ferrous sulfate varying in dosage from 5 to 500 mg. of iron. Normal subjects showed such individual variations as to obscure the effect of dosage on absorption. However, in phlebotomized and iron deficient subjects, the higher absorption with increases in iron ingestion was better defined. The 3 groups studied appeared to differ largely in the capacity of the mucosa to transport iron. The absorption ratio between the normal, phlebotomized, and iron deficient subjects was approximately 1 : 3 : 9.

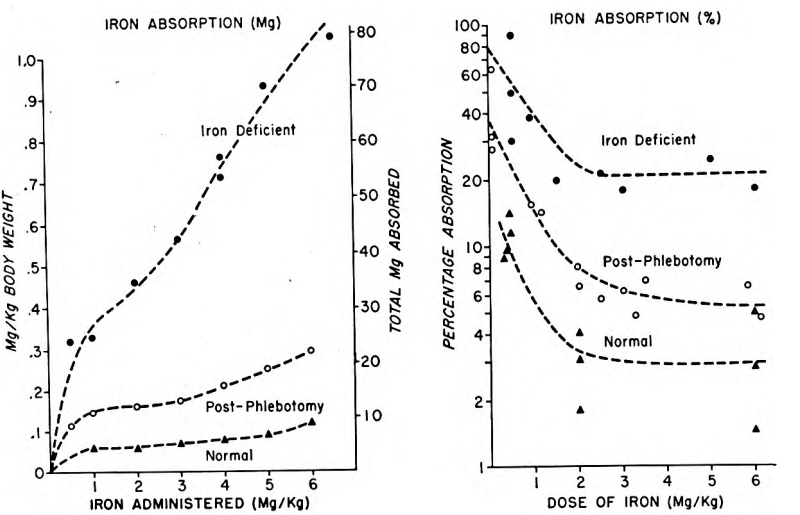


Fig. 4.—The absorption of varying dosages of iron in normal, phlebotomized, and iron deficient subjects.

3. The effect of ascorbic acid and cobalt on iron absorption: In animals given a constant amount of ascorbic acid and increments of oral iron (Fig. 5), it was possible to demonstrate an enhancement in iron absorption. In man, smaller dosages of ascorbic acid, in keeping with amounts feasible for clinical administration, were given. The dosage of ascorbic acid varied from 100 to 1,000 mg., and iron was given in amounts of either 1 or 2 mg./Kg. The subjects were

*These were obtained through the courtesy of Smith, Kline & French Laboratories, Philadelphia, Pa.

†This material, ferrous calcium citrate with tricalcium citrate, distributed as Rarical, was obtained through the courtesy of the Ortho Pharmaceutical Corporation, Raritan, N. J.

phlebotomized prior to absorption tests and studied either fasting or postprandial. The effect of ascorbic acid was not pronounced, as indicated by an average absorption of 12 per cent in all studies as compared with control values of 9 per cent (Table VII).

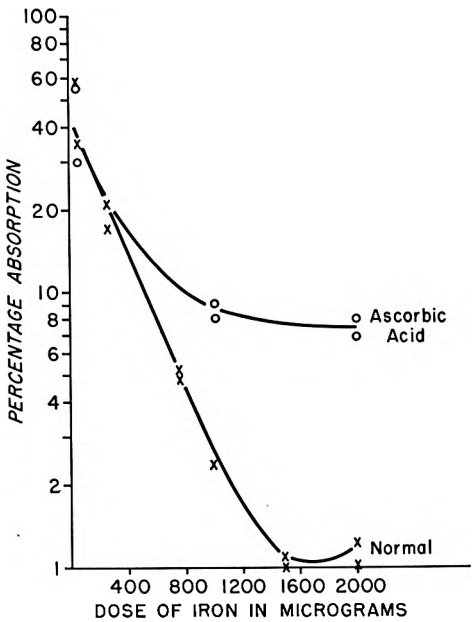


Fig. 5.—The effect of 10 mg. of ascorbic acid given with varying doses of iron was compared with absorption of iron alone.

TABLE VII. EFFECTS OF ASCORBIC ACID ON IRON ABSORPTION IN PHLEBOTOMIZED NORMAL SUBJECTS

METHOD OF Fe ADMINISTRATION	DOSE OF ASCORBIC ACID (MG.)	ABSORPTION (PER CENT)	
		CONTROL	WITH ASCORBIC ACID
Fasting	100	10	11.6
Dose of 1 mg./Kg.	200	17	22.0
	200	8	13.0
	500	6	8.0
	1,000	12	16.0
Fasting	100	6.9	4.9
Dose of 2 mg./Kg.	200	7.3	6.3
	400	3.6	7.4
	600	6.1	7.2
	1,000	8.2	6.1
After Meals	100	8.0	11.0
Dose of 1 mg./Kg.	200	13.0	15.0
	500	18.0	24.0
	1,000	7.0	14.0

The effect on iron absorption of 80 to 120 mg. per day of cobalt given as cobalt chloride was also determined. In two subjects absorption studies were carried out after 7 days and in two others after 14 days of cobalt administration. Absorptions before and after cobalt were 3.7 versus 9.8 per cent, 2.3 versus 5.5 per cent, 9.4 versus 9.2 per cent, and 9.8 versus 8.1 per cent.

DISCUSSION

Ingested iron is absorbed in the ferrous form largely by the duodenum. The absorbed iron passes through the mucosal cells into the blood stream where it is bound by a beta-1 globulin of the plasma. Normally about 90 per cent of this iron is incorporated into the hemoglobin and is found in the circulating red cell mass within 2 or 3 weeks; in iron deficiency anemia absorbed iron may be almost quantitatively recovered from circulating erythrocytes.

The most accurate early information in the literature of iron absorption related to iron deficiency anemia, where the amounts of iron absorbed were great, and absorption could be measured by the increase in circulating hemoglobin. Initial measurements employing radioiron followed the same principle of measuring the radioactivity in the circulating red cell mass.[12] This measurement was found to be unreliable in certain diseases due to a significant and variable distribution of radioactive iron into tissues other than the erythrocyte. This led to the development of a stool recovery method where absorption was determined from the difference between iron administered and that recovered in the feces.[13]

While there is no reason to question the validity of this method on theoretical grounds, since excretion of absorbed iron into the gut is negligible, there are practical difficulties. Not only is it a problem to ensure complete collection over a period of 8 to 12 days, but the per cent absorption is often so small that technical errors are magnified.* At these low levels it is possible to determine absorption more accurately by a double isotope method, whereby the second isotope measures distribution between tissues and red cells. The ratio of the two isotopes then indicates the total amount absorbed without the necessity of a blood volume determination. The reliability of this method at a dose range of 0.5 mg./Kg. iron in measuring isotope distribution between red cells and other tissues has been previously reported.[6] These studies have been extended over a wide dose range up to 10 mg./Kg. with similar results.[16] Combined stool and double isotope measurements on 4 subjects gave an average of 12 per cent and 9 per cent, respectively (Table I). In another subject given radioiron every 5 days over a 42-day period, average daily absorption was 5.4 per cent by the stool method and 3.9 per cent by the double isotope technique (Fig. 1). A further indication of the general reproducibility of the double isotope technique was found in examination of the consistency in repeat measurements in the same individual where the two sets of conditions produced similar absorption (Tables V, VI, and VII).

*In certain conditions, e.g., infections, lymphomas and hemolytic anemias,[13] and rheumatoid arthritis,[14] absorption as judged by stool recoveries has been found sometimes to be a good deal greater than would have been apparent from blood levels alone. Such results would presume that only a small portion of the radioiron entering the plasma is utilized for hemoglobin production in these conditions. In recent years, however, it has been possible, using iron of high specific activity, to tag the plasma iron-binding globulin without exceeding its binding capacity and so find out what proportion of the plasma iron is concerned with red cell production.[15] Under these circumstances, with the exception of certain hemolytic anemias, it has been shown that red cell utilization is only rarely below 50 per cent and that it is usually a good deal higher.[16] These findings suggest the need for re-evaluating some of the earlier results for absorption obtained by stool recovery techniques.

It is apparent that the method employed to measure iron absorption must be modified depending on the problem at hand. When the per cent incorporation of radioiron is 80 to 100 as in the normal or iron deficient subject, a determination of total red cell activity at two weeks will suffice. Comparison can then be made of two absorption tests employing different isotopes. If the per cent utilization of radioiron for red cell production is not predictable, either the double isotope method previously described or the stool recovery method must be employed. While the double isotope method has been preferred for most of the studies reported here because of its simplicity and greater accuracy, this method is not feasible when there is a complete failure of erythropoiesis. Accordingly, in these studies a variety of methods have been employed according to the experimental situation.

A further adjunct to the evaluation of absorption in man has been the use of phlebotomy. This has proved particularly useful in testing iron preparations of unknown value, since the amount of iron absorbed in normal subjects is often too small for definitive results. The absorption by phlebotomized subjects over a range of iron dosage was 2 to 3 times normal (Fig. 4) and appeared somewhat more uniform from subject to subject.

Normal man was taken as a reference point in studies of iron absorption. It seemed important to define normality insofar as possible as it is related to erythropoiesis and body iron content including normal red cell, plasma, and marrow iron values. In such subjects, mean absorption of ferrous salts ranged between 2 and 8 mg. as the dose of iron was increased from 20 to 400 mg. (Fig. 3). One point of interest was the variation in absorption between individual normal subjects. While the explanation for this may lie in changes too subtle to measure, it does emphasize the difficulty of evaluating absorption in the normal subject unless comparative measurements between two compounds are made.

Both the status of iron stores and the level of erythropoiesis have been shown to influence iron absorption. The increased absorption of both iron salts and dietary iron in iron deficiency has been well established.[2, 17] Studies here in animals also indicated depressed iron absorption when iron stores were increased above normal. To what extent this may apply within the narrower limits of expansion and contraction of the normal iron stores is not clear. Available evidence suggests that once the anemia of iron deficiency has been corrected, iron absorption returns to a normal base line despite depleted stores.[18, 19] More data are required on this point.

The second important factor influencing absorption is the activity of erythroid marrow. In the present study, absorption has been shown to be either increased or decreased in direct relationship to the level of erythropoiesis in experimental animals given iron salts. These findings support previous reports of increased absorption related to increased erythropoiesis in experimental animals.[20, 21] In the phlebotomized but otherwise normal subject, absorption has

been shown to be consistently elevated (Fig. 1). The lack of any relationship between iron absorption and anemia per se is indicated by clinical studies in a following report.[22]

In summary, a variety of experiments has provided abundant evidence to indicate that interplay between body iron content and erythropoietic activity affect iron absorption.

The effectiveness of oral iron in treatment of iron deficiency anemia is indicated by comparing the amount of iron which may be absorbed with the needs of the marrow for erythropoiesis. These marrow requirements, estimated at 25 to 50 mg. per day,[23] are met by a single dose of 150 to 200 mg. of iron. Comparative studies of different forms of iron indicate that iron must be in a form available to the upper intestinal tract, and that ascorbic acid* in doses of 100 to 1,000 mg. and cobalt in daily dosage of 80 to 120 mg. over one to two weeks do not offer any significant advantage in iron therapy. A citrate-calcium-chelate of iron was shown to be nearly as well absorbed as ferrous sulfate. While these absorption data have no bearing on the tolerance of various preparations, they are consistent with many observations in the literature that ferrous salts in a form available to the upper intestine are the most suitable forms of medicinal iron, and a daily dosage of 0.2 to 0.3 mg. iron per kilogram, equivalent to 0.2 Gm. exsiccated ferrous sulfate 3 times per day, is usually adequate.

Certain speculations concerning the mechanism of iron absorption seem justified. The role of iron stores and erythropoiesis in modifying absorption has been shown. The time relationship of erythroid marrow and increased iron absorption is of particular interest. Following phlebotomy marrow iron uptake increases within 24 hours,[8] while absorption shows a lag of several days. The connection between marrow and intestinal mucosa is not through the plasma iron or hemoglobin level. Another mechanism whereby the needs of the bone marrow are felt by the mucosa must be operative, although this has not been directly demonstrated.

SUMMARY

Iron absorption has been quantitated by a double isotope method, by stool recovery and by comparative measurements of iron absorption from two compounds as they appeared within the red cell mass. Employing these isotope measuring techniques, the regulation of absorption of small and large doses of iron has been studied.

Both the size of iron stores and the rate of erythropoiesis have been shown to influence the absorption of iron. The larger the iron stores the less iron absorbed whereas the greater the red cell production, the greater the amount of iron absorbed.

Absorption in normal and phlebotomized subjects and in patients with iron deficiency was measured. The effects on absorption of the amount of iron, the form in which it is administered, and of supplements were also evaluated.

*The effect of ascorbic acid would appear different when employed with therapeutic doses of iron salt as compared to its administration with food iron, when it has been shown to have a clear-cut effect in enhancing absorption.[22]

REFERENCES

1. McCance, R. A., and Widdowson, E. M.: Absorption and Excretion of Iron Following Oral and Intravenous Administration, J. Physiol. **94**: 148-154, 1938.
2. Hahn, P. F., Ross, J. F., Balfour, W. M., and Whipple, G. H.: Radioactive Iron Absorption by Gastro-Intestinal Tract, Influence of Anemia, Anoxia and Antecedent Feeding Distribution in Growing Dogs, J. Exper. Med. **78**: 169-188, 1943.
3. Huestis, R. R., Anderson, R. S., and Motulsky, A. G.: Hereditary Spherocytosis in Peromyscus I Genetic Studies, J. Hered. **47**: 225-228, 1956.
4. Giblett, E. R., Motulsky, A. G., Casserd, F., Houghton, B., and Finch, C. A.: Studies on the Pathogenesis of Splenic Anemia, Blood **11**: 1118-1131, 1956.
5. Brodish, A., and Long, C. M. H.: A Technique of Cross Circulation in the Rat Which Permits Accurate Control of Blood Volume Transfers, Yale J. Biol. & Med. **28**: 644-649, 1956.
6. Saylor, L., and Finch, C. A.: Determination of Iron Absorption Using Two Isotopes of Iron, Am. J. Physiol. **172**: 372-376, 1953.
7. Peacock, W. C., Evans, R. D., Irvine, J. W., Good, W. M., Kip, A. F., Weise, S., and Gibson, J. G., II: The Use of Two Radioactive Isotopes of Iron in Tracer Studies of Erythrocytes, J. Clin. Invest. **25**: 605-615, 1946.
8. Bothwell, T. H., Hurtado, A. V., Donohue, D. M., and Finch, C. A.: Erythrokinetics. IV. Plasma Iron Turnover as a Measure of Erythropoiesis, Blood **12**: 409-427, 1957.
9. Bothwell, T. H., and Mallett, B.: The Determination of Iron in Plasma or Serum, Biochem. J. **59**: 599-602, 1955.
10. Kitzes, G., Elvehjem, C. A., and Schuette, H. A.: Determination of Blood Plasma Iron, J. Biol. Chem. **155**: 653-660, 1944.
11. Rath, C. E., and Finch, C. A.: Sternal Marrow Hemosiderin, J. Lab. & Clin. Med. **33**: 81-86, 1948.
12. Hahn, P. F., Bale, W. F., Lawrence, E. O., and Whipple, G. H.: Radioactive Iron and Its Metabolism in Anemia: Its Absorption, Transportation and Utilization, J. Exper. Med. **69**: 739-753, 1939.
13. Dubach, R., Callender, S. T. E., and Moore, C. V.: Studies in Iron Transportation and Metabolism; Absorption of Radioactive Iron in Patients With Fever and With Anemias of Varied Etiology, Blood **3**: 526-540, 1948.
14. Jeffrey, M. R., Freundlich, H. F., Jackson, E. B., and Watson, D.: The Absorption and Utilization of Radioiron in Rheumatoid Disease, Clin. Sc. **14**: 395-406, 1955.
15. Bothwell, T. H., Callender, S., Mallett, B., and Witts, L. J.: The Study of Erythropoiesis Using Tracer Quantities of Radioactive Iron, Brit. J. Haemat. **2**: 1-16, 1956.
16. Finch, C. A., Coleman, D., and Bothwell, T. H.: Unpublished observations.
17. Moore, C. V.: The Importance of Nutritional Factors in the Pathogenesis of Iron-Deficiency Anemia, Am. J. Clin. Nutrition **3**: 3-10, 1955.
18. Finch, S., Haskins, D., and Finch, C. A.: Iron Metabolism. Hematopoiesis Following Phlebotomy. Iron as a Limiting Factor, J. Clin. Invest. **29**: 1078-1086, 1950.
19. Beutler, E., Drennan, W., and Bloch, M.: The Bone Marrow and Liver in Iron-Deficiency Anemia, a Histopathologic Study of Sections With Special Reference to the Stainable Iron Content, J. Lab. & Clin. Med. **43**: 427-439, 1954.
20. Stewart, W. B., Vassar, P. S., and Stone, R. S.: Iron Absorption in Dogs During Anemia Due to Acetylphenylhydrazine, J. Clin. Invest. **32**: 1225-1228, 1953.
21. Chappelle, E., Gabrio, B. W., Stevens, A. R., and Finch, C. A.: Regulation of Body Iron Content Through Excretion in the Mouse, Am. J. Physiol. **182**: 390-392, 1955.
22. Pirzio-Biroli, G., Bothwell, T. H., and Finch, C. A.: Iron Absorption. II. The Absorption of Radioiron Administered With a Standard Meal in Man, J. Lab. & Clin. Med. **51**: 37, 1958.
23. Coleman, D. H., Stevens, A. R., Dodge, H. T., and Finch, C. A.: Rate of Blood Regeneration After Blood Loss, A. M. A. Arch. Int. Med. **92**: 341-349, 1953.

Reprinted from THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, St. Louis.
Vol. 51, No. 1, Pages 37-48, January, 1958. (Printed in the U. S. A.)

IRON ABSORPTION

II. THE ABSORPTION OF RADIOIRON ADMINISTERED WITH A STANDARD MEAL IN MAN

G. PIRZIO-BIROLI, M.D., T. H. BOTHWELL, M.D., AND C. A. FINCH, M.D.
SEATTLE, WASH.

IN THE preceding report[1] evidence has been presented that iron absorption is influenced by at least two factors, i.e., the state of the body iron stores and the erythropoietic activity of the marrow. These conclusions were drawn from studies performed in both laboratory animals and human subjects. In an attempt to minimize the numerous variables attendant upon such an investigation, all experiments were done with subjects in the fasting state and soluble iron salts were used. With this general information on iron absorption in hand, it seemed of importance to evaluate the absorption of iron in food by individuals in a healthy state and in patients suffering from any one of a variety of diseases affecting iron metabolism.

MATERIALS AND METHODS

Subjects.—The *normal group* consisted of 8 young men between the ages of 20 and 40 years, 6 patients with psychoses, and 6 patients with other illness not affecting hematopoiesis. These patients were chosen on the basis of a normal history relative to iron metabolism, their state of good health, normal hematologic studies, and, when possible, the demonstration of adequate iron stores by marrow examination.

An *iron deficient group* consisted of 19 anemic patients who fulfilled the diagnostic criteria of having an absence of marrow hemosiderin and a depressed plasma iron level. Fourteen had microcytosis and 14 of 17 had hypochromia. Plasma iron was below 50 mg. per cent in all except one (subject 2) who had been placed on iron therapy 3 days preceding the determination. These patients were further divided into two groups, an uncomplicated and a complicated group. The uncomplicated was composed of those patients with no associated illness which might have altered iron metabolism and whose anemia responded promptly to oral iron. The complicated group consisted of those patients whose iron deficiency state, while fulfilling the criteria for iron deficiency, was refractory to oral iron therapy or those who had some disease which might be anticipated to alter absorption.

The *group with increased erythropoiesis* included 15 patients with various hematologic disorders, all having in common increased red cell marrow activity. Reticulocyte counts are included in this group to indicate the level of effective erythropoietic activity.

Decreased erythropoiesis was represented by 4 patients, all with marked reticulocytopenia and a significant degree of anemia; 3 of the 4 patients required transfusions.

Iron storage disease (idiopathic hemochromatosis) was represented by 5 patients who had been so diagnosed through histologic examination of liver biopsies.

Procedure.—Subjects were questioned concerning prior oral iron intake, diet, blood donations or transfusions, and associated disease. Blood studies were performed which included red cell count, hematocrit, hemoglobin, reticulocyte count, serum iron, and, when possible, marrow aspiration for hemosiderin.

From the Department of Medicine, School of Medicine, University of Washington, Seattle, Wash.

This investigation was supported by a research grant from the United States Atomic Energy Commission (Contract AT-[45-1]-218).

Received for publication May 6, 1957.

The iron absorption study was done at noon. Canned food materials were obtained for the test, and the same manufacturer's lot was used throughout the experiment. The test meal consisted of corned beef hash (200 Gm.), applesauce (200 Gm.), tomato juice (200 Gm.), soda crackers (13 Gm.), and cocoa (3 Gm.). The iron content of the meal was found by analysis to be 4.6 mg. A tracer dose of 50 μc Fe^{55} Cl^{+++} was administered halfway through the meal.

The absorption of radioiron from this meal was measured by the double isotope method of Saylor and Finch[2] in all studies except for those on 2 patients (61 and 50) who had negligible erythropoietic activity. The method consisted of administering one isotope of iron by mouth as ferric chloride and the other, bound to fresh plasma, intravenously. Two weeks after the test meal, samples of blood were obtained and a quantity sufficient to give adequate counts was electroplated on copper planchettes. The per cent of absorption was determined by comparing the relative amounts of the two isotopes in the blood to the doses administered.

A second method, designed to determine comparative absorption of iron on two occasions, was employed. Here, one isotope of iron was given with a test meal on one day, and a second isotope was administered with a test meal on the next day. This permitted comparison between a control absorption study and the situation to be evaluated. Further details of these methods and the stool recovery technique of measuring absorption are provided in the preceding article.[1]

Plasma iron was estimated by the method of Bothwell and Mallett,[3] or Kitzes, Elvehjem, and Schuette.[4] Bone marrow iron was estimated from sternal aspirate by the method of Rath and Finch.[5] Hemoglobin was determined as oxyhemoglobin in an Evelyn colorimeter and hematocrits were spun at 1,800 g for 30 minutes. Reticulocytes were stained with cresyl blue and the number per 1,000 erthrocytes enumerated.

TABLE I. ABSORPTION OF IRON GIVEN WITH A TEST MEAL IN NORMAL SUBJECTS

PATIENT NO.	SEX	PCV (PER CENT)	MCV CUμ	MCHC (PER CENT)	RETICULOCYTES (%)	PLASMA IRON (μG%)	MARROW FE (GRADE)	IRON ABSORPTION (PER CENT)
A. Normal Students								
35	M	48	89	33	1.5	115	-	16.6
34	M	47	89	33	1.7	154	1	2.6
31	M	46	87	36	1.4	137	1	12.8
32	M	45	84	34	1.4	102	-	3.6
109	M	47	86	33	1.0	125	-	2.9
110	M	46	91	30	1.2	109	-	4.1
111	M	46	98	31	1.8	135	-	1.7
112	M	47	98	32	0.2	91	-	6.5
B. Patients Convalescent From Nonhematological Disorders								
1	M	47	90	33	0.8	90	1	10.5
15	F	37	86	31	0.7	114	3	0.6
16	F	46	94	33	2.1	90	2	2.0
17	F	42	80	33	0.7	114	1	0.5
18	F	43	74	31	2.0	70	3	3.8
9	M	45	84	32	0.4	90	2	1.5
*C. Subjects With Psychoses**								
21	M	46	85	34	1.1	95	-	3.3
12	M	46	88	35	-	62	-	7.1
22	M	47	85	34	0.9	160	-	6.2
14	M	48	96	30	0.7	105	-	6.1
20	M	48	83	33	1.1	101	-	6.9
13	M	48	99	33	2.2	136	-	7.0
D. Patients With Pernicious Anemia in Remission								
54	M	40	88	38	1.2	123	3	5.5
57	F	43	80	33	1.1	74	-	0.6
56	F	38	78	35	1.3	82	-	6.5
55	F	38	89	37	1.1	59	-	8.6

*In contrast to other groups when radioiron was given with meals, in Group C radioiron was administered immediately preceding the regular hospital meal.

RESULTS

The absorption of radioiron given with a standard meal to 20 patients considered normal and 4 patients with pernicious anemia in remission is summarized in Table I. The average absorption was 5.3 per cent (S.D. ± 4 per cent). The maximal absorption was 16.6 per cent and the minimal absorption 0.5 per cent. There was no indication that variations from the mean in this group of 20 patients were related to age, sex, hematocrit, reticulocyte count, plasma iron, or marrow iron. No decrease of absorption was found in 4 patients with gastric achlorhydria (Group D).

To indicate to what extent composition of the diet might alter iron absorption from this test meal, supplements of ascorbic acid in amounts of 200 to 1,000 mg. were given to 4 subjects. While absorption was not appreciably increased with the 200 mg. dose, with amounts of 500 to 1,000 mg., absorption was doubled when compared to the control value of the same subjects (Table II).

TABLE II

SUBJECT	ASCORBIC ACID (MG.)	ABSORPTION (% OF ADMIN. IRON)	
		CONTROL	WITH ASCORBIC
Lo.	200	3.2	4.1
Ho.	500	4.2	9.0
Ha.	750	1.9	4.3
Bo.	1000	10.6	22.1

Iron Deficiency.—Absorption of radioiron from the test meal was studied in 7 female patients and in 1 male patient who had uncomplicated iron deficiency anemia (Table III, *A*). Absorption was increased above the average normal of 5.3 per cent in all these patients. The average absorption in the uncomplicated iron deficient group was 21.6 per cent.

A second group of 11 subjects was of interest in that absorption was depressed despite iron deficiency anemia (Table III, *B* and Fig. 1). Complicating conditions included malabsorption syndrome (nontropical sprue), myxedema, infection, gastrointestinal neoplasm, and previous gastrointestinal surgery. In 3 patients there was no response to oral iron but response did follow intravenous saccharated iron. In two patients who had had partial gastrectomy there was a response to a therapeutic dosage of oral iron salts. One patient (75) with no detectable associated disorder and with moderately severe iron deficiency anemia did not respond to oral iron, but had a normal response to intravenous therapy.

Increased Erythropoiesis.—Fifteen patients with increased marrow erythroid activity were studied (Table IV). Eleven of these had not been given oral or parenteral iron or transfused blood. They were considered to have normal body iron stores although it was appreciated that a shift in iron had occurred from red cells to tissues. The first 7 patients had pernicious anemia in relapse. More detailed hematopoietic studies, reported elsewhere on similar patients who also had B_{12} deficiency, indicated that despite low circulating reticulocyte counts total marrow erythropoiesis was approximately three times normal.[6] Absorption in the group of 7 patients averaged 5.7 per cent. The additional 4 patients with hereditary hemolytic anemia averaged 9.7 per cent

TABLE III. IRON DEFICIENCY

PATIENT NO.	DIAGNOSIS	SEX	PCV	MCV	MCHC	RETICULOCYTES	PLASMA P.I.	MARROW	ABSORPTION	RESPONSE TO ORAL FE THERAPY	RESPONSE TO ORAL FE I.V.
A. Uncomplicated											
58	Intestinal bleeding	F	38	80	25	0.9	27	0	38.7	+	
47	Hypermenorrhea	F	28	71	-	2.8	35	0	9.9	+	
1	Hypermenorrhea	F	27	78	29	2.7	20	0	22.0	+	
80	Bleeding peptic ulcer	M	26	114	33	1.2	25	0	22.0	+	
2	Multiple pregnancies on iron therapy	F	29	74	28	6.9	69	0	21.0	+	
76	Hypermenorrhea	F	35	55	25	-	7	0	17.6	+	
90	Bleeding ulcer	F	26	60		1.3	27	0	23.6	+	
70	Hypermenorrhea	F	29	89	25	5.6	10	1	17.7	+	
B. Complicated											
5	Idiopathic malabsorption	F	29	67	27	22.0	17	0	0.6	0	+
	Idiopathic malabsorption	F	25	71	28	2.1	29	0-1	0.5	0	+
49	Cancer of colon	F	27	70	30	2.0	20	0	2.7		
25	Low grade infection	F	24	82	30	1.2	36	0	2.6	0	+
39	Myxedema	F	30	65	33	1.6	20	0	2.7		+ (after thyroxin)
7	Postgastrectomy	F	33	61	28	-	32	-	0.6		+
68	Postgastrectomy	M	28	60	30	1.2	13	0	6.5 (suboptimal)		+
59	Postgastrectomy	M	28	71	28	0.8	18	0	5.0		
74	Postgastrectomy	M	21	54	35	2.2	12	0	1.3 (transfused)		
73	Postgastrectomy and gastrointestinal bleeding	F	28	80	30	0.6	24	0	2.3 (suboptimal)	+	+
75	Malabsorption of iron and uterine myomata	F	35	66	19	1.3	22	0	2.3	0	+
131	Myxedema	F	28	69		1.8			1.4		

TABLE IV. INCREASED ERYTHROPOIESIS

PATIENT NO.	DIAGNOSIS	SEX	PCV (%)	MCV	MCHC	RETICULOCYTES	P.I. MCG %	MARROW GRADE	ABSORP. (%)
A. Without Associated Iron Loading									
96	Pernicious anemia in relapse	F	36	118	--	0.6	80	-	0.3
102	Pernicious anemia in relapse	F	29	112	34	0.5	124	2+	3.4
108	Pernicious anemia in relapse	M	17	102	30	0.4	141		6.8
106	Pernicious anemia in relapse	F	34	-	--	0.8	42	2+	0.8
94	Pernicious anemia in relapse	M	26	126	30	0.7	197	2+	22.5
93	Pernicious anemia in relapse	F	15	134	37	-	162	3+	5.7
120	Pernicious anemia in relapse	M	26.5	101	36	0.3	137		0.6
121	Hereditary nonspherocytic anemia	M	41.4	-	--	7.2	60	3+	8.4
117	Hereditary nonspherocytic anemia	M	--	-	--	1.9	159		5.6
114	Hereditary spherocytic anemia	M	37	86	--	2.1	147		3.5
101	Hereditary nonspherocytic anemia	M	37	85	34	12.0	129		21.3
B. With Associated Iron Loading									
98	Sickle cell anemia (last transfusion 2 months prior)	F	29	82	33	5.6	166	5+	13.5
122	Paroxysmal nocturnal hemoglobinuria	M	36.5	96.7	31.8	13.6	191	5+	2.5
115*	Thalassemia minor	F	37	76	28	1.9	84	4+	1.0
51*	Thalassemia minor	F	29	71	31	2.6	70	5+	1.1

*Survival time by Cr^{51} technique was ½ time of 13½ days and 14½ days.

TABLE V. DECREASED ERYTHROPOIESIS

PATIENT NO.	DIAGNOSIS	SEX	PCV (%)	MCV	MCHC	RETICULOCYTES	PLASMA IRON (μg %)	MARROW FE GRADE	ABSORP. (%)
A. Without Associated Iron Loading									
61	Aplastic anemia	M	15	81	33	0.1	87		1.4*
B. With Associated Iron Loading									
46	Uremia 44 Tx	M	19	101	36	0.1	85	4	0.13
116	Refractory anemia 7 Tx	F	30	90	30	0.3	46	4	0.59
50	Refractory anemia 200 Tx	M	22	81	39	0.2	260	-	0.0*

*Determination done by stool method. (These patients had no demonstrable activity in circulating blood.)

absorption. Four patients had been repeatedly transfused and had heavy hemosiderin deposits in the marrow. Absorption studies in these patients showed a low absorption in 3 and a somewhat elevated absorption in the fourth, with a group average of 4.5 per cent. As shown in Fig. 2, the absorption in this group of 15 patients with hemolytic anemia was only slightly higher than the normal patients studied.

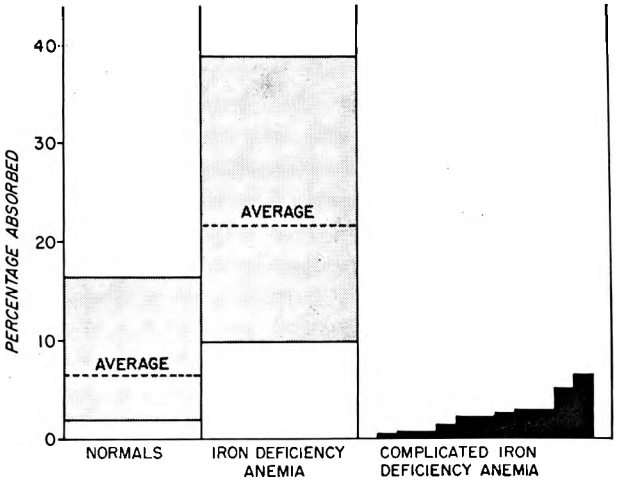


Fig. 1.—Absorption in complicated patients with iron deficiency anemia is shown by the solid areas on the right, as compared with absorption observed in normal subjects and patients with uncomplicated iron deficiency anemia.

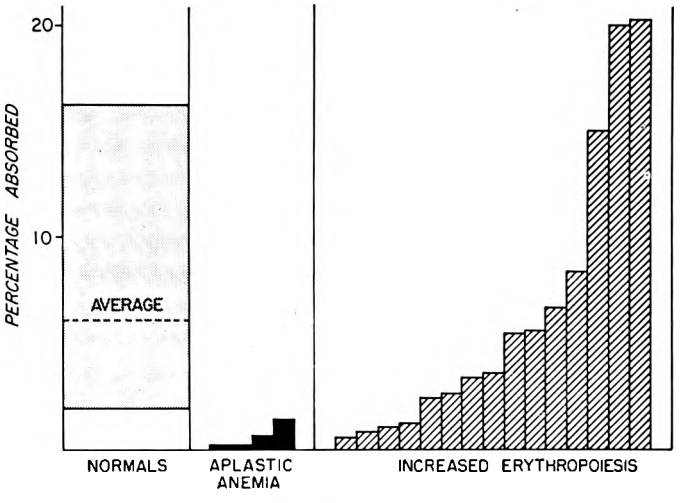


Fig. 2.—Relation of iron absorption to erythropoiesis. The normal range of absorption is shown on the left. Solid black areas indicate the per cent of absorption in 4 patients with aplastic anemia. Absorption of iron by 15 patients with increased erythropoiesis is indicated by the cross-hatched columns on the right.

Decreased Erythropoiesis.—Four patients with decreased red cell production were studied (Table V). One of these patients had had no previous transfusions. The others had been transfused frequently. Absorption, measured by

the double isotope method in all and the stool method as well in the 2 patients who had a negligible red cell function (61 and 50), indicated marked depression in iron absorption in all 4 patients.

Idiopathic Hemochromatosis.—Absorption studies (Table VI) were carried out before treatment in 5 patients with iron storage disease. Their absorption varied from 4.7 to 17 per cent (average of 10.6 per cent). While this is not greatly above the normal it becomes particularly significant when the large iron stores of these patients are considered. Furthermore, absorption was increased to 29.7 per cent following multiple phlebotomies at a time when the serum iron was still elevated or normal and tissue iron stores were still greatly increased. This increase was greater than that seen in cases of noncomplicated iron deficiency anemia or in the group of phlebotomized normal subjects mentioned in the previous article.

TABLE VI. HEMOCHROMATOSIS

PATIENT	DIAGNOSIS	SEX	PCV (%)	MCV	MCHC	RETICULOCYTES	P.I. MCG %	MARROW FE GRADE	ABSORP.
A. Before Phlebotomy Treatment									
48		M	42	90	33	1.0	318	–	4.8
	Met.	M	42	102	33	1.9	245	4	12.9
81		M	47	92	33	1.1	260	4	17.0
124		M	46	–	–	1.2	212	–	11.4
38		M	76	90	36	0.7	285	4	7.0
B. After Repeated Bleeding, but Not Iron Depleted									
38A		M	43	87	34	2.6	105		29.7
48A		M	38	89	30	2.0	262		29.7
88		M	42	87	34	2.0	192		29.4

DISCUSSION

It is generally accepted that the amount of iron absorbed from food is dependent on both the individual's capacity to absorb iron and the "availability" of the dietary iron ingested. The absorption of food iron may differ greatly according to the type of food in which it is found. This is well documented by the studies of Chodos and associates,[7] Walsh and associates,[8] and Moore and associates[9] in which radioiron was incorporated into several food articles in its natural form and its absorption measured. However, these reports indicate the absorption of iron from only a few foods and no attempt has been made to label an entire diet. Beyond this, the blocking effect of such substances as phosphates[10] and phytates[11] and the enhancement of absorption by reducing substances such as ascorbic acid must be considered.[12]

It becomes apparent, if one assumes that isotope methods are essential for precise measurement of absorption, that not only is the isotope labeling of an entire diet a formidable task, but that an arbitrary diet must be selected regardless of the extent of labeling. After considering these problems it was decided to employ a standard meal to which Fe^{55} Cl was added in tracer amount. It was assumed that this iron would mix with a considerable portion of food iron which would be converted to chloride salt within the stomach. Thus, while not representing all of the dietary iron, this would represent a large portion of ingested food iron.

The average absorption in normal subjects given this tagged test meal (Table I) was 5.3 per cent, which might be extrapolated to a daily absorption of 0.8 mg. from a diet containing a total of 16 mg. of iron. The results are in general similar to other studies employing food and radioiron techniques. Sharpe and associates[11] found an average absorption of 4.4 per cent in adolescent boys fed a meal of milk, rolled oats, tomato juice, white bread, omelet, and radioiron. Chodos and associates[7] found 0.5 to 5 per cent absorption of radioactive iron from eggs, 0.8 to 1.6 per cent from Swiss chard, and 0.8 to 0.9 per cent from beet greens. Moore and Dubach[9] found 1 to 8 per cent from tagged eggs and an average of 6 per cent from liver. Walsh and associates[8] found 3 per cent from hemoglobin iron and 8 per cent from liver and kidneys. These figures are also in general agreement with the excretion studies of Dubach and co-workers[13] and the careful balance studies of Johnston and associates[14] which estimate an iron turnover in man of approximately 0.5 to 1.5 mg. per day.

A comparison of the present results with those obtained previously when iron alone was given[1] indicates that the presence of food reduces absorption by approximately one half. This effect has been pointed out by others.[7, 10, 11] Studies with ascorbic acid reported here (Table II) and presented by others[12] suggest that the action of ascorbic acid is concerned at least in part with this blocking mechanism of food since ascorbic acid is less effective with iron salts alone.[1]

Results in 4 subjects with histamine-fast achlorhydria show no significant deviation from the normal, lending further support to the contention of Moore and Dubach[9] that gastric acidity plays no demonstrable role in the absorption of iron from food.

Variations from 0.5 to 16.6 per cent in absorption from this test meal observed between individuals in the normal group are unexplained, since no correlation was observed between these results and the hematologic measurements, plasma iron level, or marrow iron. Such differences, however, do point out the difficulty of evaluating factors affecting iron absorption in the normal subject.

Erythropoietic activity has been shown to affect absorption of iron salts.[1] With decreased red cell production, iron absorption was depressed, whereas increased erythropoiesis was associated with enhanced iron uptake. Consistent with these results the absorption of iron from food was depressed in 4 subjects with aregenerative anemia to between 0 and 1.4 per cent and had an associated increase in iron stores. Patients with increased erythropoiesis showed less consistent results (Fig. 2). Discounting the subjects who had increased body iron stores due to transfusion, the remainder showed an absorption of 7.2 per cent, only slightly above the normal average. This group was heavily weighted with patients having pernicious anemia in relapse. Their erythroid marrow is know to be hyperactive and their plasma iron turnover three times normal,[6] yet iron absorption was not significantly increased (Table IV, *A*).

Patients with other types of hemolytic anemia had an average absorption somewhat above the normal range (9.7 per cent as compared to the normal average of 5.3 per cent). While in better controlled animal studies it has

been possible to demonstrate in a variety of hemolytic anemias a significant increase in absorption, results in man are less convincing. It is possible that the present clinical data are inadequate, since relatively few patients with maximal erythropoiesis were studied; yet there was no obvious correlation between degree of erythropoiesis and the level of absorption (see Patients 122, 101, 94, and 106). It is also likely that increased iron stores, which have been shown to be able to counteract the effect of increased erythropoiesis on iron absorption, might be of importance in keeping absorption within normal range. In an attempt to evaluate this point, a comparison was made between patients where iron stores were not known to be enlarged through therapy (Group A of Table IV), and patients having had previous transfusions or oral iron (Group B). While absorption in Group B was 4.5 per cent as compared to 7.2 per cent in Group A, individual variations were so great as to make this of little significance. Further attempts by history and marrow examination for hemosiderin were not helpful in bringing individual variations into line. These studies, however, would suggest that iron overload from diet is unlikely in hemolytic anemia and that it should not occur in anemias with hypoplastic marrow. It follows that those cases reported to show such overload may be presumed to be the result of long continued oral iron therapy or blood transfusions.

The second internal factor of some consequence in the regulation of iron absorption is the state of iron stores.[1] In the presence of iron deficiency anemia, where there was considered to be both depleted iron stores and increased marrow capacity to assimilate iron, absorption of radioiron from the test meal was increased. The increased absorption of iron in iron deficiency has been consistently demonstrated both with iron salts[15, 16] and with food iron.[7, 12] In our own studies[1] absorption of 4 to 5 mg. of iron salts in the normal subject was estimated at about 15 per cent as compared to 80 per cent in the iron deficient subject. At a similar iron dose, absorption from food was 5.3 per cent in the normal and approximately 22 per cent in the iron deficient patient. It is also of interest to estimate daily food iron absorption from the later data. Assuming a total dietary iron intake of 15 mg. per day and an absorption of 22 per cent, daily absorption would be 3.3 mg. This is quite similar to the figure of 3 to 4 mg. per day calculated previously from the iron balance of phlebotomized patients with polycythemia vera.[17]

While experimental evidence is conclusive in showing a depression of absorption as a result of iron overload, no uncomplicated patients were available on whom such observations could be made. However, the data on patients with combined hemolytic anemia and iron overload or decreased erythropoiesis and iron overload tend to substantiate this. Information now available does not permit a conclusion as to whether absorption is altered by small increases or decreases in iron stores.

While an extreme degree of iron overload exists in idiopathic hemochromatosis, this must be regarded as a special condition. Here it has been recognized that excessive absorption is responsible for the iron surplus, and this excessive absorption has been demonstrated.[7, 8, 18] Observations on the 5 subjects studied here indicate that despite massive iron stores and elevated plasma iron, the

average absorption was over 10 per cent. Following phlebotomy with resultant increase in erythropoiesis, the average absorption was increased to 29 per cent in spite of the fact that iron stores were still excessive and that plasma iron level was normal or elevated. It should be noted that this figure exceeds that found in uncomplicated iron deficiency anemia or in phlebotomized subjects. These studies, in keeping with observations of Chodos and associates[7] and Peterson and co-workers,[19] indicate that the state of iron stores and the level of erythropoietic activity may influence absorption in the patient with hemochromatosis as well as in the normal subject. The basic level of absorption in idiopathic hemochromatosis, however, is considerably above that found in normal man and accounts for the gradual accumulation of excessive iron in the body.

Factors other than decreased erythropoiesis or increased iron stores may decrease iron absorption. Such decreases are more clearly demonstrated in the phlebotomized, or better yet, in the iron deficient subject. In this study there were 11 patients who did not show the expected increase characteristic of iron deficiency. This was a selected group and does not in any way reflect the incidence of malabsorption of iron. Etiologic factors included (1) idiopathic steatorrhea, which has been previously demonstrated to interfere with iron absorption,[20] and to be responsible for perhaps one half of the instances of refractiveness to oral iron therapy; (2) infection, which has been shown to block not only mucosal transfer of iron,[21, 22] but the transportation of iron across the placenta and reticuloendothelial cells[23]; (3) myxedema, where the defect is reversible with therapy; and (4) the partial gastrectomy state, where a curious discrepancy exists between response to oral iron salts[24] and lack of absorption of food iron.[7] In view of the importance of time of exposure to the mucosa,[25] it might be suggested that the rapid transit of iron in a bolus of food might produce this abnormality.

Finally, a study of the absorption of iron in the various patients studied showed no correlation with the hemoglobin level of the patient. Thus it may be concluded that anemia per se plays no role in the regulation of iron absorption.

SUMMARY

The absorption of iron in food was studied by the administration of a test meal to which a tracer dose of $FeCl_3$ was added. A double isotope technique was employed to quantitate absorption.

The absorption of iron from food by both normal and iron deficient subjects was lower than that seen when iron salts alone were given. Supplemental ascorbic acid diminished this difference.

In keeping with previous studies employing iron salts, it was found that absorption from diet also varied inversely with iron stores and directly with the degree of erythropoiesis. However, increases in hemolytic anemia were usually not significant, and it seems unlikely that iron overload from dietary sources will occur in either aplastic or hemolytic anemia.

Studies in idiopathic hemochromatosis before and after phlebotomies indicate excessive absorption in these patients under all circumstances.

In addition to excessive iron stores and decreased erythropoiesis there are conditions which may act at the mucosal level to impair iron absorption. Idiopathic steatorrhea, infection, myxedema, and the postgastrectomy state have all been shown to result in decreased absorption by the iron deficient patient.

REFERENCES

1. Bothwell, T. H., Pirzio-Biroli, G., and Finch, C. A.: Iron Absorption. I. Factors Influencing Absorption, J. Lab. & Clin. Med. 51: 24, 1958.
2. Saylor, L., and Finch, C. A.: Determination of Iron Absorption Using Two Isotopes of Iron, Am. J. Physiol. 172: 372-376, 1953.
3. Bothwell, T. H., and Mallett, B.: The Determination of Iron in Plasma or Serum, Biochem. J. 59: 599-602, 1955.
4. Kitzes, G., Elvehjem, C. A., and Schuette, H. A.: Determination of Blood Plasma Iron, J. Biol. Chem. 155: 653-660, 1944.
5. Rath, C. E., and Finch, C. A.: Sternal Marrow Hemosiderin, J. Lab. & Clin. Med. 33: 81-86, 1948.
6. Finch, C. A., Coleman, D. H., Motulsky, A. G., Donohue, D. M., and Reiff, R. H.: Erythrokinetics in Pernicious Anemia, Blood 11: 807-820, 1956.
7. Chodos, R. B., Ross, J. F., Apt, L., Pollycove, M., and Halkett, J. A. E.: The Absorption of Radioiron Labelled Foods and Iron Salts in Normal and Iron Deficient Subjects and in Idiopathic Hemochromatosis, J. Clin. Invest. 36: 314-326, 1957.
8. Walsh, R. J., Kaldon, I., Brading, I., and George, E. P.: The Availability of Iron in Meat: Some Experiments With Radioactive Iron, Australasian Ann. Med. 4: 272-276, 1955.
9. Moore, C. V., and Dubach, R.: Observations on the Absorption of Iron From Foods Tagged With Radioiron, Tr. A. Am. Physicians 64: 245-256, 1951.
10. Hegsted, D. M., Finch, C. A., and Kinney, T. D.: The Influence of Diet on Iron Absorption. II. The Interrelation of Iron and Phosphorous, J. Exper. Med. 90: 147-156, 1949.
11. Sharpe, L. M., Peacock, W. C., Cooke, R., and Harris, R. S.: The Effect of Phytate and Other Food Factors on Iron Absorption, J. Nutrition 41: 433-446, 1950.
12. Moore, C. V.: The Importance of Nutritional Factors in the Pathogenesis of Iron-Deficiency Anemia, Am. J. Clin. Nutrition 3: 3-10, 1955.
13. Dubach, R., Moore, C. V., and Callender, S. T.: Studies in Iron Transportation and Metabolism. IX. The Excretion of Iron as Measured by the Isotope Technique, J. Lab. & Clin. Med. 45: 599-615, 1955.
14. Johnston, F. A., Frenchman, R., Boroughs, E. D.: The Iron Metabolism of Young Women on Two Levels of Intake, J. Nutrition 38: 479-487, 1949.
15. Dubach, R., Callender, S. T., and Moore, C. V.: Studies in Iron Transportation and Metabolism; Absorption of Radioactive Iron in Patients With Fever and With Anemias of Varied Etiology, Blood 3: 526-540, 1948.
16. Balfour, W. M., Hahn, P. F., Bale, W. F., Pommerenke, W. T., and Whipple, G. H.: Radioactive Iron Absorption in Clinical Conditions: Normal, Pregnancy, Anemia, and Hemochromatosis, J. Exper. Med. 76: 15-30, 1942.
17. Finch, S., Haskins, D., and Finch, C. A.: Iron Metabolism. Hematopoiesis Following Phlebotomy. Iron as a Limiting Factor, J. Clin. Invest. 29: 1078-1086, 1950.
18. Bothwell, T. H., van Doorn-Wittkampf, H. V., Du Preez, M. L. and Alper, T.: The Absorption of Iron; Radioiron Studies in Idiopathic Hemochromatosis, Malnutritional Cytosiderosis, and Transfusional Hemosiderosis, J. Lab. & Clin. Med. 41: 836-848, 1953.
19. Peterson, R. E., and Ettinger, R. H.: Radioactive Iron Absorption in Siderosis (Hemochromatosis) of the Liver, Am. J. Med. 15: 518-524, 1953.
20. Badenoch, J., and Callender, S. T.: Iron Metabolism in Steatorrhea; The Use of Radioactive Iron in Studies of Absorption and Utilization, Blood 9: 123-133, 1954.
21. Gubler, C. J., Cartwright, G. E., and Wintrobe, M. M.: The Anemia of Infection. X. The Effect of Infection on the Absorption and Storage of Iron by the Rat, J. Biol. Chem. 184: 563-574, 1950.
22. Hahn, P. F., Bale, W. F., and Whipple, G. H.: Effects of Inflammation (Turpentine Abscess) on Iron Absorption, Proc. Soc. Exper. Biol. & Med. 61: 405-406, 1946.
23. Bothwell, T. H., Noyes, W. D., and Finch, C. A.: Reticuloendothelial Handling of Iron. (To be published.)
24. Callender, S. T.: Personal Communication.
25. Hahn, P. F., Ross, J. F., Balfour, W. M., and Whipple, G. H.: Radioactive Iron Absorption by Gastro-Intestinal Tract, Influence of Anemia, Anoxia and Antecedent Feeding Distribution in Growing Dogs, J. Exper. Med. 78: 169-188, 1943.

THE ROLE OF FERRITIN IN IRON ABSORPTION

R. W. CHARLTON
M.D., B.Sc. W'srand, M.R.C.P.E.

P. JACOBS
M.B. W'srand

J. D. TORRANCE
M.B. W'srand

T. H. BOTHWELL
M.B. W'srand, M.R.C.P.

Preliminary Communication

reprinted from THE LANCET, *October* 12, 1963, *pp.* 762-764

THE ROLE OF FERRITIN IN IRON ABSORPTION

SEVERAL workers have shown that ferritin accumulates in the mucosal cells of the gastrointestinal tract when large amounts of iron salts are given to laboratory animals.[1–3] On this basis, Granick [1] proposed that the accumulation of ferritin prevented excessive absorption from the lumen. Although attractive, the theory has fallen into disrepute because later workers have reported significant absorption even when large amounts of mucosal ferritin are present.[3] In view of these findings, it has been suggested that ferritin may actually augment absorption by temporarily storing iron before delivering it into the plasma.[3] These different concepts, however, are based on experimental data obtained when pharmacological amounts of iron were given, and attempts to identify the presence of ferritin with physiological quantities have so far been unsuccessful.[4 5] In view of these difficulties, a study was undertaken using a combination of immunological and isotopic techniques to determine whether ferritin is formed in mucosal cells during the absorption of small doses of iron, and, if so, to attempt to define its role in the normal absorptive process.

MATERIAL AND METHODS

Iron salts, labelled with ^{59}Fe and dissolved in 1 ml. of distilled water, were administered to fasting rats of the Wistar strain by œsophageal cannula. The animals were killed at various intervals after administration of the iron, and, after the whole gastrointestinal canal had been removed intact, absorption into the plasma was assessed by whole-body counting. The small intestine was washed with saline and homogenised in 0·1 *M* edetic-acid solution buffered to pH 7·0. (Preliminary studies established that the chelate prevented the binding of free ^{59}Fe to ferritin in vitro.) The radioactivity of an aliquot of the intestinal homogenate was measured. After heating to 70°C

1. Granick, S. *Science*, 1946, **103**, 107.
2. Gabrio, B. W., Salomon, K. *Proc. Soc. exp. Biol. Med., N.Y.* 1950, **75**, 124.
3. Heilmeyer, L. *in* Iron in Clinical Medicine (edited by R. O. Wallerstein and S. R. Mettier). Berkeley, 1958.
4. Brown, E. B., Rother, M. *Blood*, 1961, **18**, 780.
5. Beutler, E. Clinical Disorders of Iron Metabolism. New York, 1963.

and centrifuging at 9000*g* for ten minutes, the ferritin present in the heat supernatant solution was precipitated with an antiserum to horse-spleen ferritin prepared in rabbits.[6] (The cross-specificity of horse-spleen ferritin and rat-liver ferritin has been established.)[7] To facilitate the manipulation of the very small amounts of labelled ferritin present, purified horse-spleen ferritin was added as a carrier. After incubation at 37°C for an hour and centrifugation, the deposit was washed twice with distilled water. The radioactivity of the washed deposit then provided a measure of the ^{59}Fe-labelled ferritin.

A development of the agar-gel-diffusion plate technique[8] was also used to demonstrate the presence of radioactive ferritin in the duodenal homogenate. The antiserum was placed in the central well, and duodenal homogenate in two of the peripheral wells, while the remaining four wells were filled with a solution of radioactive horse-spleen ferritin. After the precipitation bands between the ferritin solution and the antiserum had formed, the gel was washed for three days with frequent changes of distilled water, and then it was allowed to dry, forming a paper-thin sheet. An autoradiograph was then prepared.

RESULTS

The presence of radioactive ferritin in duodenal homogenate one hour after the administration of 1 μg. of iron as $^{59}FeCl_3$ was established with the gel-diffusion technique. Although no visible lines of precipitation could be seen opposite the wells containing the duodenal homogenate, the autoradiograph revealed the presence of bands of radioactivity which coalesced with the bands opposite the ferritin cups (fig. 1). It was

6. Proom, H. *J. Path. Bact.* 1943, **55**, 419.
7. Mazur, A., Green, S., Carleton, A. *J. biol. Chem.* 1960, **235**, 595.
8. Bodman, J. *in* Chromatographic and Electrophoretic Techniques (edited by I. Smith). New York, 1960.

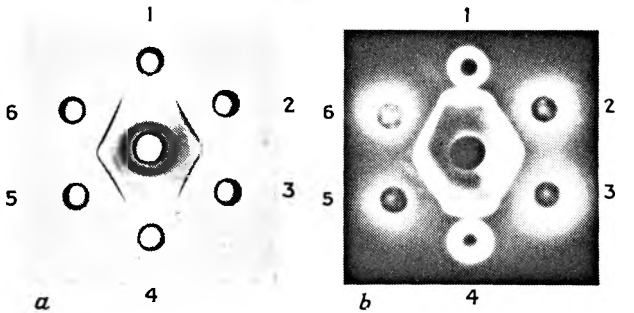


Fig. 1—Agar-gel-diffusion plate.

Wells 1 and 4 contain duodenal homogenate; wells 2, 3, 5, and 6 contain ferritin. Although no lines of precipitation can be seen opposite the wells containing the heat supernatant solution from the duodenal homogenate (*a*), the autoradiograph (*b*) reveals bands of radioactivity coalescing with the bands opposite the ferritin wells.

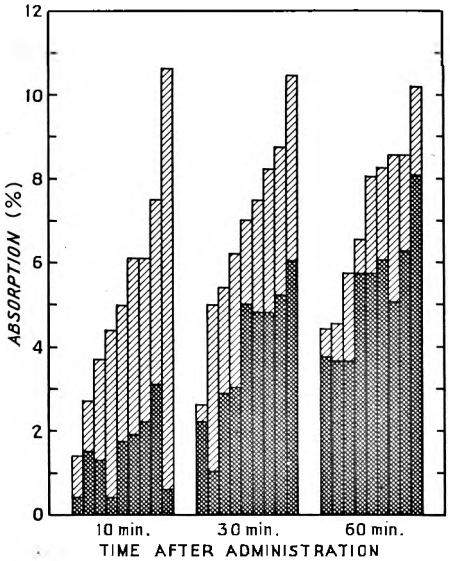


Fig. 2—Proportion of administered dose in duodenal mucosæ of a group of rats at various times after the administration of 10 μg. iron as $^{59}FeCl_3$.

Stippled = ferritin; hatched = non-ferritin iron.

concluded that ferritin labelled with isotope was present in duodenal cells after the administration of only 1 μg. of iron.

The presence of ferritin in duodenal cells at various times after administration of the iron solution was then characterised. To be able to compare the ferritin content soon after ingestion with the findings at later stages, only the upper 10 cm. of small intestine was examined. (Preliminary studies revealed that about 60% of the total mucosal uptake of iron was from this area.) When a dose of 10 μg. of iron as ferric chloride was administered, the mean proportion of the labelled iron within the washed cells precipitable with the ferritin antiserum at ten minutes was 31%. By thirty minutes it had risen to 58%, and at sixty minutes to 75%. During this period there was an equivalent decrease in the non-ferritin component (fig. 2). After four hours there was little change in either the total mucosal content of ^{59}Fe or in the proportion identifiable as ferritin, but by twelve hours the total activity had fallen to a mean of 27% of the one-hour value with a proportional fall in ferritin activity. Twenty-four hours after administration there was little residual labelled iron in the mucosa. Between four and twenty-four hours there was little, if any, increase in the amount of labelled iron which had been transferred across the mucosa into the plasma. A similar pattern of absorption was

DISTRIBUTION OF LABELLED IRON 2 HOURS AND 24 HOURS AFTER AN ORAL DOSE OF 10 μg. IN RATS PREVIOUSLY OVERLOADED WITH PARENTERAL IRON

Time	% absorbed	% in mucosa as ferritin
After 2 hours	2·6	17·3
	3·0	16·0
	1·8	14·4
	5·7	18·4
	(Mean 3·3)	(Mean 16·5)
After 24 hours	5·7	1·5
	4·1	1·5
	3·2	1·3
	5·9	1·2
	(Mean 4·7)	(Mean 1·4)

observed when 100 μg. of iron as ferric chloride was administered (fig. 3). When 1000 and 10,000 μg. doses were studied, however, the amount of radioiron absorbed into the plasma at two to four hours was much greater in proportion to the mucosal ferritin content (ratio 20/1) than with the smaller quantities (ratio 2/1).

To try to define the role of mucosal ferritin in the absorptive process more accurately, absorption was studied in animals made avid for iron by venesection. Thirty minutes after the administration of 10 μg. of iron to this group a mean of 43% of the ingested dose had been transferred into the plasma (compared with 12·7% in the control animals), but little or no labelled ferritin could be identified in the avidly absorbing mucosa.

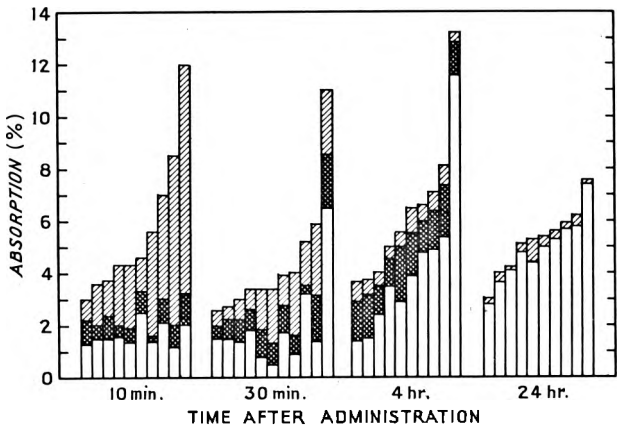


Fig. 3—Distribution of labelled iron in groups of rats at various times after the administration of 100 μg. as $^{59}FeCl_3$.

White = already absorbed; stippled = ferritin in duodenal mucosa; hatched = non-ferritin iron in duodenal mucosa

Because it appeared from these studies that mucosal ferritin was not directly involved in the transfer of iron into the plasma, it seemed feasible that a major portion of the ferritin iron might ultimately be lost with the exfoliation of the mucosal cells. This possibility was examined in rats whose avidity for iron had been decreased by parenteral iron loading. In these animals a mean of only 3·3% of a 10 μg. dose had passed into the plasma two hours after ingestion, although as much as 16·5% was present in the mucosa of the whole small intestine as ferritin at this time. Twenty-four hours after administration the mean activity in plasma was still only 4·7%, even though the mucosal ferritin content had fallen to 1·4% (see table). We concluded that the decrease in ferritin iron was due to loss into the intestinal lumen rather than to a gradual release into the plasma.

DISCUSSION

These results indicate that the iron taken up by mucosal cells when physiological amounts are administered exists in at least two forms: as ferritin and as a non-ferritin complex. Although the total activity present in mucosal cells remains relatively constant over the first hour there is a progressive change in the distribution of iron within the cell, with a fall in the concentration of the non-ferritin fraction and a corresponding rise in ferritin. Since absorption into the plasma is most active during this period it seems reasonable to suppose that the non-ferritin fraction is the component actively transporting iron through the cell. Although we did not study the nature of this fraction in the present investigation, other workers have suggested that it represents iron complexed with the aminoacids serine and glycine.[4] The iron present as ferritin remains constant between one and four hours and then steadily declines until at twenty-four hours almost all the activity has disappeared from mucosal cells. While it is possible that a proportion of the iron stored as ferritin is normally released slowly into the plasma over this period, the evidence suggests that in some circumstances at least this does not happen. In groups of rats in which the avidity for iron had been suppressed by parenteral iron loading, about 17% of an oral dose was incorporated into ferritin at two hours. At twenty-four hours almost all the ferritin had disappeared from mucosal cells, although the total amount absorbed into the animals had not altered significantly (from a mean of 3·3% to 4·7%). Since the rate of loss of labelled ferritin was similar to the known turnover-rate of intestinal cells in the rat,[9] it seems likely that the decline in ferritin activity reflected the exfoliation of the cells containing it.

9. Messier, B., Leblond, C. P. *Amer. J. Anat.* 1960, **106**, 247.

The present findings indicate that the incorporation of absorbed iron into ferritin within mucosal cells may subserve an important regulatory function when physiological amounts are given. In situations where the need for iron is increased (e.g., after repeated venesections), absorption is increased but no ferritin is formed in mucosal cells. In contrast, increased amounts of iron are incorporated into ferritin when the need for iron is reduced, and this ferritin is lost from the body as the mucosal cells exfoliate. While it is not yet clear what factors influence the particular pathway taken by iron within mucosal cells, it may well relate to the iron concentration within the cell at the time. This, in turn, may be influenced by both luminal and plasma factors. The importance of luminal factors has been emphasised by the recent observation that the absorption-rate rises in iron-overloaded animals within two days if iron is excluded from the diet.[10] Presumably this change relates to a new population of upper intestinal cells with a lower iron content. In addition, the concentration of iron in mucosal cells must also depend on the rate at which the transferrin of plasma removes iron from the membranes of mucosal cells. On this basis, it is possible that any build-up of iron in mucosal cells promotes ferritin formation, and so prevents excessive absorption. It is tempting to postulate that ferritin, when present in sufficient concentrations, may then inhibit the formation of the complex or complexes which are responsible for active transport through the cell. Whatever the explanation for the present findings, this mechanism appears to be ineffective when large quantities of iron are presented to the mucosa, since with doses of 1000 and 10,000 μg. the amount of iron reaching the plasma was tenfold greater relative to mucosal ferritin formation than with physiological doses.

This study was supported in part by a grant from the National Institutes of Health, U.S.A., and in part by grants from the Council for Scientific and Industrial Research and the Atomic Energy Board, South Africa.

R. W. CHARLTON
M.D., B.SC. W'srand, M.R.C.P.E.
P. JACOBS
M.B. W'srand
J. D. TORRANCE
M.SC. W'srand
T. H. BOTHWELL
M.D. W'srand, M.R.C.P.

Department of Medicine,
University of the Witwatersrand,
Johannesburg, South Africa

10. Bannerman, R. M., O'Brien, J. R. P., Witts, L. J. *Blood*, 1962, **20**, 532.

Printed in
Great Britain

The Lancet Office,
7, Adam Street, Adelphi, London, W.C.2

Reprinted from JOURNAL OF APPLIED PHYSIOLOGY
Vol. 19, No. 2, March, 1964
Printed in U.S.A.

Role of hydrochloric acid in iron absorption[1]

PETER JACOBS, THOMAS BOTHWELL, AND ROBERT W. CHARLTON
Department of Medicine, University of the Witwatersrand Medical School, Johannesburg, South Africa

JACOBS, PETER, THOMAS BOTHWELL, AND ROBERT W. CHARLTON. *Role of hydrochloric acid in iron absorption.* J. Appl. Physiol. 19(2): 187–188. 1964.—The absorption of 5 mg iron administered in 300 ml water was compared with absorption from 300 ml 0.05 N HCl in 24 subjects with histamine-fast achlorhydria, using a double isotope technique. In ten individuals given ferric chloride the mean absorptions were 2.2% with water and 9.2% in acid; the difference was less marked in the case of the seven subjects to whom ferrous ascorbate was administered (10.9% and 19.3%, respectively). In contrast, acid did not increase the absorption of hemoglobin iron, the mean figures being 13.4% with water and 9.6% with HCl.

pernicious anemia achlorhydria gastric pH
Fe^{55} and Fe^{59} radioactive hemoglobin iron

THERE IS STILL CONTROVERSY as to the possible effects of gastric hydrochloric acid on iron absorption. Although the results of early studies suggested that acid potentiates the clinical response in patients with iron-deficiency anemia (7, 8), several subsequent isotopic investigations have yielded conflicting results. Pirzio-Biroli et al. (10) found that the absorption of a tracer amount of radioiron administered with food was normal in subjects with pernicious anemia, while Moore and Dubach (9) were unable to enhance the absorption of eggs labeled with Fe^{59} in achlorhydric subjects when hydrochloric acid was added. In addition, Biggs et al. (1) found that the absorption of Fe^{59}-labeled hemoglobin was normal in achlorhydric individuals. In contrast, Williams (13) demonstrated defective absorption of radioiron incorporated into bread in patients with histamine-fast achlorhydria, while Goldberg et al. (4) found a significant depression in the absorption of a tracer quantity of iron administered with food in achlorhydric subjects with iron-deficiency anemia.

The present investigation was undertaken because studies in this laboratory using an in vitro preparation of rat intestine indicated that the pH of the luminal contents has a marked effect on the absorption of iron salts. It was therefore decided to note the effects of hydrochloric acid on the absorption of inorganic and organically bound iron in achlorhydric subjects. In order to overcome difficulties resulting from individual variations in absorption a double isotope technique was used, so that the absorption of iron administered with and without acid was compared in the same subject.

MATERIALS AND METHODS

Twenty-four patients with pernicious anemia in remission were studied. The absence of free hydrochloric acid was established in each subject by the augmented histamine test (5). Iron, 5 mg, was administered by nasogastric tube to the fasting subjects over a period of 1 hr on two consecutive mornings. The iron was made up in 300 ml water on the one day and in the same volume of 0.05 N hydrochloric acid on the other day (15 mEq HCl). (This quantity of acid is slightly more than half the mean output of normal subjects after maximal histamine stimulation (6).) In each instance the iron was labeled with 5 μc Fe^{59} on the one occasion and with 125 μc Fe^{55} on the other. In ten subjects the iron was given as ferric chloride, in seven as ferrous ascorbate (50 mg ascorbic acid was added to the ferric chloride immediately prior to administration), and in the remainder as hemoglobin. The radioactive hemoglobin was prepared from rat blood 14 days after the intravenous administration of a tracer quantity of radioiron.

Samples of blood were collected from all subjects 14 days after the second dose. Aliquots were then digested, electroplated onto copper discs, and differentially counted for the two isotopes as previously described (2). The relative percentage absorptions of Fe^{55} and Fe^{59} were then calculated on the assumption that the blood volume was 70 ml/kg and that 80% of the absorbed iron was in circulation at 14 days.

Validation of double isotope technique. Preliminary studies were done on convalescent hospital patients to establish the validity of the double isotope technique. Iron in a dosage of 5 mg was administered on two consecutive mornings, the label being Fe^{55} on the one day and Fe^{59} on the other. The mean absorptions with ferric chloride

Received for publication 17 June 1963.
[1] This study was supported in part by Grant AM04912-02 from the National Institutes of Health, and in part by grants from the Council for Scientific and Industrial Research and the Atomic Energy Board, South Africa.

TABLE 1. *Comparison of the percentage absorption of a 5-mg dose iron given to fasting subjects on two consecutive days*

	Day 1	Day 2
Ferric chloride, 5 mg	0.6	0.8
	3.6	5.7
	0.5	0.8
	3.8	5.6
Mean	2.1	3.2
Ferrous ascorbate, 5 mg	10.5	8.7
	51.8	47.9
	22.1	21.9
	7.8	6.2
	67.1	55.7
Mean	31.8	28.1

were 2.1 % and 3.2 %, and with ferrous ascorbate 31.8 % and 28.1 % (Table 1).

RESULTS

The mean absorption of ferric chloride in water was considerably less than when it was administered in 0.05 N hydrochloric acid (2.2 % as compared with 9.2 %) (Fig. 1*A*). Results were less striking with ferrous ascorbate, since acid only caused a marked increase in three of seven subjects (Fig. 1*B*). The mean absorption with water was 10.9 % as compared with 19.3 % with acid. The absorption of hemoglobin iron was not enhanced when hydrochloric acid was present and, in fact, there was suggestive evidence that it caused some depression of absorption in six of the seven subjects (Fig. 1*C*). The mean absorptions of hemoglobin were 13.4 % with water and 9.6 % with acid.

DISCUSSION

Although previous workers have demonstrated diminished absorption of ferric iron in achlorhydric subjects (4), they were unable to establish whether this was due to the absence of hydrochloric acid or to the associated mucosal changes. The enhancement of ferric iron absorption caused by the addition of hydrochloric acid alone in the present study indicates that this is the important factor. Presumably the acid exerts its effect by keeping the ferric iron in solution until it reaches the absorbing area of the duodenum. The fact that the absorption of a ferrous salt was influenced to a lesser degree by the presence of acid is quite compatible with this thesis, since it is known that ferrous ions remain in solution at a much higher pH than do ferric ions (11).

The results obtained with hemoglobin iron are of interest from two points of view. First, it was noteworthy how well hemoglobin iron was absorbed in achlorhydric subjects; and second, it was apparent that hydrochloric acid did not potentiate absorption. Taken in conjunction with previous similar findings (1) these results suggest that the absorption of hemoglobin iron is not dependent on any gastric factor, and lend further support to the thesis that hemoglobin iron is absorbed by a mechanism different from that which operates for inorganic salts. This thesis has resulted from several observations. These include the fact that the peak of plasma radioactivity occurs later after the giving of hemoglobin than it does after salts, and on the finding that its absorption is not increased by ascorbic acid or depressed by food or phytate (3, 12). Although it has been suggested that hemoglobin iron may be absorbed as a porphyrin complex (12), there is no evidence to support or refute this speculation at the present time.

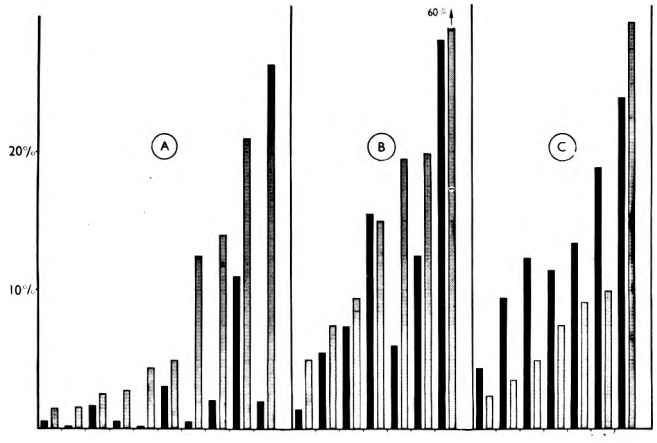


FIG. 1. A comparison of the percentage absorption of 5 mg iron dissolved in 300 ml water (black) with the same dose of iron in 300 ml 0.05 N hydrochloric acid (cross-hatched). The iron was given as ferric chloride (*A*), ferrous ascorbate (*B*), and as hemoglobin (*C*).

REFERENCES

1. BIGGS, J. C., R. M. BANNERMAN, AND S. T. CALLENDER. *Proc. 8th Congr. Europ. Soc. Haemat.* Basle.
2. BOTHWELL, T. H., AND C. A. FINCH. *Iron Metabolism.* Boston: Little, Brown, 1962. p. 30.
3. CALLENDER, S. T., B. J. MALLETT, AND M. D. SMITH. *Brit. J. Haematol.* 3: 186, 1957.
4. GOLDBERG, A., A. LOCHHEAD, AND J. H. DAGG. *Lancet* 1: 848, 1963.
5. KAY, A. W. *Brit. Med. J.* 2: 77, 1953.
6. MARKS, I. N., S. BANK, J. H. LOUW, AND B. H. VAN EMBDEN. *S. African Med. J.* 36: 807, 1962.
7. METTIER, S. R., AND G. R. MINOT. *Am. J. Med. Sci.* 181: 25, 1931.
8. MINOT, G. R., AND C. W. HEATH. *Am. J. Med. Sci.* 183: 110, 1932.
9. MOORE, C. V., AND R. DUBACH. *J. Am. Med. Assoc.* 162: 197, 1956.
10. PIRZIO-BIROLI, G., T. H. BOTHWELL, AND C. A. FINCH. *J. Lab. Clin. Med.* 51: 37, 1958.
11. THORNE, P. C. L., AND E. R. ROBERTS. *Inorganic Chemistry.* London: Oliver & Boyd, 1954, p. 477.
12. TURNBULL, A., F. CLETON, AND C. A. FINCH. *J. Clin. Invest.* 41: 1897, 1962.
13. WILLIAMS, J. *Clin. Sci.* 18: 521, 1959.

THE EFFECT OF ALCOHOL ON IRON ABSORPTION.

*R.W. CHARLTON, M.D., B.Sc., M.R.C.P.ED.

*P. JACOBS, M.B.

+H. SEFTEL, B.Sc., Dip. Med.

*T.H. BOTHWELL, M.D., M.R.C.P.

*Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa.

+Baragwanath Hospital, Johannesburg, South Africa.

There are several observations which suggest a relationship between excessive consumption of alcohol and the development of iron overload. For example, approximately a third of patients exhibiting the full-blown clinical manifestations of idiopathic haemochromatosis have a significant alcoholic background (Sheldon, 1935; Finch and Finch, 1955). Furthermore, there is evidence that alcoholics as a group tend to have iron stores that are greater than normal (Herbut and Tamaki, 1946; MacDonald and Mallory, 1960). Finally, there is suggestive evidence that the severity of the tissue siderosis so commonly noted in the adult Bantu males of South Africa is closely related to the drinking habits of these subjects (Seftel et al., 1961).

There are several possible reasons for these various associations. In the first place, analyses of the home-brewed alcoholic beverages consumed by the Bantu have revealed high iron concentrations (Walker and Arvidsson, 1953), and it has been calculated that many males ingest between 50 and 100 mg. iron daily from this source alone (Bothwell et al., 1964). A similar, though less striking association between iron and alcoholic intakes has recently been pointed out by MacDonald (1963), who has shown that most European and United States wines contain significant amounts of iron. (For example, the average iron concentrations in French wines is 8.8 mg./1000 ml., and in United States wines, 4.9 mg./1000 ml.). A second possible way in which excessive consumption

of alcohol may lead to iron overload is through its pathological sequelae, such as chronic pancreatitis and chronic liver disease. Deficiency of the exocrine secretions of the pancreas has been shown to be associated with increased absorption of iron from the gut (Taylor et al., 1931; Davis and Badenoch, 1962), and there is recent evidence indicating that some subjects with cirrhosis of the liver absorb iron excessively (Conrad et al., 1962; Callender and Malpas, 1963).

There are therefore several possible reasons why so many heavy drinkers have increased iron stores. However, one possibility has not been investigated: namely, that alcohol itself directly enhances iron absorption. The present investigation was carried out to try and answer this point.

MATERIAL AND METHODS

The absorption of 5 mg. iron was assessed in 31 haematologically normal subjects and in 4 patients with histamine-fast achlorhydria. Because of the well-known variability in iron absorption from subject to subject, a double isotope technique was used in which each individual served as his own control. (The validity of this approach has been demonstrated in a previous communication (Jacobs et al., 1964)). The iron was administered in the fasting state. On one day it was dissolved in 250 ml. water, and on the following day in 60 ml. whisky or brandy, made up to a final

volume of 250 ml. with water. In half the experiments the order of administration was reversed. Isotope labelling was with 70 μc Fe^{55} on the one occasion and with 5 μc Fe^{59} on the other. In different experiments the iron was administered as ferric chloride, as ferrous ascorbate (with 50 mg. ascorbic acid), and as rat haemoglobin. Duplicate blood samples collected two weeks later were digested, electroplated onto copper discs and differentially counted for the two isotopes as described previously (Bothwell and Finch, 1962). The relative percentage absorptions of the two doses were then calculated on the assumption that 80% of the iron absorbed from the gut was present in the red cell mass at two weeks, and that the blood volume was 70 ml./kg. Results were therefore corrected by a factor of $^{10}/_{8}$.

RESULTS

The absorption of iron given as ferrous ascorbate was assessed in 6 normal subjects. The mean absorption was 18.8% in water and 17.5% in alcohol (see table). There was also no enhancement of the absorption of haemoglobin iron in the presence of alcohol, the mean figures in 8 subjects being 14.9% in water and 13.4% in alcohol. In contrast, alcohol caused a marked increase in the absorption of iron given as ferric chloride. In 17 subjects the mean absorption in alcohol was 22.6% as compared with 4.1% in water (see figure).

This effect could not, however, be demonstrated in 4 subjects with histamine-fast achlorhydria (a mean of 3.5% in water and 5.2% in alcohol).

DISCUSSION.

In a previous investigation the effect of hydrochloric acid on iron absorption was studied in subjects with histamine-fast achlorhydria (Jacobs et al., 1964). Hydrochloric acid was found to enhance markedly the absorption of ferric iron and to cause a modest increase in the absorption of ferrous iron. In contrast, haemoglobin iron absorption was not increased by acid. Although the reasons for these differences were not established, it seems likely that hydrochloric acid acts by maintaining ferric iron in solution until it reaches the absorbing area of the duodenum. The relatively small effect observed with ferrous iron is compatible with this thesis, since it is known that ferrous ions remain in solution at a much higher pH than do ferric ions (Hodgman et al., 1958). The fact that the absorption of haemoglobin iron is not increased by acid adds further weight to evidence obtained by other workers which suggests that this organic iron complex is absorbed in a manner different from simple iron salts (Turnbull et al., 1962).

In the present study alcohol was found to potentiate the absorption of ferric iron considerably, but to have no effect on the absorption of ferrous or haemoglobin iron. The similarity between these results and those obtained

previously with hydrochloric acid suggested that alcohol might be exerting its effect by stimulating the production of gastric acid. The fact that the administration of alcohol did not increase the absorption of iron in 4 subjects with histamine-fast achlorhydria would be compatible with this hypothesis.

Whether the present findings bear any relation to the pathogenesis of iron overload is still not clear. While it is known that excessive amounts of iron are present in the fermented beverages consumed by the Bantu (Walker and Arvidsson, 1953), it has not been possible to show an enhancement of absorption when iron has been administered in Bantu beer (Bothwell et al., 1964). However, it should be noted that the type of beer which was tested had a very low alcohol content (± 3%), and it remains possible that the more potent adulterated drinks consumed by many of these subjects may enhance iron absorption significantly.

Recent evidence suggests that the present findings may have a wider relevance. It has been pointed out that many wines from different parts of the world contain significant amounts of iron, and it has been suggested that the high incidence of iron overload in alcoholic subjects is a result of the excessive iron intake (MacDonald, 1963). It should, however, be noted that wines contain 10-20% alcohol, and the absorption of iron taken in this way may thus be

enhanced.

These considerations do not exclude the possibility that other factors may contribute to the association between iron overload and the excessive consumption of alcohol. Pancreatitis is common in alcoholics, and there is now good evidence that deficiency of the exocrine secretions of the pancreas is associated with excessive absorption of iron. This has been shown in animals after pancreatectomy (Taylor et al., 1931), ligation of the pancreatic duct (Taylor et al., 1935; Gillman et al., 1947; Kinney et al., 1950), ethionine-induced pancreatic damage (Kinney et al., 1955), and in patients with chronic pancreatitis (Davis and Badenoch, 1962). Furthermore, there is evidence that the increased absorption can be reduced by the giving of pancreatic extract (Kinney et al., 1950; Davis and Badenoch, 1962; Saunders et al., 1963). The fact that cirrhosis is common in alcoholics may also be of relevance, since it has been shown that some cirrhotic subjects absorb excessive amounts of iron even when the body stores are normal or increased (Conrad et al., 1962). It is, however, still not clear whether these effects are secondary to the cirrhosis itself or to associated pancreatic damage (Callender and Malpas, 1963). Lastly, alcoholics as a group eat suboptimal diets, and there are several experimental studies showing increased absorption of iron when the diet is

low in certain nutrients but high in iron. These diets have included low phosphate (Hegsted et al., 1949), low protein, high fat (Kaufman et al., 1958), and choline-deficient (MacDonald, 1960) regimes.

With all these factors contributing to the excessive absorption of iron it is not surprising that doubt has been cast on whether idiopathic haemochromatosis occurs as a distinct entity. The arguments against its existence have been presented by MacDonald (1961, 1963), who believes the condition to be a variant of alcoholic cirrhosis occurring in subjects exposed to a high iron diet. Some support for this suggestion is provided by the fact that many patients presenting with the disease have background of alcoholism (Sheldon, 1935; Finch and Finch, 1955). There is, however, a strong body of evidence, accumulated from many sources, which indicates the existence of a specific metabolic disorder in which excessive amounts of iron are absorbed from a normal diet. Firstly, it should be emphasised that a large number of reported patients with the disease have not been alcoholic, and no other source of excessive dietary iron has been uncovered in these cases. Secondly, there are several family studies in which varying degrees of iron overload have been demonstrated in relatives of patients with haemochromatosis, thus supporting the concept of an inherited metabolic disorder (Bothwell et al., 1959; Williams et al., 1962). The fact that only a proportion of siblings living

in the same household have shown increased stores can also be taken as evidence against the idea that some environmental factor, such as a high dietary intake, is essential in pathogenesis. Thirdly, it has been possible to show an abnormally high absorption of radioiron in some patients with the fully developed disease (Alper et al., 1951; Bothwell et al., 1953). This is especially so in young subjects, and the failure to demonstrate increased absorption in some older patients may well be due to the inhibitory effect exerted by the enormous iron stores. Support for this contention has been provided by the finding of very high absorption rates in such individuals after reduction of stores by repeated phlebotomy. This has been noted at a time when the haemoglobin level was normal and the serum iron normal or even raised (Pirzio-Biroli et al., 1958).

Although consideration of these various findings suggests the existence of a specific metabolic disorder resulting in excessive iron absorption, it is probable that only a proportion of affected individuals develop the clinical manifestations of haemochromatosis. The size of the accumulated iron deposits in any particular case must depend upon a number of factors. These include the degree to which the absorptive rate deviates from the normal, the amount of iron in the diet, the duration of the period of increased absorption, and the quantity of

iron lost from the body. On this basis the excessive intake of iron, particularly when present in alcohol, might be expected to accelerate the development of the full clinical syndrome in subjects who would otherwise have remained asymptomatic.

SUMMARY

Alcohol in the form of whisky or brandy markedly increased the absorption of ferric chloride in normal subjects, but had no effect upon the absorption of ferrous ascorbate or haemoglobin iron. Some evidence was obtained to suggest that this action was due to the stimulation of hydrochloric acid production in the stomach.

These findings may have some relevance to the well-established association between excessive consumption of alcohol and the development of iron overload.

****

This study was supported in part by a grant (AM 04912-02) from the National Institutes of Health, U.S.A., and in part by grants from the Council for Scientific and Industrial Research and the Atomic Energy Board, South Africa.

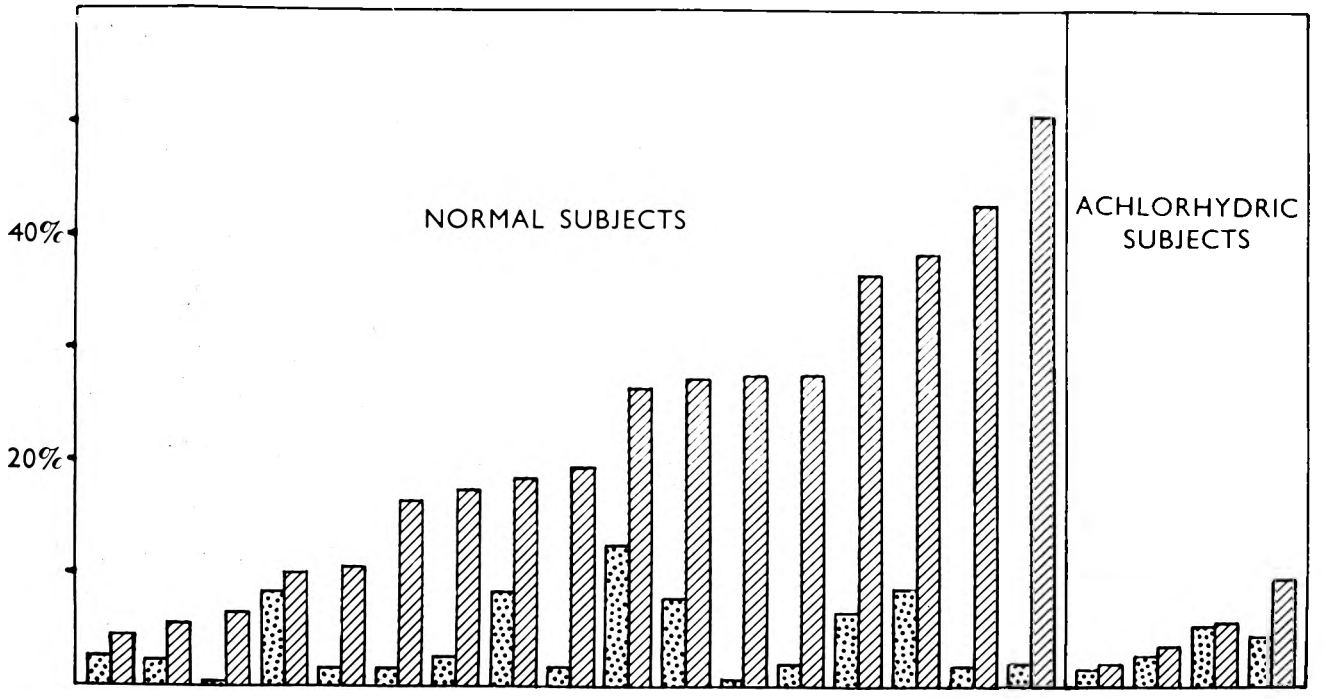
THE EFFECT OF ALCOHOL UPON THE ABSORPTION OF 5 MG. IRON GIVEN AS (A) FERROUS ASCORBATE AND (B) HAEMOGLOBIN

	Ferrous Ascorbate		Haemoglobin	
	Without alcohol	With alcohol	Without alcohol	With alcohol
rcentage sorption	8.3	4.6	12.8	9.7
	21.7	14.5	6.1	8.2
	20.5	16.1	11.4	14.1
	22.5	28.3	10.6	8.4
	20.2	14.7	21.6	15.3
	19.5	27.0	19.0	18.6
			20.5	8.9
			17.1	23.6
Mean	18.8	17.5	14.9	13.4

'DH.

FIGURE

Effect of alcohol on the absorption of 5 mg. iron given as ferric chloride. Each pair of columns represents absorption with alcohol (hatched) and without alcohol (stippled) in the same individual. In normal subjects mean absorptions were 4.1% with water and 22.6% with alcohol, while in achlorhydric subjects the corresponding figures were 3.5% and 5.2%.



REFERENCES

Alper, T., Savage, D.V., and Bothwell, T.H. (1951). J. Lab. clin.Med., 37, 665.

Bothwell, T.H., Cohen, I., Abrahams, O.L., and Perold, S.M. (1959). Amer. J.Med., 27, 730.

Bothwell, T.H., van Doorn-Wittkampf, H. van W., du Preez, M.L., and Alper, T. (1953). J.Lab. clin.Med., 41, 836.

Bothwell, T.H., and Finch, C.A. (1962). Iron Metabolism. p. 32. Little, Brown, Boston.

Bothwell, T.H., Seftel, H., Jacobs, P., Torrance, J.D. and Baumslag, N. (1964). Amer.J.clin.Nutr., 14, 47.

Callender, S.T., and Malpas, J.S. (1963). Brit.Med.J., ii, 1516.

Conrad, M.E., Berman, A., and Crosby, W.H. (1962). Gastroenterology, 43, 385.

Davis, A.E., and Badenoch, J. (1962). Lancet, ii, 6.

Finch, S.C., and Finch, C.A. (1955). Medicine, 34, 381.

Gillman, J., Gillman, T., Mandelstam, J., and Gilbert, C.(1947) Nature, 159, 875.

Hegsted, D.M., Finch, C.A., and Kinney, T.D. (1949). J. exp. Med., 90, 147.

Herbut, P.A., and Tamaki, H.T. (1946). Amer.J.clin.Path., 16, 640.

Hodgman, C.D., Weast, R.C. and Selby, S.M. (1958). Handbook of Chemistry and Physics. p.1740. Chemical Rubber Publishing Company, Cleveland, Ohio.

Jacobs, P., Bothwell, T.H., and Charlton, R.W. (1964). J. applied Physiol., 19, 187.

Kaufman, N., Klavins, J.V., and Kinney, T.D. (1958). Lab. Invest., 7, 369.

Kinney, T.D., Finch, C.A., Kaufman, N., Hegsted, M., and Partington, P.F. (1950). Amer.J.Path., 26, 746.

Kinney, T.D., Kaufman, N., and Klavins, J. (1955). J. exp.Med., 102, 151.

MacDonald, R.A. (1960). Amer.J.Path., 36, 499.

MacDonald, R.A. (1961). Arch.int.Med., 107, 606.

MacDonald, R.A. (1963). ibid., 112, 184.

MacDonald, R.A., and Mallory, G.K. (1960). ibid., 105, 686.

Pirzio-Birolo, G., Bothwell, T.H., and Finch, C.A. (1958). J.Lab.clin.Med., 51, 37.

Saunders, S.J., Bank, S., Airth, E., and Williams, J. (1963). S.Afr.Med.J., 37, 1106.

Seftel, H.C., Keeley, K.J., Isaacson, C., and Bothwell, T.H. (1961). J.Lab.clin.Med., 58, 837.

Sheldon, J.H. (1935). Haemochromatosis. p. 274. Oxford University Press, London.

Taylor, J., Stiven, D., and Reid, E.W. (1931). J.Path.Bact., 34, 793.

Taylor, J., Stiven, D., and Reid, E.W. (1935). ibid., 41, 397.

Turnbull, A., Cleton, F., and Finch, C.A. (1962). J.clin. Invest., 41, 1897.

Walker, A.R.P., and Arvidsson, U.B. (1953). Trans.R.Soc.trop. Med.Hyg., 47, 536.

Williams, R., Scheuer, P.J., and Sherlock, S. (1962). Quart.J. Med., 31, 249.

/DH.

FERROKINETIC MEASUREMENTS

Reprinted from THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, St. Louis.
Vol. 45, No. 2, Pages 167-178, February, 1955. (Printed in the U. S. A.)

RADIOIRON STUDIES IN HEMOCHROMATOSIS

THE EFFECTS OF REPEATED PHLEBOTOMIES

T. H. BOTHWELL, M.D., M.R.C.P.,* B. C. ELLIS, M.B., B.CH.,** H. VAN W. VAN DOORN-WITTKAMPF, M.D., AND O. L. ABRAHAMS, B.SC.**
JOHANNESBURG, SOUTH AFRICA

RECENTLY Huff and co-workers[14] have described a method for estimating the plasma and red cell iron turnovers in the body. The present investigation was undertaken to determine such turnovers in hemochromatosis. The results were compared with those obtained in a control group of healthy subjects and in one case of malnutritional cytosiderosis.[10] In addition, the effects of repeated phlebotomies on iron metabolism in hemochromatosis were investigated. At the time these studies were done the radioiron used was of a lower specific activity than is now available and this made the analysis of data more difficult. In spite of this qualification the results obtained were considered interesting and worthy of publication.

MATERIAL AND METHODS

Clinical Material.—Two male and two female cases of hemochromatosis were studied. In each the diagnosis was confirmed by liver aspiration biopsy. Of the females Case 15, aged 55 years, was postmenopausal and the other (Case 14), aged 23 years, had amenorrhea of 4 years' duration. Skin pigmentation and cirrhosis of the liver were present in all, none were anemic, and Cases 12, 13, and 15 had diabetes.

The control group consisted of 8 healthy men and 3 healthy women, two of whom were postmenopausal (Cases 9 and 10). Their ages ranged from 20 to 65 years.

The one case of malnutritional cytosiderosis studied was a South African Negro, aged 64 years, who presented with diabetes. Histologic examination of a liver biopsy showed heavy hepatocellular and portal iron deposits with portal fibrosis and bile duct hyperplasia.

Preparation of Radioiron.—The methods were similar to those used previously.[1] Radioiron derived from the Szilard-Chalmers reaction was supplied by the Atomic Energy Research Establishment (Harwell, England) as Fe^{59} of specific activity varying from 10 to 30 μg per microcurie. The iron was in the form of a ferric chloride stock solution and tracer doses made

This work was assisted by a grant from the Council for Scientific and Industrial Research, South Africa.
Received for publication Jan. 19, 1954.
*Department of Medicine, University of the Witwatersrand.
**Department of Clinical Pathology, University of the Witwatersrand.

up from this had activities varying from 4 to 10 μc on the day of administration. Each dose was autoclaved in rubber-capped bottles at a pressure of 20 pounds p.s.i. for 20 minutes. On the day of study 15 to 20 ml. of normal plasma was rapidly added to this solution and allowed to stand for about 10 minutes to enable the iron to attach itself to the β_1 globulin. In earlier studies the stock doses of about 3 ml. of ferric chloride solution were buffered to pH 6 with 3.8 per cent sodium citrate. However, it was found early that the solution was effectively buffered merely by the addition of the plasma, and this method was used in all later studies. The amount of elemental iron administered varied from 50 to 300 μg (mean 135 μg).

Administration of Radioiron.—All subjects were basal at the time of study. About 20 ml. of the plasma-iron dose was injected intravenously, the weight administered being determined by difference. An aliquot of 1 to 2 ml. of this solution was retained for subsequent analysis in order to determine the exact dose of radioiron and elemental iron given. Thereafter blood samples of about 3 ml. were taken serially into weighed tubes each containing 6 mg. of mixed oxalate. The tubes were again weighed and the weight of each blood sample determined by difference. In the initial 6 hours samples were taken at 30-minute intervals, but when a rapid plasma-iron turnover was expected, more frequent sampling was carried out within the first 3 hours. The red cell iron uptake was determined in most subjects by taking daily blood samples between the seventh and fourteenth days. In the earlier studies the plasma from each blood sample was pipetted off and added to 3 red cell washings. Separate analysis for radioactivity could then be made on plasma and red cells. However, it became apparent that the amount of radioiron that could be recovered from red cells in the early samples and from the plasma in the later samples was so low as to be of no account. In subsequent studies, therefore, no separation was done except in patients to whom a previous dose of radioiron had been given. In addition, in such cases basal blood samples were taken for radioiron analysis prior to giving the next radioiron dose. It was assumed that any residual radioactivity in the blood from previous tests was constant throughout the period of the subsequent study and corrections for red cell uptakes were made on this assumption.

In the hemochromatotic subjects repeat radioiron studies were done after the removal of various quantities of blood (Fig. 1). Phlebotomies (300 to 800 ml.) were performed at 3- to 10-day intervals and frequent hemoglobin, serum iron, and iron-binding capacity values were estimated. In Case 12 repeat radioiron studies were done after the removal of 5,190 ml. and again after a total of 13,110 ml. had been taken off. Case 13 had a repeat study performed after 1,800 ml. blood had been removed, and 3,650 ml. was taken off prior to the second study in Case 14. In Case 15 radioiron tests were done after 5,060 ml. and 9,610 ml. blood, respectively, had been removed. The time intervals between the commencement of the phlebotomies and the subsequent radioiron studies in each case can be seen in Fig. 1. In two normal subjects (4 and 11) repeat plasma iron turnovers were performed 48 hours after the initial ones.

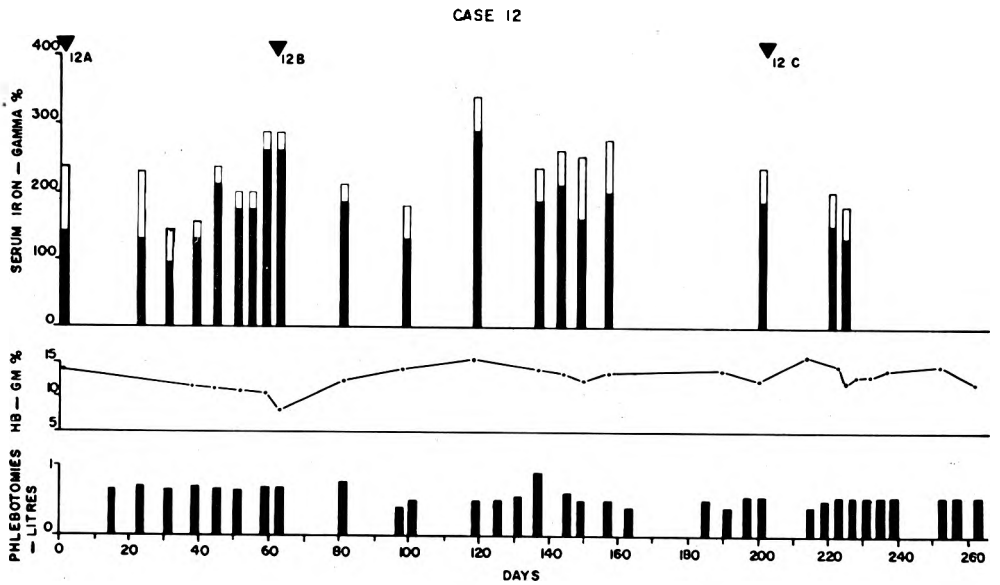
Blood Measurements.—Radioiron analyses on all blood samples were performed by electroplating them according to a slightly modified technique of Vosburgh, Flexner, and Cowie.[22] The samples were weighed, dry-ashed, chemically processed, and plated on copper discs in electroplating cells. These were then counted with a G.E.C. Counter (type E.H.M. 2-mica window, with a window thickness of 1.9 mg. per cm.^{2}) and the counts were compared with those due to plated standards prepared by the same technique from aliquots of the stock solution from which the patient's dose was derived. Activities of plated samples could thus be given in terms of the administered tracer dose. The specific gravities of the plasma and whole blood were determined by the copper sulfate method.[18] From these data the weights of plasma and red cells in each sample were converted into volumetric measurements. Serum iron values were determined in duplicate by the method of Kitzes and associates.[17] Iron-binding capacities were estimated by the method of Rath and Finch[19] modified for use with the Evelyn colorimeter. Total hemoglobin estimations were performed by a cyanhematin method[16] adapted for use with the Evelyn colorimeter. Blood volume determinations were made by a T1824-hematocrit method.[6] The values for red cell mass were reduced by 15 per cent since it has frequently been shown that such an overestimation occurs with the dye method.[20]

Analysis of Data.—Recently it has been shown that when a tracer dose of radioiron of high specific activity is added to plasma and injected intravenously it disappears exponentially, thus reflecting the normal course of the transport iron through the plasma iron pool. Thereafter, some of the radioiron reappears in the hemoglobin of the red cells. With a knowledge of the plasma iron level and of the plasma and red cell volumes it is then possible to measure both the plasma and red cell iron turnovers.[14] The present data were analyzed according to these methods. However, it was found that in four control subjects and in the majority of cases with hemochromatosis the disappearance of radioiron after injection was not exponential from 100 per cent concentration at zero time. In such cases there was a rapid loss in the first few minutes which accounted for as much as 65 per cent of the injected dose in one test (Table I). Thereafter, however, the disappearance was exponential over the next few hours, suggesting that the radioiron which had initially passed into the extravascular spaces did not re-enter the plasma in significant quantities during this period. It was, therefore, considered valid to use this slow component in the calculation of plasma iron turnovers. Huff and co-workers[14] encountered similar difficulties in cases with refractory anemia and also used this slow exponential component in the calculation of their results. The subsequent course of the radioiron which disappeared early from the circulation is uncertain. However, there is experimental evidence that at least some of this unattached ferric chloride is stored in the liver.[7] In the present study it was also found that some of this radioiron was later fed back into the circulation as the percentage of the injected dose appearing in the red cell mass of certain cases of hemochromatosis was greater than the percentage initially attached to the β_1 globulin of the plasma (Table I). The actual quantity of radioiron redelivered to the circulation from the extravascular compartment in such instances must depend on several factors, viz., the size of the iron stores, the degree of equilibrium between radioiron and nonradioactive iron in these stores, and the percentage being fed back from the extracellular spaces. As there is no way of measuring the relative importance of these factors or the proportions of radioiron re-entering the circulation from these various sites, it would seem to be invalid to calculate the red cell iron turnover (percentage radioiron in red cell mass × plasma iron turnover) in such cases. Therefore red cell iron turnovers were calculated only in studies where the initial disappearance of radioiron was exponential from 100 per cent concentration at zero time. In the others the red cell iron utilization was expressed as a percentage of the administered dose.

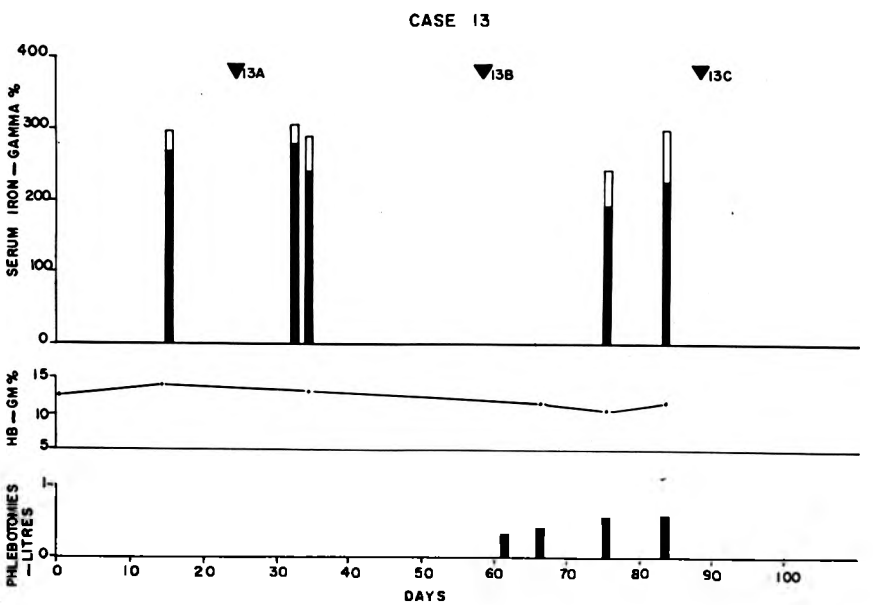
The incomplete attachment of radioiron to the plasma globulin in some cases seemed to depend on two factors. First, the quantity of iron which was injected was relatively large, the average amount being 135 μg (range 30 to 300 μg). Although the doses were prepared with normal human plasma (collected with precautions against iron contamination) there was still insufficient unsaturated β_1 globulin present to ensure that such amounts of iron would all be bound. However, it might be expected that at least some of this unbound iron would become attached intravascularly, especially as these doses were injected over a period of about 5 minutes. The second factor was probably related to the plasma iron-binding capacity which was reduced in the hemochromatotic subjects. However, in spite of the incomplete binding of the radioiron the doses could be regarded as true tracers in so far as the bound fractions did not alter the plasma iron component significantly. In these cases the average amount of radioiron which was attached to the β_1 globulin of the plasma was 80 μg (range 20 to 170 μg) and the addition of such amounts of iron could not produce any measurable change in the plasma iron level.

RESULTS

Plasma Iron Turnover.—In 13 studies on 11 control subjects the mean plasma iron turnover was 30 mg. per day (range 17 to 40 mg. per day) and 0.46 mg. per kilogram per day (range 0.29 to 0.62 mg. per kilogram per day). The turnover in milligrams per day was higher in men (9 studies) as compared with women (4 studies), but this difference was not apparent when the results

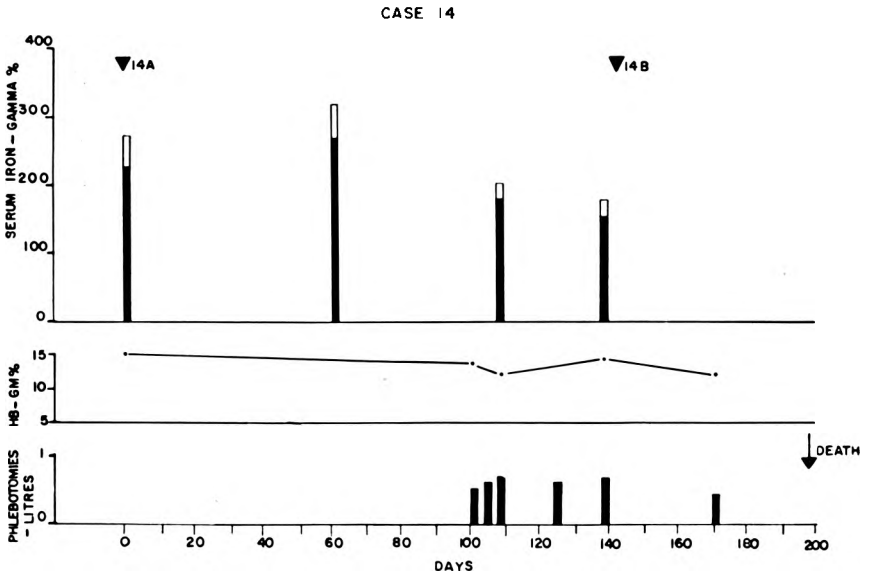


A.

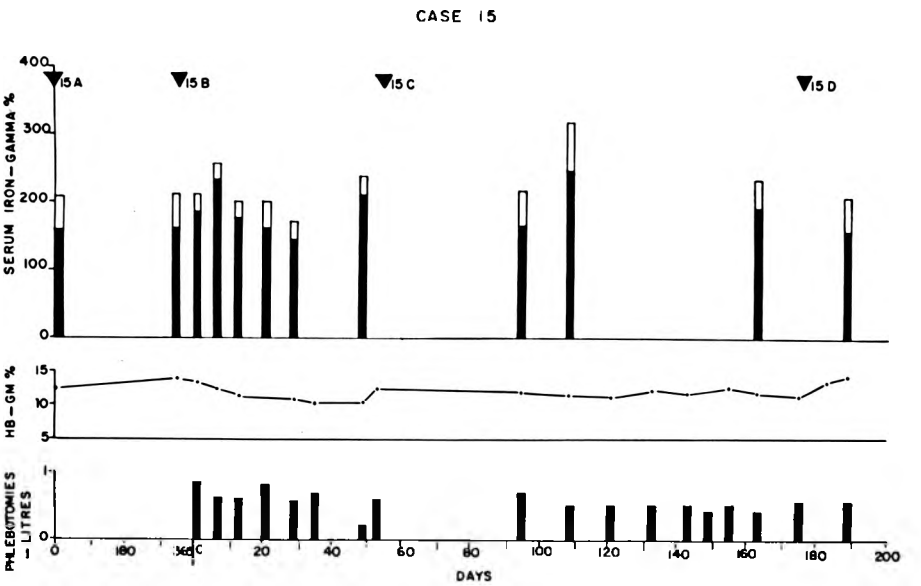


B.

Fig. 1, *A* and *B*. (*For legend see opposite page.*)



C.



D.

Fig. 1.—*A*, *B*, *C*, and *D*. The effects of repeated phlebotomies on the serum iron, iron-binding capacity, and hemoglobin levels in four cases of hemochromatosis. The times are shown at which the radioiron studies reported in Table I were done. The open areas of the serum iron columns represent the iron-binding capacity.

TABLE I

CASE NO.	SEX	AGE (YR.)	INJECTED RADIOIRON BOUND TO β_1 GLOBULIN OF PLASMA (%)†	TIME TAKEN TO REACH ½-CONCENTRATION (HR.)‡	PLASMA IRON TURNOVER (MG./DAY)	PLASMA IRON TURNOVER (MG./KG./DAY)	PLASMA IRON REMOVED (FRACTION PER HR.)	RED CELL IRON TURNOVER (MG./DAY)	RED CELL IRON TURNOVER (MG./KG./DAY)	RED CELL IRON RENEWED (FRACTION PER DAY)	RED CELL IRON UTILIZATION (%)
					Normal						
1	M	45	100	1.2	28	0.40	0.59	25	0.36	0.015	89
2	M	20	95	1.6	38	0.54	0.42	—	—	—	85
3	M	20	100	2.1	33	0.41	0.35	33	0.41	0.013	100
4 A	M	46	100	1.1	40	0.57	0.63	*	*	*	*
4 B	M	46	100	1.0	39	0.56	0.61	*	*	*	*
5	M	22	85	1.6	35	0.56	0.41	—	—	—	82
6	M	22	100	2.0	24	0.29	0.34	20	0.24	0.006	83
7	M	23	100	2.4	24	0.38	0.29	21	0.33	0.009	88
8	M	24	90	1.7	40	0.56	0.40	—	—	—	82
9	F	52	100	1.6	17	0.32	0.44	17	0.32	0.012	100
10	F	65	100	1.0	29	0.62	0.66	29	0.62	0.020	100
11 A	F	37	45	1.8	22	0.34	0.40	*	*	*	*
11 B	F	37	100	1.6	25	0.38	0.45	*	*	*	*
Mean				1.6	30	0.46	0.46	24	0.38	0.013	90
					Hemochromatosis—before phlebotomies						
12 A	M	63	100	1.2	42	0.75	0.58	21	0.38	0.016	50
13 A	M	60	100	2.0	46	1.01	0.35	25	0.55	0.019	54
13 B	M	60	35	1.9	38	0.78	0.36	*	*	*	*
14 A	F	23	55	1.7	56	0.83	0.47	—	—	—	29
15 A	F	55	60	1.6	42	0.87	0.45	—	—	—	20
15 B	F	55	40	1.9	36	0.59	0.38	—	—	—	43
Mean				1.7	43	0.80	0.43	23	0.46	0.018	39
					Hemochromatosis—after phlebotomies						
12 B	M	63	100	0.6	106	1.91	1.08	76	1.36	0.076	72
12 C	M	63	50	0.7	101	1.67	0.98	—	—	—	67
13 C	M	60	40	0.7	105	2.20	0.94	—	—	—	51
14 B	F	23	40	0.7	72	1.39	0.99	—	—	—	42
15 C	F	55	55	1.1	73	1.05	0.60	—	—	—	32
15 D	F	55	45	0.9	68	1.07	0.74	—	—	—	58
Mean				0.8	87	1.55	0.89				54
					Malnutritional cytosiderosis						
16	M	64	50	2.4	34	0.46	0.29	—	—	—	34

*Not done.

-Not calculated as the exponential plasma iron disappearance slopes did not extrapolate to 100 per cent at zero time (see text).

†These quantities represent the extrapolated concentration of the exponential plasma iron disappearance slopes at zero time and are assumed to be equivalent to the amount of injected radioiron bound to the β_1 globulin of the plasma.

‡In those cases where not all the injected radioiron was bound to the plasma β_1 globulin the extrapolated values at zero time were taken as 100 per cent and the ½-concentration times corrected accordingly (see text).

were expressed in milligrams per kilogram per day. In two subjects (Cases 4 and 11) repeat studies were performed 48 hours after the initial ones. No significant change in the plasma iron turnover was noted in either subject. The fraction of plasma iron removed per hour in normal subjects was 0.46 (range 0.29 to 0.66).

In 6 studies on 4 cases of hemochromatosis the mean plasma iron turnover was 43 mg. per day (range 36 to 56 mg. per day). Expressed in terms of body weight the mean was 0.80 mg. per kilogram per day (range 0.59 to 1.01 mg. per kilogram per day). The mean weight of the hemochromatotic subjects (before phlebotomies) was 54 kg. and that of the control group was 65 kg. This difference would account for the apparent discrepancy between these two groups of the values expressed in terms of body weight. The fraction of plasma iron removed per hour was 0.43 (range 0.35 to 0.58). Repeat basal studies performed in 2 subjects (Cases 13 and 15) gave results for plasma iron turnover in milligrams per day which approximated fairly closely those found previously. In one (Case 15), however, there was a marked reduction in the plasma iron turnover expressed in milligrams per kilogram per day from 0.87 in the first to 0.59 in the second study. This was due to a marked gain in weight (from 48 to 61 kg.) in the four-month period between the two investigations. After repeated phlebotomies the mean plasma iron turnover was raised to 87 mg. per day (range 68 to 106 mg. per day) and 1.55 mg. per kilogram per day (range 1.05 to 2.20 mg. per kilogram per day). The fraction of plasma iron removed per hour was increased to 0.89 (range 0.60 to 1.08). In Cases 12 and 15 further studies were done after the removal of additional volumes of 7,120 and 4,550 ml. blood, respectively. The results for plasma iron turnover closely approximated those obtained previously (101 mg. per day as compared with 106 mg. per day and 68 mg. per day as compared with 73 mg. per day). The one case of malnutritional cytosiderosis studied had a plasma iron turnover which was within the normal range (34 mg. per day and 0.46 mg. per kilogram per day).

Plasma Iron Disappearance Rates.—The plasma iron disappearance rates are given in Table I. In those cases where the exponential component did not extrapolate through 100 per cent at zero time, its observed extrapolated concentration was regarded as 100 per cent and the times taken to reach ½-concentration were calculated. Although this correction was not necessary in order to calculate the turnover values it was done so that the ½-concentration times could be compared. In the control group the mean time was 1.6 hr. (range 1.0 to 2.4 hr.), and in the cases with hemochromatosis it was 1.7 hr. (range 1.2 to 2.0 hr.), which was reduced to a mean of 0.8 hr. (range 0.6 to 1.1 hr.) after repeated phlebotomies. In the case of malnutritional cytosiderosis the time was 2.4 hr.

Red Cell Iron Turnover and Percentage Red Cell Utilization.—The red cell iron turnover in 6 control subjects was 24 mg. per day (range 17 to 33 mg. per day) and 0.38 mg. per kilogram per day (range 0.24 to 0.62 mg. per kilogram per day), while the fraction of red cells renewed per day was 0.013 (range 0.006 to 0.020). In only two subjects with hemochromatosis (Cases 12 and 13)

was it possible to calculate the red cell iron turnover. Results of 21 and 25 mg. per day and 0.38 and 0.55 mg. per kilogram per day were recorded with red cell renewal fractions of 0.016 and 0.019, respectively. In Case 12 further results were obtained after repeated phlebotomies. It was found that the red cell iron turnover had been raised to 76 mg. per day and 1.36 mg. per kilogram per day and the fraction of red cells renewed to 0.076 per day.

The mean red cell utilization of the injected dose of radioiron was 90 per cent (range 82 to 100 per cent) in 9 control subjects as compared with a mean of 39 per cent (range 20 to 54 per cent) in 5 studies on 4 cases with hemochromatosis (Fig. 3). This figure was raised to a mean of 54 per cent (range 32 to 72 per cent) in 6 studies done after repeated phlebotomies on 4 subjects with hemochromatosis. In the case of malnutritional cytosiderosis the red cell utilization of injected radioiron was 34 per cent.

DISCUSSION

It has been shown that iron derived from gut absorption accounts for less than 1 per cent of the plasma iron turnover in normal subjects.[14] However, iron absorption is so high in at least some cases of hemochromatosis[1, 3, 4] that an associated increase in the turnover of iron in the plasma might be expected. In addition, there is a continual internal redistribution of iron in subjects with raised iron stores.[7] As this transfer must occur via the plasma iron compartment it should also lead to a more rapid turnover of iron in the plasma. The results of the present investigation showed that there was, in fact, a significant increase in the plasma iron turnover expressed in milligrams per kilogram per day in hemochromatosis as compared with a group of control subjects ($P < 0.01$ as calculated by the t test) (Table I and Fig. 2). Previously we have demonstrated that there was a very great absorption of iron from the gut in two young subjects with hemochromatosis, and it was suggested that the metabolic defect was more severe and accounted for the poor prognosis of such cases.[4] It is of interest, therefore, that the highest turnover of 56 mg. per day was recorded in the only young subject with hemachromatosis investigated. Iron absorption studies done previously on this case had shown a retention of 72 per cent of an oral dose of 20 mg. of iron.[4] The increased plasma iron turnover in hemochromatosis seems to be a function of the large plasma iron pool as the rate of removal of iron from the plasma was normal (Table I). In only two of these subjects was it possible to calculate the red cell iron turnover and in both it was well within the normal range. This finding might have been expected as there is usually no anemia in hemochromatosis[21] and no evidence of a shortened red cell life. It has been shown previously that the percentage red cell utilization of a small dose of injected radioiron is low in hemochromatosis as compared with normal subjects.[8] This finding was confirmed in the present study (Fig. 3). It merely reflects, however, the high level of iron stores in the disease and does not indicate diminished red cell production.

Only one case of malnutritional cytosiderosis was investigated. This condition has been found to be present in a large percentage of South African Negro pellagrins.[9] The histologic features in the livers in some of these cases

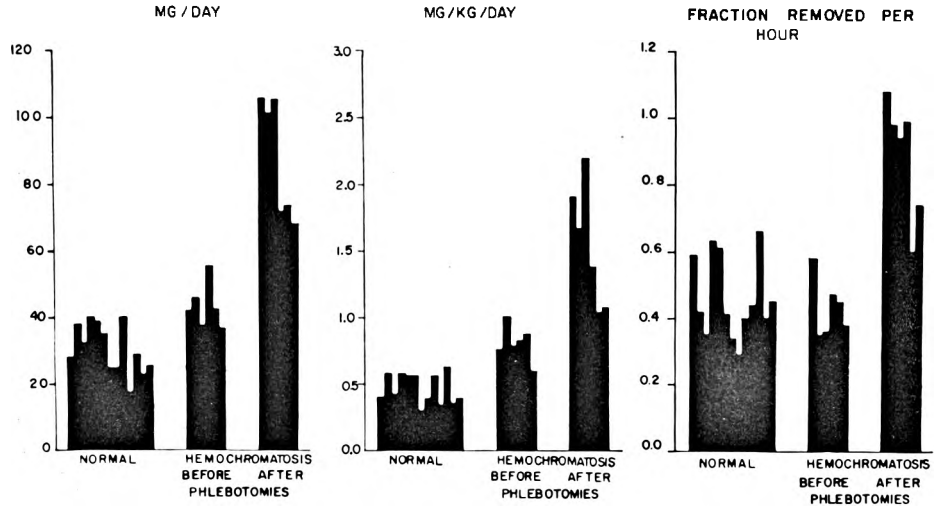


Fig. 2.—Plasma iron turnover values in normal subjects and in patients with hemochromatosis before and after repeated phlebotomies.

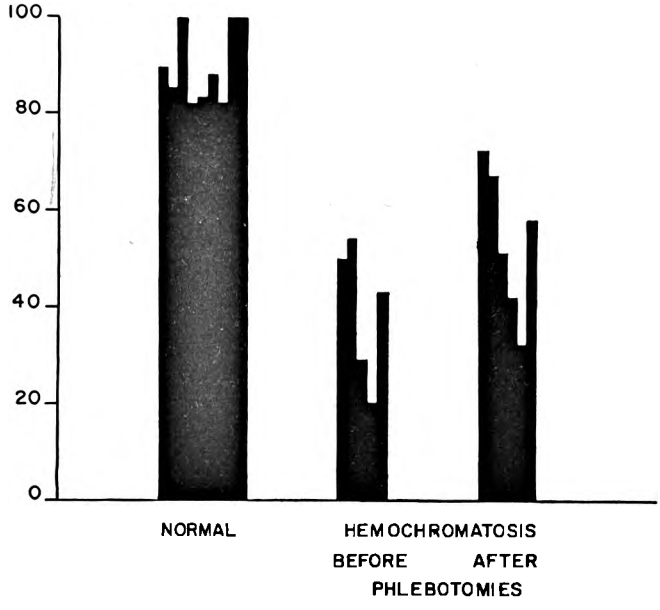


Fig. 3.—The percentage red cell utilization of an intravenous dose of radioiron in normal subjects and in patients with hemochromatosis before and after repeated phlebotomies.

were indistinguishable from those seen in hemochromatosis, and it was concluded that chronic malnutrition leads to a derangement in intracellular metabolism which interferes with the normal "mucosal block"[12] and so allows an abnormally large absorption of iron from the gut.[10] In previous oral radioiron studies on 5 subjects with malnutritional cytosiderosis, absorption from the gut was shown to be very low in 4 subjects.[3] However, in the remaining one a significant blood uptake was found and there was evidence of deposition of radioiron in the liver. It was decided to reinvestigate this last subject in the present study. Although the serum iron level was high (197 μg per cent) and the plasma volume normal, the plasma iron turnover was found to be lower than in any of the subjects with hemochromatosis and was well within the normal range. This was accounted for by the slow plasma iron disappearance rate (Table I).

There was a wide individual variation in the plasma and red cell iron turnovers in normal subjects. The plasma iron turnover in milligrams per day was significantly higher in men than in women ($P < 0.05$), but this difference was not apparent when the turnover was related to body weight as milligrams per kilogram per day ($P < 0.7$). However, the groups were small and the age distributions not strictly comparable so that no valid conclusions could be drawn from these figures. The mean results for plasma iron turnover and for red cell iron turnover, where this could be calculated, approximated fairly closely those reported by Huff and co-workers[14] in normal subjects. They were slightly higher, however, but the reason for this may be related in some way to a different physiologic pattern at the Johannesburg altitude (5,700 feet). In a later paper, however, these workers reported on findings in a group of normal students in Lima.[15] In this second study they recorded a mean plasma iron turnover of 57 mg. per day and a red cell iron turnover of 47.3 mg. per day as compared with 30 mg. per day and 24 mg. per day, respectively, in the present series. On analyzing their data it seemed that the major factor producing this difference was the high serum iron level in Lima (a mean of 180 μg per cent as compared with a mean of 109 μg per cent in the present study). Huff and co-workers[15] suggested that the high serum values might have been due to the delay between the collection of blood samples and their subsequent analysis. There are no other available data for iron turnovers in normal subjects. However, Wasserman and co-workers[23] have studied the rate of disappearance of plasma iron in such a group. Calculations from their data show the mean time taken to reach ½-concentration to have been 1.5 hr., a figure which agrees closely with the one of 1.6 hr. found in the present study.

It has been suggested that repeated phlebotomies might form a rational form of therapy in hemochromatosis[11] and several investigators have subsequently treated cases in this way.[2, 5, 13] It has been found that these subjects are unique in their ability to maintain their hemoglobin levels after the repeated removal of large quantities of blood. One reported case tolerated the removal of as much as 50 L. of blood in 1 year and showed evidence of both subjective and objective improvement.[5] In the present study 4 patients with hemochromatosis were treated by this method and the serial changes in hemoglobin and serum iron levels can be seen in Fig. 1. One case (14) died relatively soon after the commencement of this therapy, but it was felt that this

was not causally related as she had already been in congestive cardiac failure several times prior to the commencement of phlebotomies. The others tolerated repeated phlebotomies well, but so far it is not possible to say whether the ultimate prognosis has been altered in any way.

The turnover of iron in hemochromatosis was studied in each patient after repeated phlebotomies. There was a striking rise in the plasma iron turnover. Four studies were done relatively soon after the commencement of treatment. In two (Cases 12 and 15) repeat studies were performed after the removal of further large quantities of blood (Fig. 1) and it was found that the plasma iron turnover was maintained at a constant figure for the individual. However, the capacity to mobilize iron stores for increased blood formation seemed to be different in each subject and this was reflected by the different frequencies with which phlebotomies could be performed on them. The increased plasma iron turnover following repeated phlebotomies in hemochromatosis was found to be due to an increased disappearance rate of iron from the plasma (Table I). It was possible to measure the red cell iron turnover in only one subject after repeated phlebotomies. It was found that it had risen from 21 mg. per day to 76 mg. per day. The plasma iron turnover at this time was 106 mg. per day. It is, therefore, evident that although the patient was being maintained in a negative iron balance by repeated phlebotomies, only about 75 per cent of the tagged plasma iron was being utilized for hemoglobin formation. Presumably the remainder was being deposited in various body stores. It is interesting to speculate as to whether this finding is an indication of an increased avidity of the tissues for iron as originally postulated by Sheldon.[21] If this is true then the increased absorption from the gut would seem to be a secondary factor and would not represent the primary defect in the disease.

SUMMARY AND CONCLUSIONS

Radioiron turnover studies were carried out on 4 patients with hemochromatosis, 1 patient with malnutritional cytosiderosis, and 11 normal subjects. A total of 26 studies were done. In addition, the effects of repeated phlebotomies in hemochromatosis were investigated.

The mean plasma iron turnover was found to be significantly raised in hemochromatosis, while the red cell iron turnover was normal in the two patients in whom it could be measured. The plasma iron turnover in the one case of malnutritional cytosiderosis fell within the normal range. The percentage of an administered dose of radioiron utilized for hemoglobin formation was reduced in hemochromatosis and in the subject with malnutritional cytosiderosis.

Repeated phlebotomies were well tolerated in hemochromatosis. They caused a marked and sustained increase in the plasma iron turnover. The degree to which the turnover could be raised varied in each subject but repeat studies in two cases seemed to show that it was constant for the individual. The red cell iron turnover was increased after repeated phlebotomies in the one subject in whom it was possible to measure it and the percentage red cell utilization of the injected dose of radioiron was raised although it was still lower than normal.

We wish to express our indebtednes to Professor G. A. Elliott, Head of the Department of Medicine, University of the Witwatersrand, for his generous assistance and encouragement throughout the study. In addition we are grateful to Dr. H. B. Stein, Acting Head of the Department of Clinical Pathology, for his co-operation and helpful advice and to Dr. B. van Lingen for criticizing the manuscript. Finally we wish to thank Dr. J. Stafford for facilities in the Biophysics Laboratories of the Council for Scientific and Industrial Research, Pretoria, and Mr. J. E. Kerrich, Department of Mathematics, University of the Witwatersrand, for advice on the statistical analysis.

REFERENCES

1. Alper, T., Savage, D. J., and Bothwell, T. H.: Radioiron Studies in a Case of Hemochromatosis, J. Lab. & Clin. Med. 37: 665, 1951.
2. Beyers, M. R., and Gitlow, S. E.: Metabolism of Iron in Hemochromatosis, Am. J. Clin. Path. 21: 349, 1951.
3. Bothwell, T. H., van Doorn-Wittkampf, H. van W., du Preez, M. L., and Alper, T.: The Absorption of Iron. Radioiron Studies in Idiopathic Hemochromatosis Malnutritional Cytosiderosis and Transfusional Hemosiderosis, J. Lab. & Clin. Med. 41: 836, 1953.
4. Bothwell, T. H., van Lingen, B., Alper, T., and du Preez, M. L.: The Cardiac Complications of Hemochromatosis; Report of a Case Including Radioiron Studies and a Note on Etiology, Am. Heart J. 43: 333, 1952.
5. Davis, W. D., Jr., and Arrowsmith, W. R.: The Effects of Repeated Phlebotomies in Hemochromatosis, J. Lab. & Clin. Med. 39: 526, 1952.
6. Ellis, B. C.: Plasma and Total Blood Volume Estimations by the T1824-Haematocrit Method, South African J. M. Sc. 18: 45, 1953.
7. Finch, C. A., Hegsted, M., Kinney, T. D., Thomas, E. D., Rath, C. E., Haskins, D., Finch, S., and Fluharty, R. G.: Iron Metabolism: The Pathophysiology of Iron Storage, Blood 5: 983, 1950.
8. Finch, C. A., Gibson, J. G., Peacock, W. C., and Fluharty, R. G.: Iron Metabolism. Utilisation of Intravenous Radioactive Iron, Blood 4: 905, 1949.
9. Gillman, J., and Gillman, T.: Structure of Liver in Pellagra, Arch. Path. 40: 239, 1945.
10. Gillman, J., and Gillman, T.: Pathogenesis of Cytosiderosis as Evidenced in Malnourished Africans, Gastroenterology 8: 19, 1947.
11. Granick, S.: Iron Metabolism and Hemochromatosis, Bull. New York Acad. Med. 25: 403, 1949.
12. Hahn, P. F., Bale, W. F., Ross, J. F., Balfour, W. M., and Whipple, G. H.: Radioactive Iron Absorption by Gastro-Intestinal Tract; Influence of Anemia, Anoxia, and Antecedent Feeding: Distribution in Growing Dogs, J. Exper. Med. 78: 169, 1943.
13. Houston, J. C.: Phlebotomy for Hemochromatosis, Lancet 1: 766, 1953.
14. Huff, R., Hennessy, T. G., Austin, R. E., Garcia, J. F., Roberts, B. M., and Lawrence, J. H.: Plasma and Red Cell Iron Turnover in Normal Subjects and in Patients Having Various Hematopoeitic Disorders, J. Clin. Invest. 29: 1041, 1950.
15. Huff, R. L., Lawrence, J. H., Siri, W. E., Wasserman, L. R., and Hennessy, T. G.: Effects of Changes of Altitude on Hematopoeitic Activity, Medicine 30: 197, 1951.
16. King, E. J.: Micro-Analysis in Medical Biochemistry, London, 1951, J. and A. Churchill, Ltd.
17. Kitzes, G., Elvehjem, C. A., and Schvette, H. A.: The Determination of Blood Plasma Iron, J. Biol. Chem. 155: 653, 1944.
18. Phillips, R. A., Van Slyke, D. D., Emerson, K., Jr., Hamilton, P. B., and Archibald, R. M.: Copper Sulfate Method for Measuring Specific Gravities of Whole Blood and Plasma, New York, 1944, 1945, Josiah Macy, Jr., Foundation.
19. Rath, C. E., and Finch, C. A.: Chemical, Clinical and Immunological Studies on the Products of Human Plasma Fractionation. XXXVIII. Serum Iron Transport. Measurement of the Iron Binding Capacity of Serum in Man, J. Clin. Invest. 28: 79, 1949.
20. Reeve, E. B.: Methods of Estimating Plasma and Total Red Cell Volume, Nutrition Abstr. & Rev. 17: 811, 1948.
21. Sheldon, J. H.: Hemochromatosis, New York and London, 1935, Oxford University Press.
22. Vosburgh, G. J., Flexner, L. B., and Cowie, D. B.: The Determination of Radioactive Iron in Biological Material, J. Biol. Chem. 175: 391, 1948.
23. Wasserman, L. R., Rashkoff, I. A., Leavitt, D., Mayer, J., and Port, S.: The Rate of Removal of Radioactive Iron From the Plasma—An Index of Erythropoeisis, J. Clin. Invest. 31: 32, 1952.

DIURNAL VARIATION IN THE TURNOVER OF IRON THROUGH THE PLASMA.

By T. H. BOTHWELL and BARBARA MALLETT.

[*Reprinted from* "*Clinical Science.*"
Vol. 14, No. 2, May, 1955.]

[*Reprinted from* "*Clinical Science.*"
Vol. 14, No. 2, May, 1955.]

DIURNAL VARIATION IN THE TURNOVER OF IRON THROUGH THE PLASMA.

By T. H. BOTHWELL* and BARBARA MALLETT†

(*From the Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford*).

Received November 29, 1954.

RECENTLY Huff and co-workers have shown that when a tracer dose of radioactive iron of high specific activity is added to plasma or β_1 globulin and injected intravenously it disappears exponentially from the circulation (8). From the rate of disappearance and with a knowledge of the plasma iron concentration and the plasma volume, it is possible to obtain a quantitative measure of the amount of iron passing through the plasma at any one time (8). Using such a technique, several studies have been made in a variety of conditions (8, 9, 10, 11). In each of these the turnover of iron through the plasma has been expressed in mg./24 hours. In the presentation of such data, however, little attempt has been made to allow for the considerable diurnal variations in the concentration of plasma iron which have been shown to occur (4, 5, 6, 7, 12, 13, 14).

The present study was undertaken to find out if changes in the plasma iron level affected the plasma iron turnover significantly.

METHODS.

Twenty patients in hospital were studied twice in a 12–14-hour period (Table I).

^{59}Fe of high specific activity (1–2 μc./μg. Fe) was obtained from an American source through A.E.R.E., Harwell,‡ in the form of a sterile solution of ferric chloride. 0·1 to 0·2 ml. (5 to 10 μc.) of the radioiron solution was added to 2 ml. of a 1% sterile solution of β_1 globulin (supplied by the Cutter Laboratories, U.S.A.) buffered with 10 to 20 ml. of the patient's plasma. After incubation for 30 minutes at 37°C. the mixture was injected intravenously from a calibrated syringe, a small quantity being kept for the preparation of the standard solutions. Fifteen ml. blood samples were taken at intervals following the injection. Normally 5 samples were withdrawn after 10, 20, 40, 60 and 90 minutes.

In each subject two studies were made, one at 10 a.m. and the other at 8 p.m.. In 11 cases the morning study was carried out first, while the order was reversed in the remaining 9 cases. In each instance plasma was removed prior to the second study in order to estimate any residual radioactivity.

---

* Nuffield Travelling Fellow in Medicine.

† We wish to thank Mr. J. R. P. O'Brien and Dr. Rosemary Biggs for helpful criticisms. We are also grateful to Professor L. J. Witts and Dr. Sheila Callender for advice and encouragement.

This work was in part supported by a grant to Professor L. J. Witts from the Medical Research Council.

‡ Atomic Energy Research Establishment.

Radioactivity was measured by a scintillation counter using the method described by Badenoch and Callender (1). Five ml. of plasma were counted from each sample of blood and the results plotted on semilogarithmic paper. The total radioactivity administered was estimated from standards prepared in duplicate from the radioiron plasma mixture given. 0·02 to 0·1 ml. of this mixture was used for each standard and made up to 5 ml. with isotonic saline.

Values for plasma iron turnover per hour were calculated from the Huff formula (8):

Plasma iron turnover (μg./hour) = [fraction of radioiron removed per hour × plasma volume (ml.) × plasma iron concentration (μg./ml.)].

The fraction of radioiron removed/hour was calculated by dividing the natural logarithm of two by the time taken for circulating radioiron to reach one-half concentration. Plasma volumes were calculated by extrapolation to zero time of the exponential disappearance line of radioiron from the plasma, while plasma iron levels were estimated in duplicate by a thioglycollic acid, 2 : 2′-dipyridyl technique (2). With this method for plasma iron determination the mean difference between 30 duplicate estimations was 2·6 μg./100 ml. (SD 3·9).

RESULTS.

Data from the 20 subjects investigated are shown in Table I. It can be seen that there were considerable fluctuations between the morning and evening results. These fluctuations were not found to be dependent on the order in which the two tests were done.

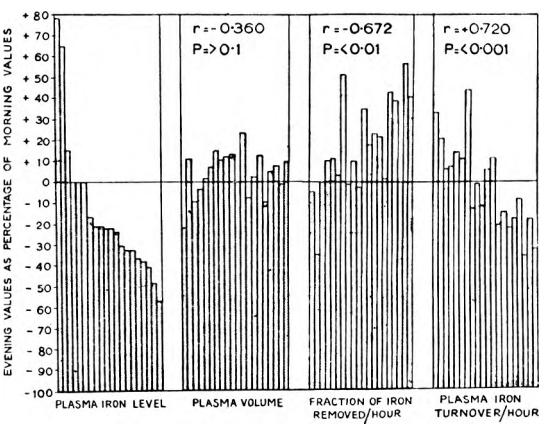


Fig. 1. The correlations between diurnal fluctuations in plasma iron level and changes in (*a*) plasma volume, (*b*) fraction of iron removed/hour and (*c*) plasma iron turnover/hour.

Major interest centred on the relationship between variations in plasma iron concentration and the other factors concerned in iron turnover. In order to assess these relationships all evening values for plasma iron, plasma volume, fraction of iron removed/hour and plasma iron turnover were expressed as percentages of their morning values (Fig. 1). It can be seen that changes in plasma volume were not large; those which did occur were not related to changes in plasma iron

TABLE I.

Results of radioiron studies in 20 subjects.

Diagnosis	Order of tests	Time	Plasma volume ml.	Plasma iron µg./100 ml.	Fraction of iron removed/ hour	Plasma iron turnover µg./hour
(1) Terminal " Hodgkins "	1	10 a.m.	5200	33	2·310	3964
	2	8 p.m.	4730	38	2·310	4151
(2) Post-hæmorrhagic anæmia	2	10 a.m.	2708	11	1·980	590
	1	8 p.m.	2773	11	2·188	667
(3) Hæmolytic anæmia	1	10 a.m.	3584	44	1·890	2980
	2	8 p.m.	2808	78	1·800	3942
(4) Gastro-colic fistula with anæmia	1	10 a.m.	2993	22	1·188	782
	2	8 p.m.	3206	22	1·222	862
(5) Thrombophlebitis	2	10 a.m.	4841	46	0·756	1684
	1	8 p.m.	5323	20	1·066	1135
(6) Myelosclerosis	2	10 a.m.	5344	170	0·904	8213
	1	8 p.m.	5194	170	0·990	8742
(7) Pernicious anæmia and thrombophlebitis	1	10 a.m.	2523	92	0·884	2052
	2	8 p.m.	2718	54	0·884	1298
(8) Coronary artery disease early CNS syphilis	1	10 a.m.	2139	68	0·770	1120
	2	8 p.m.	2372	54	0·756	968
(9) Bleeding peptic ulcer	1	10 a.m.	2814	102	0·770	2210
	2	8 p.m.	3172	80	0·756	1918
(10) Iron deficiency and relative polycythæmia	2	10 a.m.	2491	29	0·717	518
	1	8 p.m.	2801	23	0·785	506
(11) Convalescent bacterial endocarditis	1	10 a.m.	2913	86	0·693	1736
	2	8 p.m.	3271	58	0·705	1337
(12) Peptic ulcer	1	10 a.m.	2066	49	0·630	638
	2	8 p.m.	2071	38	0·848	667
(13) Carcinoma of lung	2	10 a.m.	3205	94	0·520	1567
	1	8 p.m.	3676	78	0·784	2248
(14) Convalescent cardiac failure	2	10 a.m.	3675	60	0·784	1729
	1	8 p.m.	4093	99	0·513	2079
(15) Convalescent T.B. pericarditis	2	10 a.m.	2077	113	0·570	1338
	1	8 p.m.	2126	76	0·693	1120
(16) Essential hypertension	1	10 a.m.	2534	92	0·416	970
	2	8 p.m.	2293	58	0·594	790
(17) Parkinson's disease	2	10 a.m.	2679	108	0·378	1094
	1	8 p.m.	2660	56	0·594	885
(18) Peptic ulcer	1	10 a.m.	3114	84	0·433	1133
	2	8 p.m.	2867	58	0·533	886
(19) Mild colitis	2	10 a.m.	2353	124	0·365	1065
	1	8 p.m.	2884	94	0·429	1163
(20) Emphysema	1	10 a.m.	2018	100	0·310	626
	2	8 p.m.	2126	62	0·430	567

concentration and may have been partly due to technical inaccuracies. There was a negative correlation between alterations in the plasma iron level and the fraction of iron removed/hour. However, alterations in the fraction of iron removed/hour were, on the whole, less than changes in plasma iron concentration with the result that there was still a positive correlation between changes in iron concentration and the turnover/hour.

Although the group as a whole showed these trends it can be seen from Table I that there were considerable variations in individual results. It appeared that the fraction of iron removed/hour was less likely to change in cases where this fraction was greater than normal. (Using the same technique as in the present study the upper limit of normality for the fraction of iron removed/hour in 18

healthy subjects was 0·693 (3).) The data were therefore analysed further and subjects were divided into two groups, one (cases 1 to 10) in which the figures obtained in both tests fell outside this range and the other (cases 11 to 20) in which one or both were within the normal range. It can be seen (Fig. 2) that changes in plasma iron concentration in the first group were directly reflected in the turnover/hour ($p<0·001$) as there was little change in the fraction of iron removed/hour regardless of changes in plasma iron concentration. In the second group, however, the plasma iron turnover/hour was not significantly altered ($p>0·05$) by changes in plasma iron level as the changes were in part or completely corrected by corresponding alterations in the fraction of iron removed/hour.

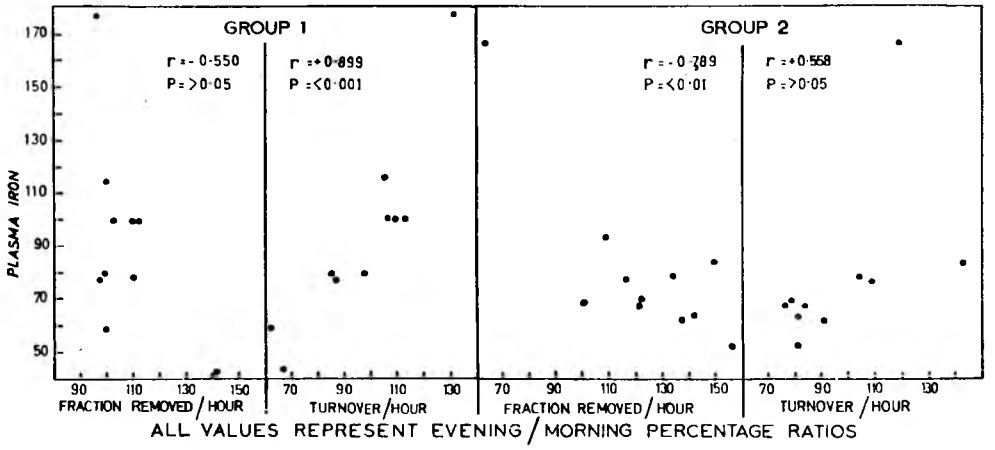


Fig. 2. The correlations between diurnal fluctuations in plasma iron level and changes in (*a*) fraction of iron removed/hour and (*b*) plasma iron turnover/hour. Group I includes all cases in which the fraction of iron removed/hour was greater than normal and Group II those in which it was within the normal range.

Discussion.

Plasma iron turnover studies repeated within a period of 12 to 14 hours in 20 subjects showed that diurnal variations in the level of iron in the plasma may significantly alter such results. However, the pattern of response was not uniform. In half the cases there was a tendency for the turnover through the plasma to be maintained fairly constant, any change in plasma level being partly or completely compensated by corresponding alterations in the rate at which iron was cleared from the plasma. This homeostatic mechanism became less apparent in subjects in whom the clearance of iron was a rapid one, *e.g.*, anæmias and infections. Changes in plasma iron concentration in these cases were directly reflected in the figures for plasma iron turnover.

The diurnal variation in plasma iron turnover which has been shown to occur makes the assessment of the rate of blood formation from such data difficult. There is no doubt that the technique yields useful information concerning the dynamics of iron transport. However, it seems invalid to assume, as has been done in the past, that figures obtained at any one time during the day reflect accurately the pattern of iron turnover throughout a 24-hour period. It would seem difficult to correct for the variations which have been found. The measurement of several plasma iron levels in a 24-hour period might increase the accuracy of the technique in cases where the rate of disappearance remains constant. It would, however,

only magnify the errors where fluctuations in plasma iron level are partly, or completely, compensated for by changes in the clearance rate of iron from the plasma.

SUMMARY.

(1) The turnover of iron through the plasma was estimated in the morning and evening in 20 subjects.

(2) The plasma iron turnover was significantly affected by changes in plasma iron concentration. However, the variability of individual responses makes it impossible, at the present time, to calculate accurately the daily turnover from observations on the clearance of single tracer doses of radioiron from the plasma.

REFERENCES.

(1) BADENOCH, J., AND CALLENDER, S. T. Iron metabolism in steatorrhoea. The use of radioactive iron in studies of absorption and utilisation. Blood, 1954, **9**, 123.

(2) BOTHWELL, T. H., AND MALLETT, B. J. The determination of iron in plasma or serum. Biochem. J., 1955, **59**, 599.

(3) BOTHWELL, T. H., AND MALLETT, B. J. Unpublished data.

(4) HAMILTON, L. D., GUBLER, C. J., CARTWRIGHT, G. E., AND WINTROBE, M. M. Diurnal variation in the plasma iron level in man. Proc. Soc. Exp. Biol., N.Y., 1950, **75**, 65.

(5) HEMMELER, G. Metabolism of Iron; Diurnal fluctuations in serum iron. Helv. med. Acta, 1944, **11**, 201.

(6) HØYER, K. Physiologic variations in the iron content of human blood serum: variations from week to week, from day to day and through 24 hours. Acta med. Scand., 1944, **119**, 562.

(7) HØYER, K. Physiologic variations in the iron content of human blood serum: further studies of the intra diem variations. Acta med. Scand., 1944, **119**, 577.

(8) HUFF, R. L., HENNESSEY, T. G., AUSTIN, R. E., GARCIA, J. F., ROBERTS, B. M., AND LAWRENCE, J. H. Plasma and red cell iron turnover in normal subjects and in patients having various hæmatopoietic disorders. J. clin. Invest., 1950, **29**, 1041.

(9) HUFF, R. L., LAWRENCE, J. H., SIRI, W. E., WASSERMAN, L. R., AND HENNESSEY, T. G. Effects of changes in altitude on hæmatopoietic activity. Medicine, 1950, **30**, 197.

(10) LAWRENCE, J. H., ELMLINGER, P. J., AND FULTON, G. Oxygen and control of red cell production in primary and secondary polycythæmia: effects on the iron turnover patterns with Fe^{59} as tracer. Cardiol., 1952, 21, 337.

(11) HUFF, R. L., TOBIAS, C. A., AND LAWRENCE, J. H. A test of red cell production. Acta Hæmatol., 1952, **7**, 129.

(12) PATERSON, J. C. S., MARRACK, D., AND WIGGINS, H. S. Hypoferræmia in the human subject: the importance of diurnal hypoferræmia. Clin. Sci., 1952, **11**, 417.

(13) SCHLAPHOFF, D., JOHNSTON, F. A., AND BOROUGHS, E. D. Serum iron levels in adolescent girls and the diurnal variation of serum iron and hæmoglobin. Arch. Biochem., 1950, **28**, 165.

(14) WALDENSTRÖM, J. The incidence of "iron deficiency" (sideropenia) in some rural and urban populations. Acta med. Scand., 1946, Supp. 170, p. 252.

CONTENTS.

PAGE

FURTHER STUDIES ON THE MECHANISM OF EXPERIMENTAL HYPERTENSION IN THE RAT 163
By M. A. FLOYER.

RELATION BETWEEN BLOOD SUGAR LEVEL AND THE OPTICAL PROPERTIES OF THE LENS OF THE HUMAN EYE 183
By D. W. VERE and D. VEREL.

REACTIVE HYPERÆMIA IN THE HUMAN FOREARM 197
By G. C. PATTERSON and R. F. WHELAN.

ANATOMICAL CLOSURE OF THE DUCTUS ARTERIOSUS IN THE GUINEA PIG 213
By R. G. RECORD and THOMAS McKEOWN.

THE EFFECT OF REDUCED ATMOSPHERIC PRESSURE ON CLOSURE OF THE DUCTUS ARTERIOSUS IN THE GUINEA PIG 225
By R. G. RECORD and THOMAS McKEOWN.

DIURNAL VARIATION IN THE TURNOVER OF IRON THROUGH THE PLASMA 235
By T. H. BOTHWELL and BARBARA MALLETT.

RENAL UPTAKE OF RADIOACTIVE POTASSIUM 241
By E. W. EMERY, R. HOLMES, H. E. F. DAVIES and D. A. K. BLACK.

DISTRIBUTION OF LABELLED GAMMA GLOBULIN IN HUMANS, STUDIED WITH AN EXTERNAL COUNTER 245
By N. B. MYANT.

"FIXATION" OF TUBERCULIN IN THE SKIN OF TUBERCULIN SENSITIVE HUMAN SUBJECTS 253
By J. PEPYS.

THE EFFECT OF INTRA-ARTERIAL ANTIHISTAMINES ON THE HYPERÆMIA FOLLOWING TEMPORARY ARREST OF THE CIRCULATION IN THE HUMAN FOREARM 267
By F. DUFF, G. C. PATTERSON and R. F. WHELAN.

THE RATE OF BLOOD FLOW AND THE OXYGEN SATURATION OF THE EFFLUENT BLOOD FOLLOWING CONTRACTION OF THE MUSCLES OF THE HUMAN FOREARM 275
By A. H. G. LOVE.

THE EFFECT OF VENOUS CONGESTION ON THE RATE OF HEAT ELIMINATION FROM THE FINGERS 285
By R. G. SHANKS.

THE URINARY EXCRETION OF LABELLED VITAMIN B_{12} 295
By SHEILA T. CALLENDER and J. R. EVANS.

THE RELATIONSHIP BETWEEN BLOOD FLOW, CAPILLARY SURFACE AREA AND SODIUM CLEARANCE IN MUSCLE 303
By D. N. WALDER.

THE ROLE OF OXYGEN, CARBON DIOXIDE AND LACTIC ACID IN THE VENTILATORY RESPONSE TO EXERCISE IN PATIENTS WITH MITRAL STENOSIS 317
By J. E. COTES.

CIRCULATORY DYNAMICS AT REST AND ON EXERCISE IN THE HYPERKINETIC STATES 329
By J. M. BISHOP, K. W. DONALD and O. L. WADE.

PRINTED IN GREAT BRITAIN.

Publishers:
SHAW & SONS LTD., 7, 8 & 9, FETTER LANE, FLEET STREET,
LONDON, E.C.4 ENGLAND.

THE STUDY OF ERYTHROPOIESIS USING TRACER QUANTITIES OF RADIOACTIVE IRON

BY

T. H. BOTHWELL, SHEILA CALLENDER,
BARBARA MALLETT and L. J. WITTS

Reprinted from British Journal of Haematology, Vol. II, No. 1, January 1956

OXFORD
BLACKWELL SCIENTIFIC PUBLICATIONS

Brit. J. Haemat., 1956, 2, 1.

The Study of Erythropoiesis Using Tracer Quantities of Radioactive Iron

T. H. Bothwell,* Sheila Callender, Barbara Mallett and L. J. Witts

Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford

Iron circulates in the plasma as a complex with β_1 globulin (Schade and Caroline, 1946). Although the plasma iron forms only about 0.1 per cent of the total body iron it is an extremely important fraction since it represents the sole means of transport of iron from one part of the body to another. As only negligible quantities of iron are normally lost from the body (McCance and Widdowson, 1938; Hahn, Bale, Hettig, Kamen and Whipple, 1939) the level of plasma iron must represent a dynamic equilibrium between the iron being supplied from haemoglobin breakdown, body stores and gastro-intestinal absorption, and that being removed by the bone marrow and tissues. In recent years the use of radioactive iron has helped to define the relative importance of these various factors in iron metabolism. Normally, iron derived from red-cell breakdown is reutilized for haemoglobin formation and does not appear to mix with the storage iron (Dubach, Moore and Minnich, 1946; West, Hahn, Clark and Chappelle, 1952). Likewise, small quantities of labelled iron added to the circulation, either by absorption from the gut (Dubach, Callender and Moore, 1948) or by intravenous injection (Greenberg and Wintrobe, 1946), are preferentially utilized for red-cell formation. This implies that iron throughout the body's stores is not uniformly mixed; that which is most recently added appears to be more readily available for current metabolic needs. Such findings have led to the concept of a 'labile iron pool' (Greenberg and Wintrobe, 1946).

In earlier studies with radioactive iron the isotopes were of relatively low specific activity and it was only possible to follow its fate in red cells and tissues. More recently, using radioiron with much higher specific activity, Huff and co-workers (1950) showed that the plasma iron could be labelled with tracer doses of ^{59}Fe and its subsequent course followed. It appeared that the loss of radioiron from the plasma was exponential, a finding which was reasonably held to imply that no appreciable quantities of radioiron were returned from the sites of removal, i.e., bone marrow and other stores, during the period of observation. The rate of removal of iron was found from the slope of the disappearance line and with a knowledge of the concentration of iron in the plasma and the plasma volume it was possible to calculate the quantity of iron passing through the plasma per unit time. Some of the radioiron reappeared subsequently as haemoglobin in the red cells and formulae were introduced to work out the amount of iron going into the red cells per day and the fraction of red cells renewed per day. This was thought to give a direct, though approximate, index of the rate of blood formation.

This paper reports the use of the tracer technique in sixty-four subjects, most of whom were suffering from various types of blood disorder. It has been found that the method is useful in demonstrating certain abnormal patterns of erythropoiesis and it seems to have a definite, though limited, value as a diagnostic aid. However, both the practical and theoretical problems which have been encountered serve to underline the difficulties in interpreting

* Nuffield Travelling Fellow.

results. Although it is possible to demonstrate qualitative alterations in red-cell formation by the technique, it is doubtful at the present time whether the results give a reliable quantitative measure of the rate of blood formation.

MATERIAL AND METHODS

Clinical Material

Most of the subjects were patients in hospital at the time of study. The diagnosis and relevant haematological data can be seen in Table I.

Preparation of Radioiron

In initial experiments ^{59}Fe was obtained from A.E.R.E., Harwell as a solution of ferric chloride with a specific activity of 1 μc per 10 to 30 μg. Small aliquots of this solution were incubated with samples of the patients' plasma and injected intravenously. However, results obtained with this method were not consistent. The dose of iron (50 to 300 μg.) was large relative to the binding capacity of the volumes of plasma used and there was evidence in some cases that a proportion of the iron remained in an ionic form and rapidly disappeared from the plasma. The use of a 5 per cent solution of pure iron-binding protein* instead of plasma was again not entirely satisfactory. In order to keep the amount of iron within the limits of a tracer technique, the amount of radioactivity given had to be diminished and counts obtained from blood sampling were sometimes too low to be reliable. Because of these difficulties results by these two techniques have been discarded.

Finally, ^{59}Fe of higher specific activity (1 to 2 μc/μg.) became available from an American source through A.E.R.E., Harwell. The ^{59}Fe was in the form of a sterile solution of ferric chloride and 100 μc were contained in a volume of 2 ml. at a pH of approximately 1.5. A quantity of 0.2 to 0.4 ml. of this iron solution was added to 10 to 20 ml. of the patient's plasma and 2 ml. of a 1 per cent solution of β_1 globulin. The plasma was used as a buffering agent since iron becomes dissociated from the protein at a pH below 6.0. The pH of the final mixture was checked in the earlier cases before injection. Other workers have used 3.8 per cent sodium citrate as a buffer but *in vitro* experiments showed that the presence of citrate interfered with the binding of iron to globulin, so that it was decided to rely on plasma for buffering in the present study. After incubation for 30 minutes, 10 to 20 ml. of the final mixture were injected intravenously, a small quantity being kept for the preparation of the standard solutions. Several blood samples were taken in the hours following the injection. The time intervals were varied depending on the type of case being studied, e.g., more frequent sampling was carried out in the first hour in subjects in whom a rapid clearance rate was expected. Thereafter, blood samples were taken over the next 2 weeks in most cases. In the majority these were withdrawn at daily intervals but in some sampling was less frequent. The radioiron which reappeared in red cells normally reached a plateau between the tenth and fourteenth days.

Radioactivity was measured using a scintillation counting technique as described by Badenoch and Callender (1954). Five ml. of plasma were counted from each sample taken on the first day of the study and the results plotted on semilogarithmic paper to give a straight line for the exponential fall in activity. The total radioactivity given was obtained from standards prepared in duplicate from aliquots of the injection mixture made up to volumes of 5 ml.

* Generously supplied by the Cutter Laboratories, U.S.A.

Whole-blood samples taken on subsequent days were measured for radioactivity and the results compared with the standards counted on the same day. The plasma iron concentration was estimated in duplicate on the first morning of the study by either the method of Powell (1944) or of Bothwell and Mallett (1955a).

Calculation of Results

The line representing the exponential disappearance of radioiron from the plasma was extrapolated back to zero time to give the level of maximum plasma activity. With a knowledge of the dose administered it was then possible to estimate the plasma volume. The total blood volume was obtained from the plasma volume and the packed cell volume corrected for trapped plasma (Chaplin and Mollison, 1952) and the body/venous haematocrit ratio (Chaplin, Mollison and Vetter, 1953).

The following were calculated from the above data:—

(*a*) Plasma iron concentration per kilogram =

$$\frac{\text{plasma iron (µg./ml.)} \times \text{plasma volume (ml.)}}{\text{weight (kg.)}}$$

(*b*) Fraction of iron removed per hour =

$$\frac{\text{natural logarithm of 2}}{\text{time taken for plasma radioiron to reach } \frac{1}{2} \text{ concentration}}$$

(*c*) With a knowledge of (*a*) and (*b*) it was possible to calculate the plasma iron turnover in µg. per kilo. per hour, i.e., plasma iron concentration per kilogram × fraction of iron removed per hour.

(*d*) Percentage of red-cell utilization =

$$\text{maximum circulating activity in blood per ml.} \times \frac{\text{total blood volume (ml.)} \times 100}{\text{total administered radioactivity}}$$

(*e*) Mean marrow time =
time between the injection of the radioiron and the maximum daily rise of radioactivity in the circulating red cells.

Other workers (Huff *et al.*, 1950) have estimated both the red-cell iron turnover per day (plasma iron turnover per day × maximum fraction of radioiron appearing in red cells) and the fraction of iron renewed per day $\left(\frac{\text{red-cell turnover per day}}{\text{total iron in red cells}}\right)$. This was not done in the present study for reasons which will be discussed later.

RESULTS

Data on all patients investigated are shown in Table I.

Control Subjects

Ten haematologically normal subjects were investigated (Nos. 1 to 10). The plasma iron concentration ranged from 25.5 to 68.4 µg./kg. (mean 40.4), the fraction of iron removed per hour from 0.33 to 0.69 (mean 0.48) and the plasma iron turnover from 14.6 to 24.6 µg./kg./hour (mean 18.3). In the seven cases in which it was estimated the percentage red-cell utilization ranged between 75 and 89 (mean 82), while the mean marrow time varied between 3 and 4 days.

TABLE I

HAEMATOLOGICAL AND RADIOIRON DATA ON ALL THE SUBJECTS INVESTIGATED

Case No.	*Diagnosis*	*Age*	*Sex*	*Haemoglobin (g./100 ml.)*	*Red cells ($\times 10^6$/c.mm.)*	*Haematocrit (%)*	*Reticulocytes (%)*	*Total plasma iron (µg./kg.)*	*Fraction of iron removed per hour*	*Plasma iron turnover (µg./kg./hour)*	*Red-cell utilization (%)*	*Mean marrow time (days)*
				Control subjects								
1	Diabetes mellitus	55	M	17.0	6.25	54	...	25.5	0.69	17.6	77	4
2	Normal	58	M	16.4	5.39	47	...	30.0	0.60	18.0	84	3
3	Epilepsy	26	F	14.8	4.62	46	1.2	37.4	0.47	17.6	89	4
4	Peptic ulcer	45	M	15.6	4.55	47	...	44.1	0.33	14.6	...	...
5	Peptic ulcer	73	M	14.8	4.84	45	...	42.4	0.48	20.4	87	4
6	Normal	43	M	15.3	5.04	46	...	42.2	0.44	18.6	75	4
7	Peptic ulcer	30	F	15.6	4.60	45	...	30.0	0.52	15.6	...	...
8	Normal	33	M	17.8	5.64	50	...	68.4	0.36	24.6	78	4
9	Normal	18	M	15.8	4.40	45	...	33.2	0.57	18.9	...	...
10	Normal	63	M	15.9	5.74	50	...	50.7	0.33	16.7	87	3
				Iron-deficiency anaemia								
11	Iron-deficiency anaemia + tuberculous endometritis + carcinoma of stomach	37	F	9.2	4.10	32	1.2	14.8	1.73	25.6	100	2
12	Idiopathic hypochromic anaemia	37	F	10.1	4.20	34	...	22.2	1.54	34.2	...	...
13	Hypochromic anaemia + menorrhagia	44	F	8.0	4.10	28	0.4	6.6	2.19	14.5	...	...
14	Idiopathic hypochromic anaemia	37	F	7.5	4.10	30	3.6	18.0	2.08	37.4	98	4
15	Gastro-jejuno-colic fistula with bleeding	49	M	12.8	5.00	41	1.4	10.6	1.19	12.6	...	...
16	Hypochromic anaemia + menorrhagia	54	F	8.0	4.30	30	0.2	11.5	1.66	19.1	...	...
17	Thrombocytopenic purpura + menorrhagia	23	F	7.1	2.80	23	3.0	20.8	1.30	27.0	89	3
18	Nutritional iron deficiency	17	M	4.6	3.10	18	1.6	14.9	1.67	24.9	100	3
				Hypoplastic anaemia								
19	Hypoplastic anaemia	32	M	8.3	2.80	22	1.4	96.6	0.15	14.5	...	...
20	Hypoplastic anaemia	48	M	7.8	2.38	21	0.4	61.1	0.16	9.8	15	...
21	Hypoplastic anaemia	20	M	7.7	2.40	21	1.6	141.3	0.14	19.8	21	...
22	Hypoplastic anaemia	53	M	9.2	2.64	27	1.0	47.7	0.41	19.6	49	...
23	Hypoplastic anaemia	78	F	8.5	2.26	24	1.0	51.1	0.25	12.8	49	...
24	Hypoplastic anaemia	60	M	8.4	2.82	28	1.2	75.9	0.40	30.4	19	4
25	Hypoplastic anaemia	50	M	8.6	2.84	25	0.6	63.8	0.54	34.5	39	3
				Haemolytic anaemia								
26	Hereditary spherocytosis	45	M	14.2	4.21	40	4.6	36.9	1.81	66.8	90*	3
27	Atypical congenital haemolytic anaemia	28	M	13.3	3.20	41	19.6	22.6	1.89	42.7	...	...
28	Reticulosis + secondary haemolytic anaemia	32	F	10.6	3.71	35	2.4	69.1	0.87	60.1	91	4
29	Acquired haemolytic anaemia	60	F	10.4	2.80	28	13.8	21.7	1.19	25.8	...	...
30	Acquired haemolytic anaemia	32	F	7.5	2.23	21	5.8	65.7	1.12	73.6	20†	...
31	Hereditary spherocytosis	19	M	11.1	3.20	32	11.8	62.8	1.54	96.7	68	3
32	Hereditary spherocytosis	21	M	15.4	4.62	40	9.2	57.6	1.49	85.8	59	3
33	Acquired haemolytic anaemia + iron deficiency	36	F	12.5	4.50	40	5.4	16.3	1.89	30.8	94	3
				Chronic leukaemia								
34	Chronic myeloid leukaemia	37	M	10.4	3.45	30	3.0	19.5	1.07	20.9	73	2
35	Chronic lymphatic leukaemia	44	M	15.4	3.90	47	...	67.9	0.39	26.5	85	4
36	Chronic lymphatic leukaemia	56	F	9.0	3.15	28	...	108.3	0.13	14.1	15	...
37	Chronic myeloid leukaemia	46	M	13.9	4.50	45	...	48.0	0.46	22.1	78	3

Case No.	Diagnosis	Age	Sex	Haemoglobin (g./100 ml.)	Red cells ($\times 10^6$/c.mm.)	Haematocrit (%)	Reticulocytes (%)	Total plasma iron (μg./kg.)	Fraction of iron removed per hour	Plasma iron turnover (μg./kg./hour)	Red-cell utilization (%)	Mean marrow time (days)
	Myelofibrosis and other leuco-erythroblastic anaemias											
38	Myelofibrosis	72	F	9.2	4.20	34	2.0	36.5	2.19	80.0	46.0	3
39	Myelofibrosis	50	M	4.7	1.45	14	3.8	108.1	0.99	107.0	...	...
40	Myelofibrosis	32	M	5.0	1.91	16	1.0	26.3	1.89	49.7	5‡	...
41	Myelosclerosis	62	F	12.3	3.60	36	2.0	92.8	0.76	70.5	19§	...
42	Leuco-erythroblastic anaemia (? cause)	62	M	6.7	2.40	25	13.4	92.8	0.45	41.8	54	...
43	Lymphosarcoma+leuco-erythroblastic anaemia	26	M	10.4	3.40	32	6.2	38.5	1.89	72.8	48	...
	Polycythaemia vera											
44	Polycythaemia vera	71	M	17.8	7.82	64	2.6	13.6	3.78	51.4	90	3
45	Polycythaemia vera	55	F	18.8	6.75	59	1.8	6.5	2.08	13.5	100	2
46	Polycythaemia vera	64	F	21.3	8.98	66	...	21.1	2.08	43.9	100	2
47	Polycythaemia vera	69	F	21.6	9.32	67	0.8	6.7	2.37	15.9	...	...
48	Polycythaemia vera	58	M	21.1	7.13	69	1.0	19.1	1.28	24.4	...	...
49	Polycythaemia vera	76	F	12.0	6.20	42	1.0	16.7	2.77	46.3	98	3
	Relative polycythaemia											
50	Coronary artery disease	52	M	19.0	6.30	55	0.8	31.2	0.38	11.9	91	4
51	Cerebral thrombosis (previous venesections)	60	M	17.9	6.36	52	...	9.0	0.72	6.5	...	...
52	Hiatus hernia+duodenal ulcer	63	F	17.2	5.71	51	...	12.9	0.85	11.0	87	4
53	Duodenal ulcer+splenic enlargement	49	M	19.0	5.99	55	...	31.2	0.92	28.7	80	3
54	Coronary artery disease+gastric ulcer	57	M	18.9	5.83	57	1.4	31.8	0.80	25.4	80	4
55	Coronary artery disease	61	M	18.6	6.24	55	0.6	30.4	0.58	17.6	87	3
	Infections											
56	Sub-acute bacterial endocarditis	26	F	10.3	3.65	33	...	20.2	1.26	25.5	95	4
57	Lobar pneumonia	56	M	13.7	4.13	47	...	56.1	0.40	22.4	76	4
58	Lobar pneumonia	22	M	15.8	5.11	43	...	41.9	0.42	17.6	82	4
59	Sub-phrenic abscess	38	M	15.0	4.82	44	...	28.1	0.69	19.4	92	3
60	Acute thrombophlebitis	52	M	12.3	4.30	39	0.4	22.8	0.76	17.3	...	...
	Miscellaneous disorders											
61	Inoperable carcinoma of lung	66	M	14.2	4.46	43	0.2	35.8	0.55	19.7	62	5
62	Widespread carcinomatosis	37	F	8.0	3.10	26	0.6	21.5	1.98	42.6	80	3
63	Untreated pernicious anaemia	56	F	10.9	2.70	28	1.2	43.3	0.89	38.5	60	3
64	Acute porphyria	19	F	14.6	...	41	...	47.3	0.23	10.9	85	5

* Post splenectomy.
† Splenectomy+transfusions during the period of utilization.
‡ Blood transfusion 4 days after plasma iron turnover.
§ Blood transfusion 8 days after plasma iron turnover.

Iron-Deficiency Anaemia

Eight cases (Nos. 11 to 18) were studied. The majority were secondary to blood loss and none had received iron therapy. In two patients other factors were present; case No. 11 had carcinoma of the stomach and tuberculous endometritis, while case No. 17 had menorrhagia secondary to thrombocytopenic purpura. However, these two patients showed patterns similar to the others and they have therefore been included in this group. The plasma iron concentration was lowered in all cases (mean 14.9 μg./kg., range 6.60 to 22.2), while the

fraction of iron removed per hour was considerably greater than normal (mean 1.67, range 1.19 to 2.19). The resulting plasma iron turnover was found to be either normal or greater than normal (mean 24.4 μg./kg./hour, range 12.6 to 37.4), while the percentage red-cell utilization in the four patients in whom it was measured (Nos. 11, 14, 17 and 18) was complete or almost complete.

Hypoplastic Anaemia

Seven patients (Nos. 19 to 25) were studied who were suffering from refractory anaemia. In all except one (No. 25) there was, in addition to the anaemia, a depression of both the white-cell and platelet counts and in six there was marrow hypoplasia. In one (No. 21), however, the sternal marrow was hyperplastic; it was felt that this was an isolated and unrepresentative fragment as it was impossible to obtain any marrow from other sites. The plasma iron concentration was raised in three patients (Nos. 19, 21 and 24) but was normal in the others, while the fraction removed per hour was low in four (Nos. 19, 20, 21 and 23) and normal in three. The plasma iron turnover was within or close to the normal range in all subjects. The red-cell utilization was lowered in the six patients in whom it was measured (mean 32 per cent, range 15 to 49).

Haemolytic Anaemia

Turnover studies were carried out on eight patients (Nos. 26 to 33). Analysis of the data obtained from these subjects was difficult as the group was heterogeneous. In addition some of the patients received therapy during the period of study which must have influenced the utilization of radioiron for red-cell production. Three had hereditary spherocytosis, one an atypical congenital haemolytic anaemia, three had idiopathic acquired haemolytic anaemia and one haemolytic anaemia secondary to a reticulosis. In half the cases (Nos. 28, 30, 31 and 32) the plasma iron concentration was relatively high although it was still within the normal range. In one patient (No. 33) it was low enough to suggest an associated iron deficiency, for which no cause could be found. The fraction of iron removed per hour was large in all cases (mean 1.48, range 0.87 to 1.89) with the result that the plasma iron turnover was likewise increased (mean 60.3 μg./kg./hour, range 25.8 to 96.7). The percentage red-cell utilization was followed in six patients. In three (Nos. 26, 28 and 33) the utilization was normal. In one of these (No. 26) the test was made shortly after splenectomy for hereditary spherocytosis and the results probably reflected active regeneration following the cessation of the haemolytic process. The second (No. 28) showed evidence of only a mild degree of haemolysis secondary to a reticulosis, while the third (No. 33) had been so well controlled on cortisone that it had been possible to stop therapy a month before the radioiron study. This patient had a low plasma iron concentration and the radioiron findings were, in fact, more suggestive of iron deficiency than of an active haemolytic process. In two of the subjects (Nos. 31 and 32) the red-cell utilization was adequate, although lower than normal. Such a finding is compatible with excess red-cell destruction leading to a continual loss of radioiron from the circulation, the major portion being then reutilized by the bone marrow. The last patient (No. 30) showed a very low red-cell utilization. However, the result is of doubtful significance as she was given blood transfusions and a splenectomy was performed during the period when the radioactive iron might have been expected to be incorporated in the red cells. The mean marrow time was normal in the five cases in which it was estimated.

Chronic Leukaemia

Two cases (Nos. 34 and 37) of chronic myeloid leukaemia and two (Nos. 35 and 36) of

chronic lymphatic leukaemia were investigated. In one of the myeloid and one of the lymphatic leukaemias (Nos. 35 and 37) results were within the normal limits. In the other myeloid leukaemia (No. 34) the plasma iron concentration was lowered (19.5 µg./kg.) and there was a corresponding increase in the fraction of iron removed per hour (1.07) so that the plasma iron turnover was normal (20.9 µg./kg./hour). Red-cell utilization was also normal (73 per cent). The second patient with lymphatic leukaemia (No. 36) is of interest as she had been given several courses of deep X-ray therapy in the past and had developed an anaemia requiring frequent blood transfusions. The patient had gross splenomegaly and the question was whether the anaemia was due to a generalized hypoplasia of the erythroid elements or to hypersplenism. The findings were those of hypoplastic anaemia with a high plasma iron concentration (108.3 µg./kg.), a low fraction of iron removed per hour (0.13), a relatively low plasma iron turnover (14.1 µg./kg./hour) and a very poor red-cell utilization (15 per cent). Marrow puncture revealed a hypocellular marrow which was in keeping with the turnover result.

Myelofibrosis and other Leuco-erythroblastic Anaemias

The plasma iron concentration was raised in two patients (Nos. 39 and 41) with myelofibrosis and was normal in another two (Nos. 38 and 40). In all four the fraction removed per hour was greater than normal (mean 1.46, range 0.76 to 2.19) as was the plasma iron turnover (mean 76.8 µg./kg./hour, range 49.7 to 107). A reduced red-cell utilization was found in the three (Nos. 38, 40 and 41) in whom it was measured. Two of these (Nos. 40 and 41) were given blood transfusions during the period when the radioactive iron might have been expected to be incorporated in the red cells, but in No. 41 this was at a time when it should not have made any great difference to the result. The high plasma iron turnover suggested that there was active extramedullary erythropoiesis in these cases while the low red-cell utilization was compatible with increased haemolysis.

Two patients (Nos. 42 and 43) with leuco-erythroblastic anaemia not due to myelofibrosis were also studied. In one (No. 43) the condition was secondary to lymphosarcoma and in the other the cause was not found. Both had an increased plasma iron turnover (41.8 and 72.8 µg./kg./hour respectively) while the percentage red-cell utilization was lower than normal (54 per cent and 48 per cent respectively). Such findings were thought to be compatible with increased haemolysis in these cases.

Polycythaemia Vera

Six cases (Nos. 44 to 49) all showed lowered plasma iron levels (mean 14.0 µg./kg. with a range from 6.5 to 21.1). This iron deficiency could be explained in part at least by previous venesections. The fractions of iron removed per hour were large (mean 2.39, range 1.28 to 3.78), with the result that the plasma iron turnover was either normal or greater than normal (mean 32.6 µg./kg./hour, range 13.5 to 51.4). The injected radioiron was either completely or almost completely utilized for red-cell production in the four subjects (Nos. 44, 45, 46 and 49) in which it was measured while the mean marrow time was reduced in two of these cases (Nos. 45 and 46).

Relative Polycythaemia

In addition, six patients (Nos. 50 to 55) were investigated in whom a tentative diagnosis of polycythaemia vera had been made because of the peripheral blood findings. None, however, showed the increase in red-cell mass characteristic of the disease, the mean red-cell

volume being 27.5 ml./kg. (range 25.5 to 30.3), as compared with 68.8 (range 41.0 to 81.4) in the six cases with polycythaemia vera, and the apparent polycythaemia was due to a reduction in the plasma volume. Similar cases have been described by Lawrence and Berlin (1952).

The results in this group were difficult to interpret. In two (Nos. 50 and 55) the radioiron studies were close to normal. In another two (Nos. 51 and 52) there was evidence of a complicating iron deficiency in that in both the plasma iron concentration was low. Although the fraction of iron removed per hour was greater than normal, the plasma iron turnover was still below the normal range. As one patient had been venesected previously and the other had a hiatus hernia, it is possible that the iron deficiency was secondary to blood loss. However, in the last two cases (Nos. 53 and 54) the fraction of iron removed per hour was greater than normal in spite of the fact that the plasma iron concentration was normal. No explanation for this finding was apparent.

Infections

Five subjects (Nos. 56 to 60) with various types of infection were studied. All were receiving therapy at the time of observation and only one (No. 56) was still pyrexial. In this case the plasma iron concentration was lowered (20.2 µg./kg.), the fraction removed per hour was large (1.26), the plasma iron turnover was normal (25.5 µg./kg./hour) and red-cell utilization was almost complete. In the others the results were within, or close to, the normal range. The only conclusion that may be drawn from these findings is that any possible disturbance in iron metabolism associated with acute infection is rapidly corrected when the infection is controlled.

Miscellaneous Disorders

Two patients (Nos. 61 and 62) suffering from carcinomatosis were studied. In one (No. 61) the findings were normal except for a lowered red-cell utilization (62 per cent), while in the other the plasma iron concentration was reduced (21.5 µg./kg.), the fraction of iron removed per hour was greater than normal (1.98), the plasma iron turnover was increased (42.6 µg./kg./hour) and the red-cell utilization was normal (80 per cent).

In one patient (No. 63) with untreated pernicious anaemia the plasma iron turnover was increased (38.5 µg./kg./hour) and the red-cell utilization was lower than normal (60 per cent).

The last patient (No. 64) was of special interest as she was studied during an attack of acute porphyria. The plasma iron concentration was normal (47.3 µg./kg.) but the fraction of iron removed per hour was very low (0.23), with the result that the plasma iron turnover was less than normal (10.9 µg./kg./hour). This pattern was similar to that found in hypoplastic anaemia and suggested that there was a defect in the incorporation of iron into haemoglobin. The red-cell utilization was normal (85 per cent) but the red-cell maturation time was prolonged (5 days). These findings are not incompatible with the plasma figures as the attack subsided soon after the start of the study and presumably the block to haemoglobin synthesis was corrected.

DISCUSSION

The final validation of a diagnostic technique of this kind, as of most other metabolic determinations, is a matter of extreme difficulty. It depends on the agreement of the results

with other methods of estimation, and on their parallelism with the fluctuations that might be expected from the disturbance of blood formation by experiment or disease. On the whole, the results of the present investigation agreed well with other diagnostic criteria and in some cases it was possible to obtain a clearer insight into pathological variations in blood formation than by other methods. Huff and his colleagues (1951) now combine such studies with *in vivo* counting over various organs. This was not done in the present investigation but it seems that it may show more accurately the patterns of erythropoiesis in health and disease, e.g., *in vivo* counting has been found particularly useful in differentiating splenomegaly with extramedullary erythropoiesis from that due to other causes and especially from splenomegaly with active destruction of red cells or removal of imperfect cells (Elmlinger *et al.*, 1953).

Discussion of Method

Although turnover studies have been shown to be of definite value in studying the mechanisms of blood formation there is some doubt as to whether the method is accurate enough for results to be expressed in quantitative terms. There are several practical and theoretical reasons why this is so.

In the first place it is assumed that the injected tracer behaves identically with the iron circulating in the plasma. In earlier studies the radioiron was incubated with the patient's plasma to allow combination with β_1 globulin before being injected intravenously. In subsequent investigations purified β_1 globulin has been used and the general pattern of results has been similar. However, it is possible that there may be variations in the rate at which such β_1 globulin releases the iron, especially in view of the fact that different batches are not all treated in the same way to free them from the virus of hepatitis. Such variations may be unimportant but there is as yet no published experimental proof that this is so. Another important practical point is the manner in which the iron is buffered prior to injection. In some studies sodium citrate has been used, but in the present investigation it was shown by *in vitro* experiments that citrate interferes with the binding of iron to globulin. This is probably due to the formation of a chelate between iron and citrate. If the iron citrate forms a firmer chelate than the iron globulin the rate of disappearance of the iron from the plasma may be altered significantly.

In calculating results Huff and co-workers (1950) introduced several formulae. From such formulae they calculated the plasma iron turnover per day, the red-cell iron turnover per day and the fraction of red cells renewed per day. They found that data obtained in this way agreed moderately well with the results obtained by other methods and the calculations have been used with certain modifications in later studies. The chief modification has been in the computation of the red-cell iron turnover. Initially it was calculated from the amount of radioactivity in the circulating red-cell mass when equilibrium of the radioiron in the red cell had been reached. This normally occurred 7 to 14 days after the injection of the radioiron. In subsequent studies, however, it has been calculated from the amount of radioiron in red cells at the time when *in vivo* counting shows that the fall in activity over the marrow has changed to a more gradual slope (normally about 5 days). This appears to be a valid modification as the rapid fall in marrow activity indicates that the incorporation into red cells of iron initially taken up by the marrow is almost complete at this stage. Any further increase in red-cell activity must be secondary to the incorporation in the marrow of radioiron which was initially directed to other sites.

In all such calculations the final result is obtained by relating the figures for utilization of radioiron in the formation of red cells to those obtained from the plasma iron turnover per day. There is some doubt, however, as to whether this is completely valid. There are considerable diurnal variations in the level of plasma iron (Paterson, Marrack and Wiggins, 1952). Bothwell and Mallett (1955b) have shown that in haematologically normal subjects the diurnal variations in plasma iron level do not significantly affect the rate of turnover through the plasma. There appears to be a regulatory mechanism which tends to keep the absolute turnover rate constant. However, in conditions where there is a rapid clearance of iron from the plasma, e.g., iron deficiency and haemolytic anaemias, there is a strong positive correlation between the plasma iron level and the rate of turnover through the plasma. This suggests that the normal regulatory mechanism is not apparent in conditions where there is an increased demand for iron and the absolute rate of turnover of iron through the plasma in such states may partly be determined by the availability of iron. The data on diurnal variations in plasma iron turnover were obtained during waking hours and it is possible that further significant changes may occur during sleep. It therefore seems unjustified to assume that the mean turnover rate will necessarily be the same as that obtained at any one time during the day. Because of this, plasma iron turnovers in the present study were expressed in terms of hourly rather than 24-hour periods.

Huff and co-workers (1950) thought that the exponential clearance of radioiron from the plasma implied that there was no significant feedback of radioiron from other sources during the observational period. From such data their values for plasma iron turnover in patients with polycythaemia vera were extremely high and on this and other evidence they postulated the existence in polycythaemia vera of two populations of cells, one being very short lived. However, Sharney and his colleagues (1954) have shown, by improved counting techniques, that the loss of radioiron from the circulation cannot be expressed by a single exponential function and that this is especially true in polycythaemia vera. Although the initial loss can be represented by a straight line on a semilogarithmic plot, deviation from the straight line is eventually apparent and the rate of loss becomes appreciably slower. In general such curves can be adequately represented by the sum of two exponential functions. It is not yet known in how many conditions the smaller rate constant is of sufficient magnitude to influence the results significantly. Certainly in normals it has little effect. In polycythaemia vera, however, figures for plasma iron turnover are considerably reduced and are compatible with a normal red-cell life span in the disease (Sharney *et al.*, 1954).

The biological significance of the observations of Sharney and co-workers (1954) is difficult to interpret. They considered that their findings were compatible with a system of two or more intercommunicating pools with significant interchange between them. It was felt that both pools might be contained within the plasma but differ in the chemical state or reactivity of the iron. However, they thought it more likely that the plasma iron constituted the first pool and that the other was located elsewhere. Although such a concept seems mathematically valid no attempt has yet been made to relate it to what is already known about iron transport in the body. It has been shown that the body iron stores are not uniformly mixed and that the most recently deposited iron is more readily available for current metabolic needs (Dubach, Moore and Minnich, 1946). If a proportion of the radioiron is removed initially from the plasma into such stores, feedback should be most noticeable in conditions such as polycythaemia vera where there is an increased demand by the marrow for iron. The amount of radioiron being fed back to the plasma at any one time might depend on the size of the easily mobilizable stores. An explanation of this type would fit in well with the

concept of a 'labile iron pool' (Greenberg and Wintrobe, 1946). However, there is as yet no compelling reason to suppose that there is any sharp natural distinction between 'labile' and 'fixed' iron in stores.

DISCUSSION OF RESULTS

In spite of the qualifications of the previous section there is no doubt that turnover studies have already yielded useful information about mechanisms of erythropoiesis. The data from the present investigation are inadequate for the more complicated analysis suggested by Sharney and co-workers (1954) but this does not influence the general conclusions. It is apparent from our own findings and those of others (Huff *et al.*, 1950; Wasserman *et al.*, 1952; Elmlinger *et al.*, 1953) that certain types of characteristic pattern are often recognizable in different blood disorders. Before discussing these in any detail it seems worthwhile to describe briefly these various patterns. It should, however, be emphasized that such a description must involve oversimplifications.

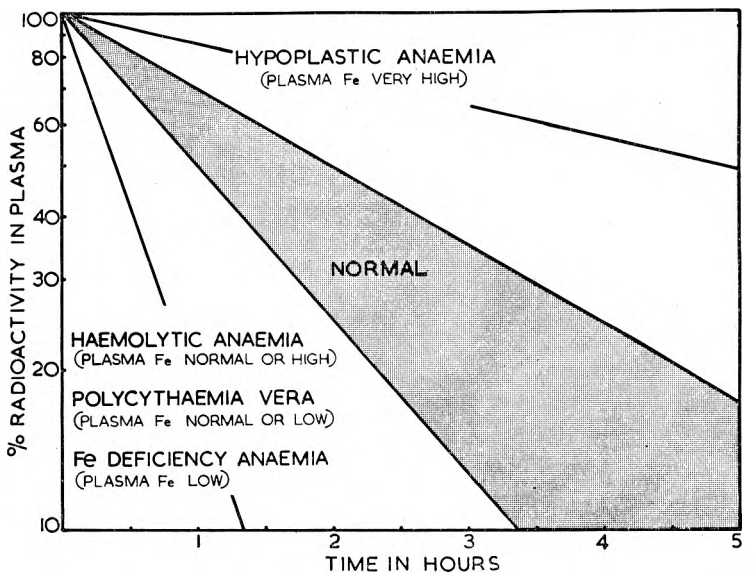


FIG. 1. The usual patterns of radioiron clearance from the plasma in normal subjects and in patients with various blood disorders.

A rapid clearance of radioiron from the plasma is seen in iron-deficiency anaemias, haemolytic anaemias and polycythaemia vera, while the converse is true in hypoplastic marrow states (Fig. 1). The utilization of injected radioiron for red-cell production is complete or almost complete in iron-deficiency anaemias, and in polycythaemia vera. In haemolytic anaemias it usually appears reduced as haemolysis leads to a concurrent loss of radioiron from labelled red cells, the major portion of which is reutilized by the bone marrow. In addition, the rapidity with which radioiron initially appears in red cells varies in different types of haemolytic anaemia. In 'compensated' cases it appears at a normal rate while in 'uncompensated' cases, where there is an accelerated maturation time, it appears more

rapidly (Finch and Coleman, 1953). Finally, in hypoplastic anaemias red-cell utilization is characteristically much decreased. These various patterns of red-cell utilization are shown in Fig. 2.

There are no published findings on iron turnover in *iron-deficiency anaemia*. In the patients included in the present study the plasma iron concentration was low, the fraction of iron removed per hour was large and red-cell utilization was complete or almost complete. A

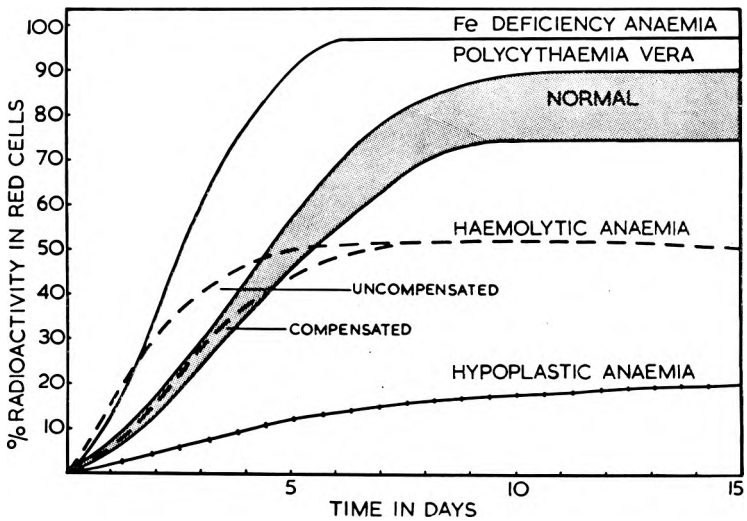


FIG. 2. The usual patterns of red-cell utilization in normal subjects and in patients with various blood disorders.

surprising finding was a normal or increased plasma iron turnover, suggesting a normal or increased amount of iron available to the bone marrow for erythropoiesis. Taken at their face value, these figures would be incompatible with a state of iron deficiency. It seems likely that the loss of radioiron from the plasma must be similar to that found in polycythaemia vera by Sharney and co-workers (1954). Unfortunately, sampling was not carried out for long enough to verify whether deviation from the initial straight line disappearance was eventually apparent.

It has been found previously (Huff *et al.*, 1950) that in patients with a *hypoplastic bone marrow* the plasma iron concentration is high and the fraction of iron removed per hour is very small, with the result that the plasma iron turnover is usually close to normal. In addition red-cell utilization is grossly inadequate. Since the absolute rate of turnover through the plasma may be normal it cannot reflect the rate of blood formation but rather the shift of iron derived from transfused cells to stores and the redistribution of iron from one store to another. There are, however, factors which might modify the pattern in hypoplastic anaemia, namely, the degree of depression of erythropoiesis and the increase in iron stores resulting from repeated transfusion. In some of our patients both the plasma iron concentration and fraction removed per hour were normal. These subjects may have had less severe degrees of hypoplasia, since red-cell utilization tended to be higher in them than in the others, but on the whole they were also those who were seen early in the disease process and hence had not received many transfusions.

Transfusion raises both the plasma iron concentration and the level of iron stores. From repeated studies in normal subjects it has been shown that the fraction of iron removed per hour tends to be less when the iron concentration is higher (Bothwell and Mallett, 1955b). The finding of a small fraction removed per hour is therefore not in itself necessarily an index of depressed erythropoiesis. The amount of iron in stores may also influence the utilization of radioiron for red-cell production (Finch *et al.*, 1949). Even when red-cell production is adequate but iron stores are increased, the mixture of injected radioiron with these stores causes less of the labelled iron to appear subsequently in red cells. It is true that these earlier studies were done with doses of iron larger than the tracer quantities currently used. It is, however, still possible that the percentage red-cell utilization of a tracer dose of radioiron may be depressed in subjects in whom the available iron pool is increased.

In *haemolytic anaemias* there is an increased breakdown of red cells and an increased demand for iron and these are reflected in the amount of iron passing through the plasma. The concentration of iron in the plasma is raised or normal and the fraction removed per hour is uniformly large. Interpretation of the patterns of red-cell utilization in haemolytic anaemias is, however, difficult in view of the random destruction of newly formed red cells and because of the possible effects of increased body stores on the utilization of radioiron (Finch *et al.*, 1949).

In contrast to our own cases of haemolytic anaemia, where the utilization was relatively good, rare cases have been described (Huff *et al.*, 1951) in which little radioiron appears in the peripheral blood although *in vivo* counting has shown a normal initial uptake in the bone marrow with a subsequent accumulation in the spleen. These findings have been interpreted as being due to an abnormality or failure in the formation of haemoglobin followed by rapid destruction of abnormal cells. It is obvious that the percentage of radioiron appearing in the red cells of these patients can give no reflection of the rate of blood formation. Finch and Coleman (1953) have suggested that the mean marrow time (i.e., the time elapsing between the injection of radioiron and the appearance of the maximum daily increment of radioactivity in circulating red cells) might be a good index of haemoglobin synthesis and red-cell delivery. In cases of compensated haemolytic anaemia the marrow mass is increased in size but the mean marrow time is normal (3 to 4 days), while in uncompensated cases there is both an increased marrow mass and a shortened mean marrow time.

Huff and co-workers (1950) have shown an increased iron turnover in *chronic leukaemia*, the findings being more marked in the myeloid form of the disease. The results were thought to be due to a combination of increased red-cell production and excessive haemolysis. Furthermore, from *in vivo* counting Elmlinger and colleagues (1953) found evidence of myeloid metaplasia in all their cases of chronic myeloid leukaemia. In contrast, both the patients in our series showed a plasma iron turnover and red-cell utilization within the normal range, as did one patient with chronic lymphatic leukaemia. The other patient with chronic lymphatic leukaemia required frequent transfusions and the study revealed a pattern indicative of erythroid hypoplasia. There are few data on acute leukaemia but what have been published (Elmlinger *et al.*, 1953) suggest that there is a decreased turnover of iron through the plasma and inadequate red-cell utilization, while *in vivo* counting has shown a relative increase in the transfer of iron from plasma to liver.

In *myelofibrosis* it is theoretically possible to get different patterns depending on the degree to which extramedullary haematopoietic foci compensate for the loss of marrow function. Where such compensation is inadequate the patterns of aplastic anaemia might be anticipated, while in cases in which an equilibrium is maintained figures within the normal range should

occur. In most cases, however, the enlarged spleen is both forming red cells and destroying them at an excessive rate. The patients investigated in this series showed an increased plasma iron turnover and a reduction in red-cell utilization, findings which suggest that excessive haemolysis was occurring. Such studies, especially if they were combined with *in vivo* counting, should help in selecting the occasional case which might be benefited by splenic irradiation or splenectomy.

Two patients with *leuco-erythroblastic anaemia* not due to myelofibrosis were also investigated. They showed an increased plasma iron turnover with depressed red-cell utilization and had other evidence compatible with increased haemolysis, namely, splenomegaly, reticulocytosis and a raised serum bilirubin.

In *polycythaemia vera* the concentration of iron in the plasma may be normal or reduced while the fraction of iron removed per hour is always large. Consequently the plasma iron turnover is usually considerably increased and the major portion of this iron is utilized for red-cell production. All the present group of cases were complicated by some degree of iron deficiency. However, it is unlikely that the large fractions of iron removed per hour were due only to this as similar findings have been reported in patients who were not iron deficient (Huff *et al.*, 1950). The results of the present study have confirmed that the method is of use in differentiating polycythaemia vera from cases in which the haemoglobin, red-cell count and haematocrit are raised but in which there is no increase in the red-cell mass. Such cases of *relative polycythaemia* have been recognized by Lawrence and Berlin (1952) and the possible significance of the condition has been discussed by Dameshek (1953). Peptic ulcer, hypertension and coronary artery disease are often present and it is noteworthy that three of the patients in the present series had peptic ulcers, five showed hypertension and three had evidence of arterial disease. It has been postulated that long continued stress may be an important aetiological factor.

Interpretation of the results obtained in the five cases with *infection* is difficult as all were on therapy at the time of study. A profound lowering in the red-cell utilization of injected radioiron has been shown previously by Wintrobe and his co-workers (1947) and by Finch and colleagues (1949). However, such results were obtained with larger doses of iron than those used in recent tracer studies and it is probable that the bulk of the injected radioiron was not initially bound to the β_1 globulin of the plasma and was taken up by the reticulo-endothelial system. Using the tracer technique Bush and Cartwright (1954) have recently demonstrated that the plasma iron turnover was high and the red-cell utilization normal in five cases of chronic infection. They felt that these data suggested that the red-cell survival time was probably somewhat shorter than normal in chronic infections and that red-cell production, although slightly increased, was not increased sufficiently to prevent the development of a mild degree of anaemia.

The findings in the *neoplastic disorders* depend on whether the anaemia is a result of bone-marrow depression, blood loss, replacement of marrow by tumour cells or increased haemolysis. In the present study one patient with an inoperable carcinoma of the lung showed a slight depression of red-cell utilization but the test was otherwise normal, while the results in another with widespread carcinomatosis suggested that iron deficiency was at least partially responsible for the anaemia.

The findings in one case of untreated *pernicious anaemia* were similar to those reported by Huff and co-workers (1950). The condition is characterized by an increased plasma iron turnover and diminished red-cell utilization and it has been suggested that these findings are compatible with the rapid destruction of abnormal red cells.

SUMMARY

Tracer studies with radioactive iron have been carried out in sixty-four subjects. The majority were patients suffering from various types of anaemia.

The quantity of iron passing through the plasma per hour has been calculated in each subject and the percentage red-cell utilization has been estimated in most of them. Difficulties in the technique and in the interpretation of results are discussed in the light of the present work and that of others.

The turnover of iron through the plasma is more complex than was initially thought; diurnal variations in the rate of turnover of plasma iron have been shown to occur, and the disappearance of radioiron from the plasma cannot always be approximated by a single exponential function. These two phenomena make it difficult to estimate quantitatively the effective daily turnover of plasma iron.

The daily red-cell iron turnover is derived from the plasma iron turnover. Its computation is further complicated by the difficulty in determining the point at which the incorporation into red cells of radioiron initially taken up by the marrow is complete.

Although the radioiron tracer technique does not yet give a reliable quantitative measure of red-cell production it has considerable qualitative value and certain definite alterations in the pattern of erythropoiesis can be recognized. The method has been found especially valuable in differentiating blood diseases associated with erythroid hyperplasia from those in which there is erythroid hypoplasia. In addition it has proved useful in separating polycythaemia vera from 'relative' polycythaemia.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the helpful criticisms given by our former colleague, Mr. E. O. Powell.

This work was supported in part by a grant to Professor L. J. Witts from the Medical Research Council.

REFERENCES

BADENOCH, J. and CALLENDER, S. T. (1954). 'Iron metabolism in steatorrhea. Use of radioactive iron in studies of absorption and utilization.' *Blood*, **9**, 123.

BOTHWELL, T. H. and MALLETT, B. (1955a). 'Determination of iron in plasma or serum.' *Biochem. J.*, **59**, 599.

BOTHWELL, T. H. and MALLETT, B. (1955b). 'Diurnal variation in the turnover of iron through the plasma.' *Clin. Sci.*, **14**, 235.

BUSH, J. A. and CARTWRIGHT, G. E. (1954). 'Ferrokinetics in anemia of infection.' *J. clin. Invest.*, **33**, 921.

CHAPLIN, H., Jr. and MOLLISON, P. L. (1952). 'Correction for plasma trapped in the red cell column of the hematocrit.' *Blood*, **7**, 1227.

CHAPLIN, H., Jr., MOLLISON, P. L. and VETTER, H. (1953). 'The body/venous hematocrit ratio: its constancy over a wide hematocrit range.' *J. clin. Invest.*, **32**. 1309.

DAMEŞHEK, W. (1953). 'Stress erythrocytosis.' *Blood*, **8**, 282.

DUBACH, R., CALLENDER, S. T. E. 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 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.

ELMLINGER, P. J., HUFF, R. L., TOBIAS, C. A. and LAWRENCE, J. H. (1953). 'Iron turnover abnormalities in patients having anemia: Serial blood and *in vivo* tissue studies with Fe^{59}.' *Acta Haemat.*, **9**, 73.

FINCH, C. A. and COLEMAN, D. H. (1953). 'Patterns of erythropoiesis in hemolytic anemia.' *J. clin. Invest.*, **32**, 567.

FINCH, C. A., GIBSON, J. G., II, PEACOCK, W. C. and FLUHARTY, R. G. (1949). 'Iron metabolism. Utilization of intravenous radioactive iron.' *Blood*, **4**, 905.

GREENBERG, G. R. and WINTROBE, M. M. (1946). 'A labile iron pool.' *J. biol. Chem.*, **165**, 397.

HAHN, P. F., BALE, W. F., HETTIG, R. A., KAMEN, M. D. and WHIPPLE, G. H. (1939). 'Radioactive iron and its excretion in urine, bile and feces.' *J. exp. Med.*, **70**, 443.

REFERENCES *(continued)*

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.

LAWRENCE, J. H. and BERLIN, N. I. (1952). 'Relative polycythemia – the polycythemia of stress.' *Yale J. Biol. Med.*, **24**, 498

MCCANCE, R. A. and WIDDOWSON, E. M. (1938). 'The absorption and excretion of iron following oral and intravenous administration.' *J. Physiol.*, **94**, 148

PATERSON, J. C. S., MARRACK, D. and WIGGINS, H. S. (1952). 'Hypoferraemia in the human subject: the importance of diurnal hypoferraemia.' *Clin. Sci.*, **11**, 417.

POWELL. J. F. (1944). 'Serum-iron in health and disease.' *Quart. J. Med.* **13**, n.s., 19.

SCHADE, A. L. and CAROLINE, L. (1946). 'An iron-binding component in human blood plasma.' *Science*, **104**, 340.

SHARNEY, L., SCHWARTZ, L., WASSERMAN, L. R., PORT, S. and LEAVITT, D. (1954). 'Pool systems in iron metabolism; with special reference to polycythemia vera.' *Proc. Soc. exp. Biol. N.Y.*, **87**, 489.

WASSERMAN, L. R. RASHKOFF, I. A., LEAVITT, D., MAYER, J. and PORT, S. (1952). 'The rate of removal of radioactive iron from the plasma – an index of erythropoiesis.' J. *clin. Invest.*, **31**, 32.

WEST, H. D., HAHN, P. F., CLARK, W. F. and CHAPPELLE, E. W. (1952). 'Labeled iron stores in study of iron liberated during red cell destruction by acetylphenylhydrazine.' *Amer. J. Physiol.*, **169**, 194.

WINTROBE, M. M., GREENBERG, G. R., HUMPHREYS, S. R., ASHENBRUCKER, H., WORTH, W. and KRAMER, R. (1947). 'The anemia of infection. III. The uptake of radioactive iron in iron-deficient and in pyridoxine-deficient pigs before and after acute inflammation.' *J. clin. Invest.*, **26**, 103.

Reprinted from Blood, The Journal of Hematology
Vol. XII, No. 5, May, 1957
Printed in U.S.A.

Erythrokinetics. IV. The Plasma Iron Turnover as a Measure of Erythropoiesis

By Thomas H. Bothwell, Arnold V. Hurtado, Dennis M. Donohue and Clement A. Finch

IRON EXCHANGE between body tissues is accomplished by a transport mechanism in which iron is attached to a plasma protein.[1] Iron entering the plasma is derived from the breakdown of heme pigments, from body stores and from gastrointestinal absorption. The major portion leaving the plasma is normally directed towards the bone marrow where it is used for hemoglobin synthesis. With the tracer technics introduced by Huff and co-workers[2] it has been possible to obtain more quantitative data on the amount of iron passing through the plasma. In such a study, the plasma iron binding protein is tagged with a tracer amount of radioiron and the rate of disappearance of radioactivity is then followed for several hours. With a knowledge of the rate of clearance and of the level of plasma iron, it is possible to obtain a quantitative measure of the amount of iron being turned over through the plasma.[2]

Data relating to this technic have been reported by a number of investigators.[2-13] While it has generally been assumed that changes in plasma iron turnover reflect changes in erythropoiesis, the possible effects of red cell destruction and altered body iron stores have not been defined. The present study was therefore undertaken in order to elucidate the role of these three factors in regulating plasma iron turnover. Experiments were done in animals and man and the findings were applied to the interpretation of plasma iron turnover data obtained in patients with a variety of blood disorders. The results of these various studies form the substance of this paper.

Material and Methods

The plasma iron turnover was determined in 14 normal male subjects, in 12 nonanemic patients of both sexes and in 59 patients with various hematologic disorders. Studies were also carried out in both animals and human subjects in whom the rate of red cell destruction or production had been experimentally altered. Increased red cell destruction was produced by injecting intravenously red cells which had been rendered nonviable by storage at 4 C. in 4 per cent citrate for 21 days. Decreased red cell production was produced by whole body

From the University of Washington School of Medicine, Department of Medicine, Seattle, Washington.

This investigation was supported by research grants from the United States Atomic Energy Commission (Contract At (45-1)-218).

Submitted July 30, 1956; accepted for publication Nov. 4, 1956.

The authors wish to thank Mary Lu Hanson, Loretta Conroy and Betty Loden for their technical assistance.

irradiation in a group of rabbits, by red cell transfusion in 1 dog and by nitrogen mustard therapy in 3 patients with lymphomas. Increased red cell production was induced by phlebotomy.

The dose of Fe^{59} given to each subject varied between 1 and 15 μc (specific activity 1–2 μc/1 μg.). It is assumed that the small quantity of elemental radioiron added to the plasma behaves as a true tracer in that it does not significantly affect the amount or concentration of iron already present in a steady state. Larger doses of iron have given results in the normal subject comparable to those found in the present study.[2, 3]

The radioiron as ferric chloride was incubated with fresh plasma prior to administration in most instances and was injected as ferrous citrate in the remainder. In eight additional subjects, it was given bound to Cohn's fraction IV_7.

Plasma iron turnovers were carried out in the following manner. Samples of plasma were taken before and at 10, 30, 60, 120, 180, and 300 minutes after the injection of the radioiron. Shorter sampling periods were employed in animals and in patients where the clearance rate was expected to be rapid. Radioactivity was determined in 2–4 ml. samples of plasma which were counted in a well-type scintillation counter. In studies in which both Fe^{59} and Fe^{55} were used, samples were wet-ashed with sulphuric and perchloric acid. They were then precipitated with addition of carrier iron to a total of 10 mg. and electroplated by the method of Peacock et al.[14] Counting was done as previously described.[15] The 10 minute sample was drawn with iron-free equipment and was analyzed for plasma iron by the method of Kitzes et al.[16] or Bothwell and Mallett.[17] Comparable results have been obtained in this laboratory with these two methods. Iron binding capacities were done according to the method of Rath and Finch.[18]

It has been customary to express plasma iron turnover in terms of total mg./day. This does not permit comparison between individuals, since it does not allow for variation in size of individuals and therefore in their erythropoietic iron requirements. It further requires an estimate of plasma volume. If the expression employed is related to body weight, data between individuals can be compared. However, blood volume must still be estimated and an additional error is introduced since red cell mass and therefore iron requirements for erythropoiesis do not correlate perfectly with body weight.[19] A simpler standard of reference, permitting comparison between individuals, relates plasma iron turnover to 100 ml. whole blood. It might be expected that a constant amount of iron would normally be required to provide the iron for the red cells in 100 ml. blood. Furthermore, whole blood volume does not change significantly in anemia, i.e., a decrease of perhaps 10–15 per cent if acute hemorrhage, burns, salt depletion are excluded.[20] Thus, plasma iron turnover may be calculated from the modified formula of Huff and associates[2] as follows:

$$\frac{\text{Plasma iron turnover*}}{\text{(mg./day/100 ml. whole blood)}} = \frac{\text{Plasma Iron } (\mu\text{g./100 ml.})}{\text{T } \tfrac{1}{2} \text{ minutes}} \times \frac{(100 - \text{hematocrit})}{100}$$

* The simplified formula for plasma iron turnover was derived as follows:

$$\frac{\text{Plasma Iron Turnover}}{\text{(mg./day/100 ml. plasma)}} = \frac{\text{Plasma Iron (mg./100 ml.)} \times 0.693 \times 1440}{\text{T } \tfrac{1}{2} \text{ (minutes)}}$$

where 0.693 = natural logarithm of 2, 1440 = minutes/day and T $\frac{1}{2}$ = time for clearance of 50 per cent of radioactivity from plasma. Since 0.693 × 1440 = approximately 1000, the expression may be further simplified to read:

$$\frac{\text{Plasma Iron Turnover}}{\text{(mg./day/100 ml. plasma)}} = \frac{\text{Plasma Iron } (\mu\text{g./100 ml.})}{\text{T } \tfrac{1}{2} \text{ (minutes)}}$$

To convert this expression to turnover per 100 ml. whole blood, the following formula was used:

$$\frac{\text{Plasma Iron Turnover}}{\text{(mg./day/100 ml. whole blood)}} = \frac{\text{Plasma Iron } (\mu\text{g./100 ml.})}{\text{T } \tfrac{1}{2} \text{ (minutes)}} \times \frac{100 - \text{hematocrit}}{100}$$

TABLE 3.—*Hematologic and Radioiron Data in Normal Subjects and in Patients with Anemia*

NORMAL SUBJECTS

No.	Diagnosis	Hematocrit	% Reticulocytes	Absolute Reticulocytes/cu. mm.	Plasma Iron µg. 100 ml.)	T 1/2 (minutes)	Plasma Iron Turnover/ 100 ml. whole blood	Production Index	
								Iron	Reticulocytes
1	Normal	47	0.7	36,000	95	84	0.60	98	60
2	Normal	44	1.0	50,000	119	98	0.68	111	83
3	Normal	41	1.1	52,000	85	65	0.77	126	86
4	Normal	46	—	—	99	74	0.72	118	—
5	Normal	42	1.6	76,000	118	156	0.44	72	126
6	Normal	44	1.2	61,000	115	120	0.53	86	101
7	Normal	47	1.2	65,000	100	72	0.74	121	108
8	Normal	47	1.3	68,000	66	92	0.38	62	113
9	Normal	51	1.3	73,600	114	93	0.60	98	122
10	Normal	48.5	1.4	75,000	162	118	0.71	116	125
11	Normal	43	1.7	82,000	37	45	0.47	77	136
12	Normal	44	0.1	4,600	56	42	0.75	122	7
13	Normal	45.5	1.9	96,000	114	134	0.46	75	160
14	Normal	41	0.8	38,000	115	105	0.65	106	63
MEAN		45	1.2	59,800	99.6	93	0.61	100	100

NON-HEMATOLOGIC DISEASES

15	Chronic alcoholism	50	2.5	139,000	28	35	0.40	65	231
16	Arteriosclerotic heart disease	40	0.6	37,000	28	45	0.37	60	61
17	Hypertensive heart disease	42	0.5	23,000	45	58	0.45	73	38
18	Arteriosclerotic heart disease	48.5	1.7	92,000	81	68	0.61	100	153
19	Arteriosclerotic heart disease	44	1.0	49,000	55	85	0.35	57	81
20	Cerebral vascular accident	50	1.0	50,000	63	50	0.63	103	83
21	Rheumatic heart disease	40	1.5	67,000	43	46	0.56	91	111
22	Rheumatic heart disease	46	0.4	20,000	34	36	0.51	83	33
23	Diabetes	46	0.8	41,000	66	77	0.46	75	68
24	Convalescent osteomyelitis	41	1.1	49,500	24	30	0.47	77	82
25	Convalescent fracture	41	2.4	109,000	26	22	0.70	115	181
26	Pulmonary emphysema	48.5	0.7	38,000	92	91	0.52	85	63
MEAN		44.8	1.2	59,500	49	54	0.50	82	99

MARROW HYPERFUNCTION

27	Hereditary Spherocytosis	38	9.7	409,000	183	29	3.91	640	682
28	Hereditary Spherocytosis	36	15.2	608,000	163	26	4.01	657	1,013

TABLE 3.—*Continued*

MARROW HYPERFUNCTION—Continued

No.	Diagnosis	Hematocrit	% Reticulocytes	Absolute Reticulocytes/cu. mm.	Plasma Iron (μg./100 ml.)	T1/2 (minutes)	Plasma Iron Turnover/100 ml. whole blood	Production Index	
								Iron	Reticulocytes
29	Hereditary Spherocytosis	35.6	11.5	430,000	82	17	3.10	508	720
30	Congenital nonspherocytic anemia	40	8.4	373,000	108	33	1.96	321	621
31	Congenital nonspherocytic anemia	41	24.9	1,120,500	46	14	1.94	318	1,867
32	Congenital nonspherocytic anemia	38	15.0	450,000	92	30	1.90	360	750
33	Congenital nonspherocytic anemia	35	15.0	570,000	108	42	1.67	273	950
34	Sickle Cell Anemia	23	10.0	223,000	151	35	3.32	544	373
35	Sickle Cell Anemia	27.6	9.7	262,000	61	25	1.76	288	438
36	Sickle Cell Anemia	30	7.0	296,000	162	52	2.18	357	493
37	Acquired Hemolytic Anemia	32	25.8	915,000	76	28	1.85	305	1,526
38	Acquired Hemolytic Anemia	30	15.0	330,000	76	17	3.13	511	550
39	Acquired Hemolytic Anemia	25	—	—	212	70	2.27	370	—
MEAN		33.2	13.9	499,000	117	32	2.54	419	832
	MARROW DYSPOIESIS								
40	Cooley's Anemia	18	2.4	48,000	167	30	4.60	754	80
41	Cooley's Anemia	20	3.6	80,000	183	29	5.00	819	133
42	Pernicious Anemia	16	0.4	5,320	130	58	1.89	309	8
43	Pernicious Anemia	10	0.3	2,670	169	68	2.23	365	4
44	Pernicious Anemia	14	0.4	4,400	190	54	3.04	498	7
45	Pernicious Anemia	20	2.0	32,000	234	53	3.54	580	53
46	Pernicious Anemia	14	1.1	12,000	176	89	1.70	278	20
47	Pernicious Anemia	18	0.8	20,000	169	621	0.22	36	34
48	Pernicious Anemia	20	2.3	37,000	130	82	1.27	208	61
49	Pernicious Anemia	30	0.8	100,000	67	35	1.32	216	166
50	Pernicious Anemia	24	1.0	25,000	192	70	2.08	340	41
51	Pernicious Anemia	18	0.1	13,800	197	79	2.03	332	23
52	Pernicious Anemia	22	2.0	42,400	63	66	0.74	121	70
53	Pernicious Anemia	19	2.5	42,750	202	41	4.00	655	71
MEAN		18.8	1.4	33,300	162	98	2.40	394	55
	MARROW HYPOFUNCTION								
54	Aplastic Anemia	27	0.1	30,000	115	202	0.42	68	50
55	Aplastic Anemia	28	0.8	24,000	140	209	0.49	80	40
56	Aplastic Anemia	20	0.1	2,000	56	209	0.21	34	3
57	Refractory Anemia	17	1.7	36,000	280	285	0.81	132	60
58	Refractory Anemia	17	0.0	0	182	671	0.22	36	0
59	Aplastic Anemia	27	0.4	14,000	216	388	0.41	67	23
60	Aplastic Anemia	32	1.3	46,000	49	59	0.56	93	77
MEAN		24	0.6	21,700	148	289	0.45	73	36

TABLE 3.—*Concluded*

No.	Diagnosis	Hematocrit	% Reticulocytes	Absolute Reticulocytes/cu. mm.	Plasma Iron (μg./100 ml.)	T 1/2 (minutes)	Plasma Iron Turnover/100 ml. whole blood	Production Index	
								Iron	Reticulocytes
	INCREASED BODY STORES								
61	Hemochromatosis	40	2.2	92,000	275	144	1.15	188	153
62	Hemochromatosis	41	1.2	53,000	224	149	0.89	145	89
63	Hemochromatosis	50	1.6	88,000	240	137	0.88	144	146
64	Hemochromatosis	46	1.4	62,000	266	123	1.17	191	103
MEAN		44	1.6	73,750	251	138	1.02	167	123
	DECREASED BODY STORES								
65	Iron Deficiency Anemia	26	1.7	87,000	13	11	0.87	142	145
66	Iron Deficiency Anemia	18	3.5	94,000	26	20	1.10	180	156
67	Iron Deficiency Anemia	26	3.6	144,000	28	22	0.92	150	240
68	Iron Deficiency Anemia	29	3.7	163,000	10	25	0.28	45	271
69	Iron Deficiency Anemia	19	2.3	73,000	33	20	1.34	219	121
70	Iron Deficiency Anemia	10	2.0	34,000	33	38	0.78	127	56
71	Iron Deficiency Anemia	39	2.2	95,000	22	22	0.33	54	158
MEAN		23.9	2.7	98,570	22	22.5	0.80	131	164
	MISCELLANEOUS ANEMIAS								
72	Juvenile Cirrhosis	30	4.2	—	78	43	1.29	211	—
73	Chronic Alcoholism	28	4.0	112,000	44	37	0.86	140	186
74	Chronic Alcoholism	27	6.8	180,000	55	22	1.82	298	301
75	Alcoholic Cirrhosis	31	1.7	—	29	57	.34	55	—
76	Myeloid Metaplasia	29	5.8	187,000	46	42	0.77	126	311
77	Myeloid Metaplasia	28	3.6	113,000	146	124	0.85	139	188
78	Myeloid Metaplasia	33	4.2	154,000	51	33	1.04	170	256
79	Myeloid Metaplasia	31	0.4	14,000	36	18	1.39	227	23
80	Subacute Myelogenous Leukemia	33	0	0	259	238	0.73	119	—
81	Subacute Myelogenous Leukemia	20.5	0.8	27,000	216	700	0.25	40	45
82	Chronic Myelogenous Leukemia	26	5.1	147,000	118	110	0.79	129	245
83	Chronic Myelogenous Leukemia	27	4.2	120,000	51	36	1.03	168	200
84	Chronic Lymphatic Leukemia	20.5	1.9	43,000	32	24	1.06	173	71
85	Chronic Lymphatic Leukemia	27.0	1.7	51,000	56	260	0.15	26	85

index of 100 per cent and all other values were related to this figure. The reticulocyte count per cu. mm., similarly expressed, was used as another standard of red cell production. Results obtained with these two erythropoietic indices are shown in figure 5.

A. NORMAL SUBJECTS. Figures for plasma iron turnover were remarkably consistent in the 14 normal subjects studied, the majority falling within 25 per cent of the mean value for this group. Reticulocyte counts showed a wider scatter.

B. NONHEMATOLOGIC DISEASES. In a group of 12 patients who were suffering from a variety of diseases but were considered hematologically normal, the mean value for plasma iron turnover and reticulocyte counts were close to normal (82 per cent and 99 per cent respectively). When the normals and this second group were analysed together, it was apparent that there was an interrelationship between the level of plasma iron and the rate of clearance of radioiron from the plasma. It can be seen in figure 6 that the lower the plasma iron level was, the faster was the clearance rate. The plasma iron turnovers in the whole series were therefore maintained within a moderately narrow range in spite of wide fluctuations in the level of plasma iron.

C. MARROW HYPERFUNCTION. The 13 patients in this group were all suffering from congenital or acquired hemolytic anemia of varying duration and severity. Red cell production, as judged by plasma iron turnover, ranged between 3 and 6 times normal. The reticulocyte index gave results which were usually disproportionately high, with values ranging from 4 to 18 times normal.

D. MARROW DYSPOIESIS. This group included two patients with severe Cooley's anemia and 12 with pernicious anemia in relapse. In all except 2, the plasma iron turnover was considerably increased, with values ranging between 2 and 8 times normal. There was, however, no comparable increase in the reticulocyte count

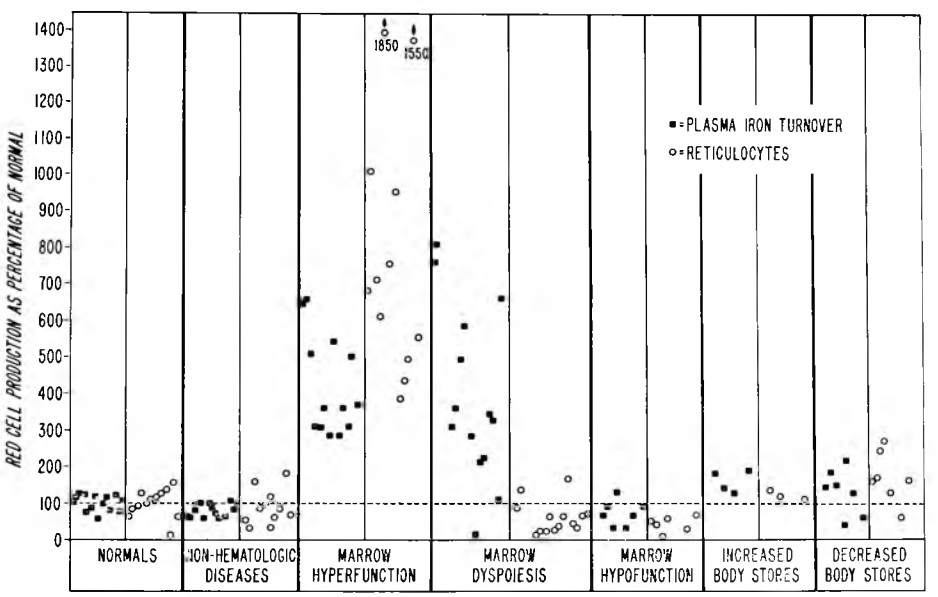


FIG. 5.—Figures for plasma iron turnover and reticulocyte counts in normal individuals and in patients with various diseases.

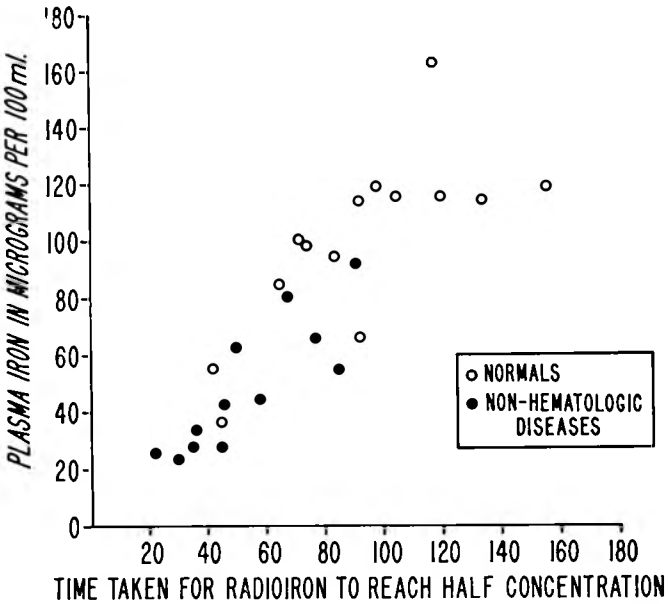


FIG. 6.—The inter-relationship between the plasma iron level and the rate of clearance of radioiron from the plasma in normal subjects and in patients with non-hematologic diseases.

which was actually less than normal in most cases. In one subject with pernicious anemia (patient 52) both indices were within normal limits while in the last one (patient 47) both were considerably reduced.

E. MARROW HYPOFUNCTION. All 7 cases in this group were patients suffering from anemia refractory to hematinics and were being maintained on repeated blood transfusions. All except two (patients 57 and 58) had hypocellular marrows. The plasma iron turnover and reticulocyte count were normal or reduced in all cases.

F. DECREASED AND INCREASED IRON STORES. Seven cases with iron deficiency were studied. The majority of these were secondary to blood loss. Although the plasma iron concentration was reduced in all cases, the plasma iron turnover was only decreased in two (patients 68 and 71) and was actually greater than normal in another 4. Likewise, there was a scatter in reticulocyte indices, with values ranging from 56 to 271 per cent.

As described in an earlier section, there was a moderate increase in plasma iron turnover (approximately 1.5 times normal) in 4 cases of untreated hemochromatosis who were studied. Reticulocyte counts were within the normal range.

G. MISCELLANEOUS ANEMIAS. The results in 14 patients with a variety of anemias have also been included. As might be expected, there was a wide scatter in both the plasma iron turnover and reticulocyte count data depending on the nature of the anemia. In only 3 patients was there evidence of decreased marrow function as judged by plasma iron turnover. One (patient 75) was a patient with alcoholic cirrhosis, another (patient 81) had subacute myelogenous leukemia and the last one (patient 85) was suffering from chronic lymphatic leukemia. The remainder showed plasma iron turnover and reticulocyte results which were normal or moderately increased.

Discussion

In previous kinetic studies employing radioiron, attempts have been made to calculate the rate of red cell formation. To do this, it would seem necessary to know the plasma iron turnover and the percentage of radioiron reappearing in red cells.[2] There are, however, two major difficulties in applying such calculations. First, it is impossible to know accurately what point on the red cell utilization curve represents that portion of radioiron which proceeds directly to the marrow as compared to the iron originally taken up by other tissues but later recircuited to the marrow. Second, in hemolytic anemias the random destruction of newly formed erythrocytes further complicates the interpretation of such red cell uptake curves. The present study was therefore undertaken to find out whether the plasma iron turnover itself might not be used as a simple index of marrow activity.

For plasma iron turnover measurements to have quantitative meaning, it was first necessary to ascertain the validity of the technics which were used. These included measurements of the plasma iron level and of the rate of clearance of radioiron from the plasma. Reproducible and comparable plasma iron values have been obtained by the method of Kitzes et al.,[16] Barkan and Walker[23] and Bothwell and Mallett[17] and it has previously been shown[17] that these methods do quantitatively measure the transport iron of the plasma. The present data indicate that physiologic binding is achieved either by incubating radioiron with fresh plasma prior to administration or simply by injecting iron citrate. While variable immediate losses of radioiron from plasma may occur in those patients with near saturated iron binding protein, this does not appear to affect the measurement of the clearance rate. In several studies Cohn's fraction IV_7 has been used for binding radioiron. The present results demonstrated that the two batches of this material tested gave significantly altered turnover rates. Since there is no in vitro test by which the suitability of a particular batch can be determined, it would seem advisable not to use fraction IV_7 for quantitative measurements of plasma iron turnover.

Another problem involves the type of data collected and its analysis. Whereas in the present study the clearance rate was determined over a few hours and expressed as a single exponential, it is known that if prolonged sampling is carried on the clearance rate becomes progressively slower and no longer can be expressed by a single component.[24] Such findings imply a biologic system in which the plasma iron is in equilibrium with one or more other pools with which there is significant interchange. The location and nature of these other pools is not known. The exchange rate between intravascular and extravascular iron binding protein may be of importance in interpretation of turnover data. The likelihood that normal marrow releases back into the plasma as much as 15 per cent of the iron initially taken up by developing red cells, and that this fraction may become much greater in certain blood dyscrasias, must also be considered.[25, 26] Whatever the biologic significance of such findings, it is clear that results calculated from the initial slope of disappearance will tend to overestimate the amount of iron being turned over through the plasma. The magnitude of error introduced will depend on the size of these late components. If within the period of study 90 per cent of the activity is cleared from the plasma without significant deviation

from the initial slope then results obtained should give a good approximation of turnover. If less than 50 per cent has cleared, serious error may be introduced by subsequent alterations in slope. Since there is no assurance that any particular pool model design will apply to different disease states, it was not considered desirable to introduce corrections for these later components in the present study.

In the past, the measurement of radioiron plasma clearance rate alone without regard to serum iron level has been employed to characterize erythropoiesis.[24] While it is true that the clearance rate is usually lengthened in aplastic anemia, and accelerated with increased erythropoiesis, it is so dependent on the level of plasma iron that any measurement of either iron level or clearance rate alone has little meaning. This is well illustrated in figure 6 where the interrelationship of these two in hematologically normal subjects is shown. These findings are further supported by the previous observations[4] that the diurnal variation in plasma iron level is not associated with any significant change of turnover in normal subjects.

Before assessing the value of the plasma iron turnover as an index of erythropoiesis, it was necessary to define the extent to which other measurable factors might influence it. Potentially the two largest factors are changes in body iron stores and increased red cell breakdown. A study of patients with hemochromatosis indicates that a gross increase in iron stores may affect the plasma iron turnover but only to a moderate degree since the results were less than twice the average normal value. It was also found that the plasma iron turnover did not drop to less than ½ normal with arrest of erythropoiesis. These findings suggest that the turnover of iron in other than blood-forming tissues may amount to as much as 50 per cent of normal turnover and may double with iron surplus. Blood destruction had even less effect on plasma iron turnover. While excessive red cell breakdown increased the plasma iron concentration (fig. 1), the turnover was not significantly altered. This emphasized the limited ability of body tissues to act as receptors where there is iron loading of the plasma. On the other hand, changes in marrow activity were rapidly reflected by alterations in plasma iron turnover. On the day following marrow stimulation (fig. 4) or marrow damage (fig. 3), significant changes were observed. These were generally comparable to observations reported by Huff et al.[8] in time and per cent response although absolute values differ. There then seemed to be adequate evidence to support the contention of others[2, 27] that *erythroid marrow activity is the dominant factor in determining plasma iron turnover.*

A series of studies were carried out in normal subjects and in patients with various anemias. It would be desirable in such studies to correct for that contribution of iron not related to erythropoiesis. This would require an analysis of the multiple components of the iron clearance curve. However, it was not considered possible to accomplish these objectives with our present limitations in knowledge. Therefore, a simplified empiric approach has been used in which patient values are compared to the normal. In assessing results for plasma iron turnover, it seemed necessary to have some other measure of erythropoiesis with which results could be compared. For this purpose, the reticulocyte count per cu. mm. of blood was used as it represented an index of the amount of blood being formed at any one time. It was realized that such an index was itself not always a reliable measure of erythropoietic activity.[28, 29]

The plasma iron turnover gave moderately consistent results (±25%) in both normal subjects and in patients with diseases unrelated to the hemopoietic system. In the group of patients with congenital or acquired hemolytic anemia, the plasma iron turnover was considerably raised, being between 3 and 6 times normal. Such figures agree well with previous findings in which it has been shown that the marrow functional capacity is capable of increasing to approximately this degree.[28] The reticulocyte count seemed a less reliable index of increased erythropoietic activity in this group. The disproportionately high figures were presumably related to the early release of young reticulocytes from the marrow which required a longer time for maturation in the peripheral blood.

In the two patients with severe Cooley's anemia and in those with pernicious anemia there was a considerable increase in the plasma iron turnover. There was not, however, an associated increase in the reticulocyte count. As described elsewhere,[28, 30, 31] the increased plasma iron turnover was associated in such cases with erythroid hyperplasia and an elevated urobilinogen excretion of similar degree. These findings were interpreted as evidence of an increase in total heme turnover. The magnitude of this increase was found to be out of proportion to the rate of blood formation as judged by the reticulocyte count and red cell utilization of radioiron and to the rate of red cell destruction as measured by cell survival studies. Such findings indicated that the hyperactivity of the marrow was largely ineffective insofar as the delivery of viable erythrocytes to the circulation was concerned. *The plasma iron turnover is therefore a reflection of total erythroid marrow activity as it includes both effective and ineffective erythropoiesis*, while the reticulocyte count reflects only effective erythropoietic activity. Findings similar to those in severe Cooley's anemia and pernicious anemia have been observed in a case where effective erythropoiesis was completely blocked by the action of an iso-antibody.[32] In such conditions of marrow dyspoiesis it is possible to visualize all grades of severity, progressing to cell damage severe enough to give rise to the picture of an aregenerative anemia.

In patients with severe marrow hypofunction, all of whom were being maintained on repeated blood transfusion, the plasma iron turnover was normal or reduced. It is probable that the major part of the plasma iron turnover represented the redistribution of iron released from hemoglobin breakdown between different body stores, although some degree of ineffective erythropoiesis may also have contributed. While it is obvious that little quantitative accuracy may be placed on the degree of reduction of plasma iron turnover, these studies demonstrate that a plasma iron turnover within normal limits in any anemia other than iron deficiency can almost certainly be regarded as indicating marrow hypofunction.

The significance of the findings in cases with decreased body stores is not clear. All were patients suffering from severe iron deficiency anemia and were not on therapy at the time of study. In spite of this, only two had a reduced plasma iron turnover and in some it was increased. It is of interest that reticulocytes were at comparable levels. These results would appear inconsistent with the impaired hematopoiesis assumed to occur in iron deficiency anemia and indicate the need for more careful measurements of production and destruction in such

cases. A reasonable explanation of the results for plasma iron turnover in iron deficiency anemia would be an abnormal clearance pattern with uptake and subsequent release of iron from other tissues.[5] Present data, however, are inadequate to provide a firm explanation for these turnover values.

Finally, a group of subjects with a variety of anemias were studied in most of whom the plasma iron turnover was normal or slightly increased as was the reticulocyte count. They included patients with liver dysfunction, myeloid metaplasia and leukemias of different types. All were moderately or severely anemic. When marrow activity in these patients is compared with the maximal response as illustrated by the hereditary hemolytic anemias, it is evident that response is suboptimal. Thus, despite the fact that most of these cases had a normal or somewhat increased rate of iron turnover, they represent examples of relative marrow hypofunction.

The results of the study support the idea that the plasma iron turnover provides a simple and useful measure of erythroid marrow activity. By correlating it with the reticulocyte count it is possible to indicate whether such activity is effective or ineffective in terms of the delivery of red cells to the circulation. While not solely a measurement of erythropoiesis, it is predominantly so and has a high degree of reproducibility. Relative to other measurements of erythrokinetics it has the advantage of being rapidly responsive to changes in erythroid marrow activity. The major difficulty in assessing the validity of the plasma iron turnover as a rough measure of total erythropoietic activity lies in the fact that there is no reliable standard for comparison. It is possible that a correlation of such data with the rate of red cell destruction, as estimated with Cr^{51} labelled cells, may be helpful in obtaining a more complete picture of the balance between red cell production and destruction at any one time.[13]

Summary and Conclusions

Experimental and clinical studies have been performed to define more clearly the significance of the plasma iron turnover.

It has been shown that the plasma iron turnover is not affected by the rate of red cell destruction and to only a limited extent by increased body stores. It does, however, reflect the degree of erythroid marrow activity and is a sensitive indicator for measuring changes in such activity.

A series of 85 studies in normal subjects and in patients with various hematologic disorders were carried out to define the range of response with anemia and to assess the clinical value of the plasma iron turnover as an index of erythropoiesis. In states of marrow hyperfunction it is increased from 3 to 6 times normal and may be depressed to approximately half normal with marrow hypofunction. The plasma iron turnover is increased with marrow dyspoiesis. This increase is a measure of total erythropoiesis and does not indicate the production of viable red cells.

Summario in Interlingua

Ha essite executate studios experimental e clinic con le objectivo de definir plus clarmente le signification del transito de ferro in le plasma.

Esseva monstrate que le transito de ferro in plasma non es afficite per le intensitate del destruction de erythrocytos e solmente a grados restringite per augmentate reservas in le corpore. Sed iste transito reflecte le grado del activitate erythropoietic del medulla e representa un sensibile indice de alterationes in ille activitate.

Esseva executate un serie de 85 studios in subjectos normal e in patientes coa varie disordines hematologic pro definir le spectro del responsas in le presentin de anemia e pro establir le valor clinic de observationes del transito de ferro in plasma como indice del activitate erythropoietic. In statos de hyperfunction medullari, le valores del transito de ferro es augmentate per 2 a 5 vices le valores normal; illo pote esser deprimite a circa un medietate del nivello normal quando il se tracta de statos de hypofunction medullari. Dyspoiese medullari es accompaniate de augmentos del transito de ferro in plasma. Iste augmento es un mesura del erythropoiese total e non indica le production de erythrocytos viabile.

REFERENCES

[1] Schade, A. L., and Caroline, L.: An iron-binding component in human blood plasma. Science *104:* 340, 1946.

[2] Huff, R. L., Hennessy, T. G., Austin, R. E., Garcia, J. F., Roberts, B. M., and Lawrence, J. H.: Plasma and red cell iron turnover in normal subjects and in patients having various hematopoietic disorders. J. Clin. Invest. *29:* 1041, 1950.

[3] Bothwell, T. H., Ellis, B. C., Van Doorn-Whitkampf, H. Van W., and Abrahams, O. L.: Radioiron studies in hemochromatosis. The effects of repeated phlebotomies. J. Lab. & Clin. Med. *45:* 167, 1955.

[4] —— and Mallett, B.: Diurnal variation in the turnover of iron through the plasma. Clin. Sc. *14:* 235, 1955.

[5] ——, Callender, S., Mallett, B., and Witts, L. J.: The study of erythropoiesis using tracer quantities of radioactive iron. Brit. J. Haemat. *2:* 1, 1956.

[6] Bush, J. A., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M.: The anemia of infection. XX. The kinetics of iron metabolism in the anemia associated with chronic infection. J. Clin. Invest. *35:* 89, 1956.

[7] Elmlinger, P. J., Huff, R. L., Tobias, C. A., and Lawrence, J. A.: Iron turnover abnormalities in patients having anemia: Serial blood and *in vivo* tissue studies with Fe^{59}. Acta Haemat. *9:* 73, 1953.

[8] Huff, R. L., Lawrence, J. H., Siri, W. E., Wasserman, L. R., and Hennessy, T. G.: Effects of changes in altitude on hematopoietic activity. Medicine *31:* 197, 1951.

[9] Hyman, G. A., and Harvey, J. E.: The pathogenesis of anemia in patients with Carcinoma. Am. J. Med. *19:* 350, 1955.

[10] Lawrence, J. H., Elmlinger, P. J., and Fulton, G.: Oxygen and the control of red cell production in primary and secondary polycythemia: Effects on the plasma iron turnover patterns with Fe^{59} as tracer. Cardiol. *21:* 337, 1952.

[11] Peterson, R. E.: Plasma radioactive iron turnover in acute viral hepatitis. Proc. Soc. Exper. Biol. & Med. *84:* 47, 1953.

[12] Sharney, L., Schwartz, L., Wasserman, L. R., Port, S., and Leavitt, D.: Pool systems in iron metabolism; with special reference to polycythemia vera. Proc. Soc. Exper. Biol. & Med. *87:* 489, 1954.

[13] Weinstein, I. M., and Beutler, E.: The use of Cr^{51} and Fe^{59} in a combined procedure to study erythrocyte production and destruction in normal subjects and in patients with hemolytic or aplastic anemia. J. Lab. & Clin. Med. *45:* 616, 1955.

[14] Peacock, W. C., Evans, R. D., Irvine, J. W., Good, W. M., Kip, A. F., Weiss, S., and Gibson 2nd, J. G.: The use of two radioactive isotopes of iron in tracer studies of erythrocytes. J. Clin. Invest. *25:* 605, 1946.

[15] Saylor, L., and Finch, C. A.: Determination of iron absorption using two isotopes of iron. Am. J. Phys. *172:* 372, 1953.

[16] KITZES, G., ELVEHJEM, C. A., AND SCHUETTE, H. A.: The determination of blood plasma iron. J. Biol. Chem. 155: 653, 1944.

[17] BOTHWELL, T. H., AND MALLETT, B.: The determination of iron in plasma or serum. Biochem. J. *59:* 599, 1955.

[18] RATH, C. E., AND FINCH, C. A.: Chemical, clinical and immunological studies on the products of human plasma fractionation. XXXVIII. Serum iron transport. Measurement of iron binding capacity of serum in man. J. Clin. Invest. *28:* 79, 1949.

[19] HUFF, R. L., AND FELLER, D. D.: Relation of circulating red cell volume to body density and obesity. J. Clin. Invest. *35:* 1, 1956.

[20] LOEB, JR., V.: Personal communication.

[21] THOMAS, E. D.: In vitro studies of erythropoiesis. I. The effect of normal serum on heme synthesis and oxygen consumption by bone marrow. Blood *10:* 600, 1955.

[22] LOEFFLER, R. K., RAPPOPORT, D. A., AND COLLINS, V. P.: Radioiron citrate as tracer to determine disappearance rate of plasma iron in normal subjects. Proc. Soc. Exper. Biol. & Med. *88:* 441, 1955.

[23] BARKAN, G., AND WALKER, B. S.: Determination of serum iron and pseudohemoglobin iron with O-phenanthroline. J. Biol. Chem. *135:* 37–42, 1940.

[24] WASSERMAN, L. R., RASHKOFF, I. A., LEAVITT, D., MAYER, J., AND PORT, S.: The rate of removal of radioactive iron from the plasma—an index of erythropoiesis. J. Clin. Invest. *31:* 32, 1952.

[25] LONDON, I. M., WEST, R., SHEMIN, D., AND RITTENBERG, D.: On the origin of the bile pigment in normal man. J. Biol. Chem. *184:* 351, 1950.

[26] —— AND ——: The formation of bile pigment in pernicious anemia. J. Biol. Chem. *184:* 359, 1950.

[27] FINCH, S. C., AND ROSS, J. F.: The significance of plasma iron turnover. J. Clin. Invest. *31:* 627, 1952.

[28] GIBLETT, E. R., COLEMAN, D. H., PIRZIO-BIROLI, G., DONOHUE, D. M., MOTULSKY, A. G., AND FINCH, C. A.: Erythrokinetics. Quantitative measurements of red cell production and destruction in normal subjects and patients with anemia. Blood *11:* 291, 1956.

[29] SEIP, M.: Reticulocyte studies. The liberation of red blood corpuscles from the bone marrow into the peripheral blood and the production of erythrocytes elucidated by reticulocyte investigations. Scandinav. Suppl. 282, 1953.

[30] FINCH, C. A., COLEMAN, D. H., MOTULSKY, A. G., DONOHUE, D. M., AND REIFF, R. H.: Erythrokinetics in pernicious anemia. Blood *11:* 807, 1956.

[31] STURGEON, P., AND FINCH, C. A.: Erythrokinetics in Cooley's Anemia. Blood *12:* 64, 1957.

[32] GIBLETT, E. R., VARELA, J. E., AND FINCH, C. A.: Bone marrow damage due to Rh antibody. Pediatrics *17:* 37, 1956.

Reprinted from THE AMERICAN JOURNAL OF PHYSIOLOGY
Vol. 193, No. 3, June 1958
Printed in U.S.A.

Iron Metabolism in the Pregnant Rabbit

Iron Transport Across the Placenta[1]

THOMAS H. BOTHWELL,[2] WALTHER F. PRIBILLA,[3] WINSTON MEBUST AND CLEMENT A. FINCH

From the Department of Medicine, School of Medicine, University of Washington, Seattle, Washington

ABSTRACT

BOTHWELL, THOMAS H., WALTHER F. PRIBILLA, WINSTON MEBUST and CLEMENT A. FINCH. *Iron metabolism in the pregnant rabbit. Iron transport across the placenta.* Am. J. Physiol. 193(3): 615–622. 1958.—Studies on iron metabolism were made in pregnant rabbits. The amount of iron transported from the maternal plasma to fetus increased progressively with age and weight of the fetus. By the end of the pregnancy 90% of the plasma iron turnover was directed to the fetus. The majority of fetal iron is deposited as nonhemoglobin iron. Placental iron transport occurs against a concentration gradient. The uptake of iron by the placenta is an active process independent of the fetus, and retrograde transfer of iron from the fetus to mother does not occur. Studies of altered maternal metabolism indicate that placental transport reflects both fetal demands and state of iron metabolism in the mother.

IT HAS BEEN SHOWN that the amount of iron passing through the plasma is dependent upon the needs of various acceptor tissues, the most important of which is the erythroid marrow (1). In pregnancy, a unique situation exists whereby two acceptor systems, namely maternal marrow and fetal tissues, compete for the available iron in the plasma. This is especially true in the rabbit, an animal in which fetal growth is rapid and whose iron content is very high at birth (2). The present paper records the ferrokinetics of the pregnant rabbit with particular attention directed toward iron transport across the placenta.

Received for publication September 11, 1957.

[1] This investigation was supported by a research grant from the U. S. Atomic Energy Commission (Contract AT-(45-1)-218); and a grant-in-aid from Smith, Kline & French Laboratories, Philadelphia, Pa.

[2] Recipient of a Lederle Traveling Fellowship for South Africa. Present address: Dept. of Medicine, University of Witwatersrand Medical School, Hospital Street, Johannesburg, South Africa.

[3] German Research Scholar under a Fulbright Grant 1955–1956. Present address: Stadt. Krankenanstalten, Haus 22, Koln-Merheim, Germany.

MATERIALS AND METHODS

All studies were performed on New Zealand white rabbits weighing between 4–5 kg in which the exact date of impregnation was known. Unless otherwise stated, the pregnant animals employed were in good health without complicating factors. In certain animals, however, an attempt was made to alter iron metabolism. Infection was simulated in two by the injection of 2 ml U.S.P. turpentine intramuscularly on the day before the study was performed. In two rabbits an attempt was made to deplete the iron reserves and stimulate maternal erythropoiesis by performing three phlebotomies of 30–40 ml each between the 21st and 27th days of pregnancy. Available iron was increased in three other animals. In two of these, the iron was supplied in the form of intramuscular iron dextran (Imferon[4]) in a dosage of 30 mg on the 20th, 21st and 22nd days of pregnancy. In the third animal, 15 ml of rabbit red cells, rendered nonviable by storage at 4°C in citrate for 24 days, were injected 2 hours before the radioiron study. Investigations performed on these animals included

[4] Kindly supplied by Benger Laboratories.

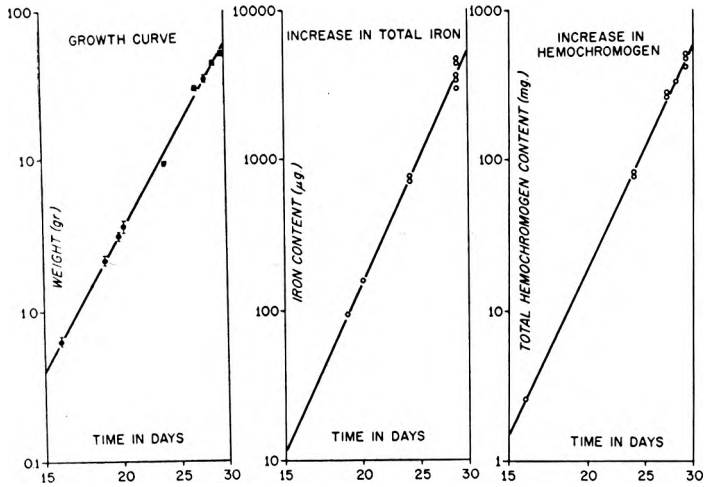


FIG. 1. Average rabbit fetus. Analyses performed on rabbit fetuses throughout pregnancy are graphed above. Standard deviation for individual fetuses is shown by brackets in the growth curve. Fetuses were pooled for other analyses during the early phase of pregnancy and individual carcass analyses of animals in later pregnancy are recorded.

TABLE 1. FERROKINETIC STUDIES IN PREGNANT RABBIT AT VARYING STAGES OF PREGNANCY

Time, days	Hematocrit	Plasma Iron, μg/100 ml	Time Taken to Reach ½ Concentration, min.	Plasma Iron Turnover/mg/day	Plasma Iron Turnover/100 ml/whole blood/day	Percentage Going to Fetuses	Amount to Fetuses, day, μg*	Amount to Average Fetus, μg/day
10	37	156	57	4.8	1.70	0		0
16	36	98	35	5.2	1.80	1.8	96	9.4
16	35	109	36	5.8	1.97	0.8	46	17.6
19	36	193	69	5.3	1.80	4.9	259	40
20	36	152	52	4.9	1.85	9.6	470	114
24	35	72	22	5.3	2.10	45	2400	368
24	36	43	16	5.4	1.73	60	2700	258
27	35	61	14	7.1	2.82	70	5000	624
29	30	46	10	10.0	3.12	85	8500	744
29	32	52	15	7.0	2.38	80	5600	724
30	39	40	12	6.5	2.10	90	5850	838

* Calculated from (plasma iron turnover × percentage going to fetus).

determination of maternal plasma iron turnover and, in some instances, manipulative procedures on fetuses *in situ*. When anesthesia was required, ether was employed. Animals were killed by the intravenous injection of air. Specific procedures employed are described in detail in the relevant sections.

The turnover of iron within the plasma was determined by injecting 3 μc of Fe^{59} chloride (specific activity 2–3 μc/μg) which had been previously incubated for one-half hour with plasma from a normal donor animal. Aliquots of this were also kept for the preparation of standards. Four to six blood samples were taken from the pregnant animals at intervals up to 90 minutes following the injection of radioiron. The plasma separated from these samples was counted for radioactivity in a well-type scintillation counter. The rate of disappearance of radioiron from plasma plotted on semilogarithmic paper approximated a straight line in most instances. Blood was removed for a plasma iron determination when the final sample was drawn. The amount of iron passing through the maternal plasma was calculated by using a modification of the formula of Huff *et al.* (3). The results were expressed: *a*) per 100 ml of whole blood (1), and *b*) per total blood volume, using an assumed blood volume of 60 ml/kg in the following formulae:

$$\text{Plasma iron turnover in mg/day/100 ml of whole blood} = \frac{\text{plasma iron in mg/100 ml}}{\text{time taken for radioiron to reach ½ concentration (days)}} \times \frac{100 - \text{hematocrit}}{100} \quad (a)$$

$$\text{Total plasma iron turnover in ml/day} = \text{plasma iron turnover/100 ml whole blood} \times \frac{\text{total blood volume in ml}}{100} \quad (b)$$

The daily fetal iron uptake was determined from the maternal plasma iron turnover data and the percentage of injected radioiron localized in the fetus. The animal was killed at 6 hours since almost all the radioactivity was found to have been cleared from the maternal

circulation at this time. The fetal uptake was estimated by counting each weighed fetus and placenta separately for radioactivity. The following formula was employed:

Iron uptake in fetuses (mg/day)

= total plasma iron turnover (mg/day)

× percentage of injected iron in fetuses

All small radioactive samples of less than 4 ml in volume were counted in a well-type scintillation counter. Because of their larger volumes, placentae and fetuses were counted in a Texas well counter. Appropriate standards were included in all such determinations. The plasma iron determination was made by the method of Bothwell and Mallett (4). Total iron content in fetuses and placentae was estimated by using a modification of the sulphosalicylic method described by Lorber (5). Hemochromogen determinations were done by a modification of the techniques of Schwartz, Zieve and Watson (6). The hemochromogen value obtained was shown to be unaffected by ferritin iron present in these tissues (Bothwell and Loden, personal observations). Reticulocytes were stained with cresyl blue and the number in 1000 red cells counted. Quantitative determinations of the nucleated red cell count were performed on femoral marrow, using a slight modification of the uniform cell suspension technique described by Thomas (7).

In electrophoretic studies of maternal and fetal plasma, a paper strip technique was used with a veronal buffer (ionic strength 0.05). The strips were run at 240 volts for 6 hours. Autoradiographs were prepared by exposing the strips to an x-ray film for 1–4 days. Radioautographic studies on histologic sections were done by fixing the tissues in Orth's fluid (2.5 gm of K_2Cr_2O, 1 gm $NaSO_4$ and 100 ml of distilled water to which 10 ml of 37–40% formalin, saturated with $CaCl_3$ was added just prior to use). Sections 10 micra thick were cut from these tissues and stained with hematoxylin and eosin. They were then coated with nitrocellulose, and a photographic emulsion (NTB$_3$ Eastman Kodak) was painted over the surface.

RESULTS

Changes in Fetal Iron Content as Related to the Duration of Pregnancy. In figure 1 the increases in weight and body iron content of the fetus during pregnancy are illustrated. It can be seen that the increase in iron roughly parallels the increase in weight, and by the 29th day of pregnancy a mean figure of 9.4 mg iron/100 gm of fetus is attained. Hemochromogen contents of fetuses increases in a fashion similar to that of the total iron. By the 29th day of pregnancy there is 1.1 gm of hemoglobin per 100 gm of fetus. Assuming a fetal hemoglobin iron concentration of 0.34%, this would indicate that only 3.7 mg of the 9.4 mg of iron at term is in the form of hemochromogen; i.e. about 40%.

Changes in Maternal and Fetal Iron Metabolism During Pregnancy. Iron going either to maternal tissues or the fetuses travels through the plasma iron compartment. Thus, determination of plasma iron turnover and its relative distribution between fetal and maternal tissues would be expected to provide information concerning the respective iron needs of mother and fetuses through pregnancy. Plasma iron turnovers performed on the 10th, 16th, 19th, 20th, 24th, 27th and 30th days of pregnancy are shown in table 1. It can be seen that total plasma iron turnover was somewhat increased in the last few days of pregnancy. During this phase, however, the percentage of iron reaching maternal tissues became pro-

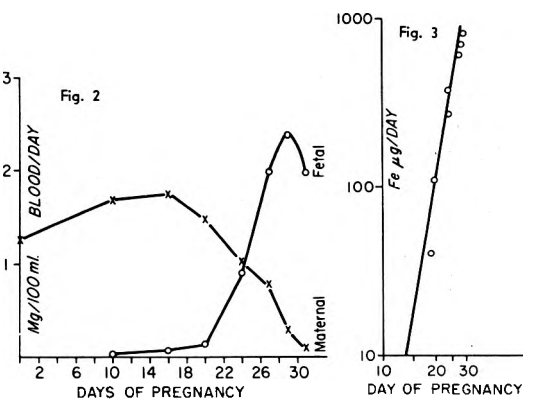


FIG. 2. Plasma iron turnover in pregnant rabbit. The amount of iron assimilated by maternal and by fetal tissues determined at intervals through pregnancy in the rabbit is plotted.

FIG. 3. Transplacental iron transport. Comparison of carcass analysis with plasma iron turnover. *Solid line* represents iron uptake of the fetus derived from carcass iron analysis data as shown in table 1. *Circled points* indicate daily fetal iron uptake derived from maternal plasma iron turnover and percentage of fetal iron uptake.

TABLE 2. EFFECT OF ALTERED MATERNAL IRON METABOLISM ON PLACENTAL IRON TRANSPORT

Diagnosis	Time, days	Hematocrit	Plasma Iron, μg/100 ml.	Time Taken to Reach ½ Concentration, min.	Plasma Iron Turnover/mg/day	Plasma Iron Turnover/100 ml/whole blood/day	Percentage Going to Fetuses	Amount to Fetuses/day, μg	Amount to Average Fetus, μg/day
Turpentine abscess	29	30	22	11	4.2	1.40	70.5	2960	403
Turpentine abscess	29	32	17	8	3.9	1.45	65.5	2550	284
Post bleeding	29	28	71	10.5	14.0	4.56	66.4	9300	1457
Post bleeding	29	23	93	15	11.73	4.77	68.7	8058	1415
Nonviable cells	29	40	215	38	8.16	3.40	33.0*	2690	1017
Intramuscular iron	29	35	85	10	16.9	5.53	85	14360	1208
Intramuscular iron	29	38	73	10	12.8	4.53	82	10496	1273
Normal pregnancy	29	32	50	12	8.5	2.8	82	6970	697

* Only 2 fetuses.

gressively less, while that going to the fetuses rose steeply. This is illustrated in figure 2. From a graphic plot of the amount of iron going to the average fetus at measured time intervals in pregnancy, it was possible to tally the cumulative amount of iron taken up by the average fetus throughout pregnancy. This result was compared with that obtained by chemical estimations of average fetal iron content at the 29th day. It was found that a figure of 4.1 mg was obtained from the plasma turnover data as against 3.9 mg by chemical estimation of fetal composition (fig. 3). The agreement of these two figures not only emphasizes the accuracy of the plasma iron turnover method, but also indicates that iron proceeding to the fetus remains there without returning to the maternal circulation.

The marked decrease in the uptake of iron by maternal tissues in the latter days of pregnancy was of considerable interest. The assumption that this reflected a curtailment of erythropoiesis was born out by the comparison of reticulocyte values on the 29th day of pregnancy with the normal value. In four pregnant animals, figures of 0.8, 1.6, 0.3 and 0.4 were obtained as compared to a normal of 4.3 (range 3.5–5.2). Studies of the total nucleated cell count of the femoral marrow also showed reduction of as much as 50% in the nucleated red cells present.

It was felt that more could be learned in terms of the distribution of iron between mother and fetus if alterations were made in maternal iron metabolism (table 2). Simulated infection, produced by turpentine abscesses, lowered both the total turnover of plasma iron and the percentage of iron going to the fetuses. When maternal demands were increased by previous bleeding, the total plasma iron turnover was considerably increased. This was largely due to a rise in the level of iron in the plasma, which presumably represented an increased mobilization from stores and gut. Under these circumstances the amounts of iron retained by maternal tissues rose to levels similar to those found in the nonpregnant animal. However, most of the plasma iron turnover was still directed to the fetuses. This also occurred when available iron was increased by supplements given in two different forms. In both instances the fetuses assimilated the major portion of the extra iron.

Nature of Placental Transfer of Iron. Observations were made on the relative plasma iron concentrations in mother and fetus. One-hundred microcuries of Fe^{59} bound to 20 ml of plasma were injected and maternal and fetal plasma samples were obtained at 25 minutes. The maternal plasma iron level was 61 μg/100 ml while the fetal was 218 μg/100 ml. Paper electrophoresis was performed on samples of fetal and maternal plasma, and autoradiographs were prepared. It was found that the radioiron migrated with the β_1 globulin in all instances. Additional iron (Fe^{59}) added to the fetal plasma prior to electrophoresis according to the method of Feinstein *et al.* (8) did not migrate with the β_1 globulin, which indicated saturation of the fetal transferrin.

If the amount of iron transferred across the placenta is known, some idea of the pool size within the placenta may be obtained from the rate of transfer from maternal to fetal plasma. Thus, if the pool were small, turnover would be rapid, while with a large pool there would

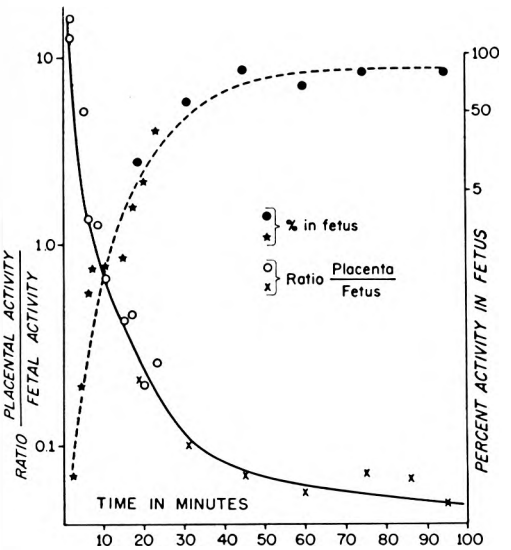


FIG. 4. Transit time through placenta. Relative amount of radioiron in placenta and fetus expressed as a ratio of placental/fetal activity over 100 min. after maternal radioiron injection as indicated by *solid line*. Fetal activity expressed as percentage of the maximal amount is illustrated by the *dashed line*.

be an appreciable delay in transit. Experiments were accordingly carried out to characterize the turnover rate across the placenta.

In the first experiment an animal 29 days pregnant was anesthetized with ether and its abdomen was opened. A known amount of plasma labeled with 3 μc Fe^{59} was injected intravenously. Thereafter, segments of uterus were opened at fixed time intervals and a placenta with its related fetus was removed. In all, nine fetuses were removed between 2 and 23 minutes after the injection of radioiron. In the second experiment the same procedure was followed except that removals were started at 18 minutes and continued beyond 100 minutes after injection. In each case the percentage of injected radioiron in placenta and fetus was measured, and the ratio of placental activity to fetal activity was calculated. The results are shown in figure 4, which also demonstrates the build-up of activity in the fetus.

In can be seen from the data from these two experiments that there was no real evidence of any significant holdup of radioiron in the placenta. Transfer of iron from maternal plasma to placenta had a T½ of 15 minutes, and uptake of iron by fetus a T½ of 25 minutes, and the ratio of placental to fetal activity dropped rapidly over 30 minutes from 12 to less than 0.1, at which level it tended to stabilize. It should be remembered that even the low activity found in the placenta at the later intervals is probably an overestimation of any metabolically active pool at this site, since some of the activity was almost certainly due to the blood in the placenta. (In 2 experiments with chromated red cells it was found that 9% and 16%, respectively, of the total fetal blood volume was present in the placenta.) From these studies it was concluded that there was no evidence to suggest that iron passing through the placenta became mixed with a pool of any measurable size at that site.

The plasma iron turnover studies already mentioned had suggested that transport from mother to fetus was in one direction, since the progressive accumulation of iron, as calculated from turnover data, agreed well with the figures obtained by chemical estimations of fetal iron content. A further study was done in which this was more directly tested. One rabbit on the 28th day of pregnancy was bled of 5 ml. Four microcuries of Fe^{59} (specific activity 2 μc/μg) was added to 1 cc of plasma separated from this blood. The rabbit was then anesthetized and the abdomen was opened. An incision was made in the uterus over one fetus and the umbilical vessels were gently exposed. One-quarter cubic centimeter of the radioactive plasma was then injected into the umbilical vein and the uterus was closed. The procedure was repeated with a second fetus. The abdomen was resutured and the animal was kept under light anesthesia for another hour. It was then killed and the fetuses and placentae were removed; all fetuses were alive and vigorous at the time of removal. All fetuses except the two which had received injections were counted for radioactivity and none was found. Since the major part of iron passing through the maternal plasma is rapidly taken up by fetuses at this stage of pregnancy, any iron escaping crom the radioactive fetuses to maternal cirfulation would be largely recaptured by the other fetuses. The absence of any evidence of uptake in this experiment was taken as strong evdence that there was no feedback of iron from fetuses to mother.

TABLE 3. PERCENTAGE INJECTED ACTIVITY LOCALIZED IN PLACENTAE AND FETUSES

Fetus No.	Receptor Tissue	Percent Injected Activity Present After 1 Hr.
1	Placenta	8.4
2	Placenta	7.0
3	Placenta	7.8
4	Placenta	5.0
5	Placenta	5.0
6	Placenta	8.0
7	Placenta	5.0
8	Fetus	10.2
	Placenta	0.4
9	Fetus	9.9
	Placenta	0.4
10	Fetus	7.3
	Placenta	0.4
11	Fetus	4.1
	Placenta	0.2
12	Fetus	4.4
	Placenta	0.2

The unidirectional transfer of iron from maternal iron-binding protein to placenta to fetal iron-binding protein raises the question as to whether this may be accomplished by three types of chelation of a progressively higher order of binding. If this were so, some difference might be observed between the release of fetal and maternal plasma iron when introduced into the same plasma pool. To test this, tagged fetal plasma was obtained from fetuses 30 minutes after injection of 100 μc of Fe^{59} bound to maternal plasma and injected into maternal circulation. The clearance rate of fetal plasma-bound Fe^{59}, and Fe^{55} bound to plasma of a normal rabbit was measured *in vivo* in a normal rabbit after intravenous injection. Curves of disappearance were similar, with a half-time of 60 minutes for Fe^{55} and 65 minutes for Fe^{59}.

The active role of the placenta in iron transport was established by a further experiment. In a 29-day pregnant rabbit under ether anesthesia, the umbilical vessels of half of the fetuses were ligated and the fetuses removed with care so as not to disturb the related placentae. The uterine and abdominal incisions were carefully closed and radioiron bound to maternal plasma was injected intravenously. The animal was killed 1 hour later. The percentage of the administered amount found in each fetus and placenta is shown in table 3. It can be seen that the mean uptake in the placentae from which the related fetuses had been removed was almost the same as that of the intact fetal-placental units which had been left *in situ* (6.6% as compared with 7.5%). It was thus apparent that the uptake of iron by the placenta is an active process which may, under experimental conditions, be separated from fetal uptake.

Macroscopically the fetal placenta is divided into two distinct zones. Attached to the uterus is a creamy membranous part, while the fetal side is succulent and mulberry red in color. Upon dividing the placentae used in the previous experiment, it was found that over 90% of the radioactivity taken up by the placenta was located on the fetal side. Microscopic studies showed iron deposits in the deeper tips of fetal villi in contact with maternal venous sinusoids. By use of radioautographs, it was also possible to demonstrate radioiron in these same areas. However, when placentae were ligated so that all radioactivity taken up over a period of two hours remained in the placentae, there was radioactive deposition and light iron staining along the whole length of the villus membranes of the placentae.

Fetal Tissue Receptors of Plasma Iron. Further studies were done to determine the localization of the radioiron which entered the fetal circulation. In three fetuses analyzed for radioactivity after six hours, 51, 50 and 59% of the total activity was found to be localized in the liver. In view of the observed difference between hemochromogen iron and total iron, further studies were performed on total fetuses to determine as a function of time the distribution of this radioiron between hemochromogen and nonhemochromogen fractions. The results are shown in figure 5 and indicate a rapid incorporation of approximately 35% of the radioiron into hemoglobin within the first 6 hours. There was no further increase during the following 18 hours. Thus the major portion of iron entering the fetus did not appear to be concerned with the immediate production of red cells. This reserve, referred to as the Bunge depot (9), would appear to be a specific adaptation of the fetus to its low iron diet after birth.

DISCUSSION

For many years it was thought that maternal red cells were the sole source of iron for the

developing fetus. However, in 1942 Pommerenke and his colleagues (10) demonstrated that when iron was fed by mouth to pregnant women it appeared in the fetal circulation within 40 minutes, an observation which suggested that the source of at least some of the fetal requirements was the iron circulating in maternal plasma. These findings were confirmed by Vosburgh and Flexner in guinea pigs (11), and from their results they calculated that the iron passing through the plasma was enough to satisfy the total needs of the fetuses. These conclusions have been confirmed in the present study. Plasma iron turnovers were done on rabbits in various stages of pregnancy and the percentage of injected radioactivity present in fetuses was calculated. In this way it was possible to show that the amount of iron going to the average fetus from the maternal plasma increased progressively throughout pregnancy. Estimations of the total iron content at term based on these radioiron data gave results very similar to those obtained by chemical means—a finding which strongly supports the idea that the only source of fetal iron is the iron circulating on the β_1 globulin of the maternal plasma.

In the rabbit, pregnancy imposes a considerable demand for iron on the mother. This is explained partly by the rapid fetal growth rate and the considerable weight of the fetuses at term in proportion to that of the mother, and also by the unusually large fraction of nonhemoglobin iron formed in the rabbit fetus. Although the maternal plasma iron turnover showed some increase in late gestation, this increase was insufficient to meet both fetal and maternal requirements. The physiologic adjustment to this situation was a sharp reduction in maternal uptake to about 10% of total plasma turnover. Since additional iron did not greatly increase maternal uptake, while production of anemia without iron supplementation resulted in a considerable increase, it seems likely that pregnancy in some way suppressed erythropoiesis during the late stage of gestation.

The question arises as to what stimulus in the fetus causes iron to be removed from the maternal plasma. Previous studies have shown that the major regulator of plasma iron turnover is the degree of erythroid marrow activity (1). It was, therefore, of interest to find out

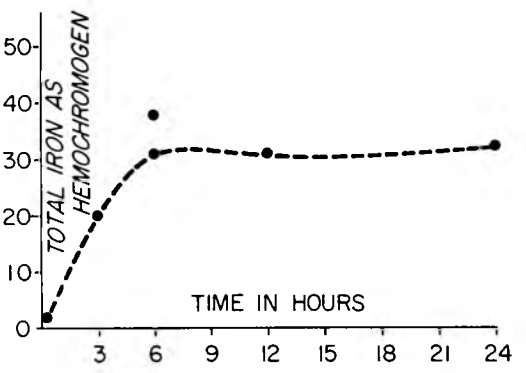


FIG. 5. Fetal liver was analyzed for hemochromogen and total iron. The percentage of radioiron in each of these fractions over a 24-hr. period after injection of radioiron into the pregnant animal is graphed.

how much of the iron going to fetuses was concerned with hemoglobin production. Although the hemochromogen content did increase in parallel with the total amount in the fetus, it formed only about 40% of this figure at all stages of pregnancy. It could, therefore, be concluded that fetal marrow activity was probably not the sole stimulus for the transplacental passage of iron.

When the mechanism of placental transfer was examined in more detail, it was found that the iron delivered from maternal plasma was rapidly transported across the placental site. The absence of any significant holdup suggested that iron entering the placenta did not become mixed with a biologic pool of any important size. The cells of the placental villi were shown by autoradiographs to be involved in iron transfer from maternal to fetal plasma. While the deeper villus tips were more heavily laden with iron, all living cells of the membrane participated. These villi obviously provided an effective cellular barrier between maternal and fetal circulations, since the plasma iron concentration was found to be higher in fetal plasma. This suggested either a higher order of binding on the fetal side or a unidirectional pumping system in the placenta. Whether maternal and fetal iron-binding globulin are identical is not known, but their electrophoretic mobility on paper appeared similar. Further, the clearance rate of iron from fetal iron-binding protein was similar in maternal circulation to that of maternal plasma iron. On direct testing it was found that no iron returned

from the fetal to the maternal circulations. It was also shown that the placenta played an active role in iron transfer, as it was capable of removing iron from the maternal circulation independent of any subsequent transport to the fetus.

These various observations allow some brief speculation regarding the nature of the mechanism of fetal iron transport. The close parallel between increased fetal size and increased amount of iron transferred implies fine regulation by the fetus. This does not occur through greater 'pull' from fetal transferrin, since this is saturated with iron at the time when fetal uptake is greatest. It also seems unlikely that the uterine blood flow or placental size accomplishes this adjustment, since changes in these do not coincide with fetal needs. The increase in fetal uptake observed on administration of supplemental iron suggests that the iron uptake by the fetus is to some extent influenced by the 'available' iron in maternal circulation. This would be consistent with studies on iron uptake of immature erythroid cells (12). It is also of interest that the placental membrane is influenced by changes in maternal metabolism. Just as infection interferes with iron transport across intestinal and reticulo-endothelial cell barriers, so, too, does it impair placental transport. Also of interest is the increased transfer which follows bleeding. In this respect the placenta seems to respond in a manner similar to that of reticulo-endothelial and mucosal cells in increasing iron transport in the face of increased erythropoiesis. Thus, placental iron transport may be visualized as a complex interaction in which the important recognizable factors are the needs of the fetus, the general state of maternal iron metabolism, and the supply of iron in maternal plasma. How these first two factors mediate their effects is not yet clear.

REFERENCES

1. BOTHWELL, T. H., A. V. HURTADO, D. M. DONOHUE AND C. A. FINCH. *Blood* 12: 409, 1957.
2. WIDDOWSON, E. M. *Nature* 166: 626, 1950.
3. HUFF, R. L., T. G. HENNESSY, R. E. AUSTIN, J. R. GARCIA, B. M. ROBERTS AND J. H. LAWRENCE. *J. Clin. Invest.* 29: 1041, 1950.
4. BOTHWELL, T. H. AND B. MALLETT. *Biochem. J.* 59: 599, 1955.
5. LORBER, L. *Biochem. Ztschr.* 181: 391, 1927.
6. SCHWARTZ, S., L. ZIEVE AND C. J. WATSON. *J. Lab. & Clin. Med.* 37: 843, 1951.
7. THOMAS, E. D. *Blood* 10: 600, 1955.
8. FEINSTEIN, A. R., W. F. BETHARD AND J. D. MCCARTHY. *J. Lab. & Clin. Med.* 42: 907, 1953.
9. BUNGE, G. *Hoppe Seylers, Z.* 16: 173, 1892.
10. POMMERENKE, W. T., P. F. HAHN, W. F. BALE AND W. M. BALFOUR. *Am. J. Physiol.* 137: 164, 1942.
11. VOSBURGH, G. J. AND L. B. FLEXNER. *Am. J. Physiol.* 161: 202, 1950.
12. JANDL, J. H., J. K. INMAN AND R. L. SIMMONS. *Clin. Res. Proc.* 5: 144, 1957.

THE ROLE OF THE RETICULO-ENDOTHELIAL CELL IN IRON METABOLISM

BY

WARD D. NOYES, THOMAS H. BOTHWELL and CLEMENT A. FINCH

Reprinted from British Journal of Haematology, Vol. VI, No. 1, January 1960

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD

Brit. J. Haemat., 1960, 6, 43.

The Role of the Reticulo-Endothelial Cell in Iron Metabolism*

WARD D. NOYES†, THOMAS H. BOTHWELL‡ AND CLEMENT A. FINCH

Department of Medicine, University of Washington School of Medicine, Seattle, Washington

SINCE exchange of iron with the external environment is very limited (Finch, 1959), body iron kinetics are largely concerned with the continuous redistribution of iron within the body. The reticulo-endothelial system is an important participant in internal iron turnover. It is believed to remove haemoglobin or non-viable erythrocytes from the circulation, process the iron from the haem moiety and return iron to the plasma for transport to the marrow and subsequent incorporation into haemoglobin. The reticulo-endothelial cells also serve as a potential reservoir for storage of iron not used for other purposes. The present studies were undertaken to examine the way in which this system processes iron derived from non-viable erythrocytes.

METHODS

The general plan of these studies involved: (1) tagging of erythrocytes with radioiron or radiochromium; (2) damaging these cells so that they would disappear rapidly from the circulation with a minimum of intravascular haemolysis; (3) administering the labelled cells to compatible recipients under a variety of experimental conditions, and (4) measuring the rate of removal of these non-viable cells from the circulation, their tissue localization and the subsequent release of radioiron from the reticulo-endothelial cells. Most of the investigations were done on normal human volunteers and on patients. In certain situations where this was not feasible, experimental animals were employed.

A. Human Studies

Erythrocytes tagged with radioiron were prepared by administering radioiron citrate in multiple intravenous injections over a 6-day period to patients with polycythaemia vera. The maximal total doses of 3 mc of ^{55}Fe or 1.5 mc of ^{59}Fe had no recognizable effect on the marrow function of the donor. Tagged cells were obtained from these donors 2 or more weeks later. Untagged erythrocytes were obtained from normal or polycythaemic donors. All donors were screened for evidence of hepatitis by liver-function tests. Blood was shown to be compatible by routine tests before transfusion and was examined by stained smear for bacterial contamination. Blood was taken into citrate and rendered non-viable by storage at 4° C. for 24–28 days, unless otherwise specified. These non-viable cells were administered in amounts varying from 0.01 ml. to 1.5 ml. of erythrocytes per kg. body weight and contained from 1 to 25 μc of ^{59}Fe, or from 5 to 30 μc of ^{55}Fe. The normal human recipients were young adult males with haematocrit values between 42 and 50 per cent and leucocyte counts between 5,000 and 10,000 per cu. mm. Also studied were patients with pernicious anaemia in early remission, iron deficiency, infection, iron-storage disease and aplastic anaemia.

* This investigation was supported by a research grant from the United States Atomic Energy Commission (Contract AT-(45-1)-218) and United States Public Health Service Research Grant No. H-2994.

† Recipient of a U.S. Public Health Service Research Fellowship from the National Heart Institute.

‡ In receipt of a Lederle Travelling Fellowship for South Africa. Present address: Department of Medicine, University of Witwatersrand Medical School, Hospital Street, Johannesburg, South Africa.

The general procedure for the study of erythrocyte disappearance and iron re-utilization was as follows. Blood was first withdrawn from the subject in whom observations were to be made for haematocrit, plasma iron and iron-binding capacity determinations. A weighed aliquot of the tagged cells to be injected was taken as a standard for determination of the haemoglobin and radioactive count. The desired amount of cells was injected intravenously into the compatible recipient within a period of 2 minutes. Following injection, frequent samples were withdrawn at appropriate intervals for determination of blood and plasma radioactivity, plasma iron, iron-binding capacity and plasma haemoglobin. In some subjects samples were taken over a 5-month period to follow the radioactivity in the recipients' erythrocytes.

The disappearance of tagged cells from the circulating erythrocyte mass was determined directly. Plasma activity, whether due to radioactive haemoglobin or radioiron bound to transferrin, was excluded. The curves of disappearance of erythrocyte radioactivity appeared to fit a simple exponential function as expressed by the formula:

$$C_t = C_o e^{-kt} \tag{I}$$

where C_t is the blood concentration at time t, C_o is the original concentration and k is the fractional disappearance of the cells per unit time. Clearance was expressed either as: (1) the fractional clearance per hour, or (2) the initial amount cleared (K_1) in mg. iron per hour per kg., according to the formula:

$$K = \frac{0.693}{T_{\frac{1}{2}}\ (\text{hours})} \tag{II}$$

$$K_1 = \frac{\text{erythrocyte iron injected} \times 0.693}{T_{\frac{1}{2}}\ (\text{hours}) \times \text{body weight (kg.)}} \tag{III}$$

The further distribution of the injected radioiron, arbitrarily assigned to: (*a*) iron remaining in reticulo-endothelial tissues, or (*b*) iron released into plasma and susequently localized in new haemoglobin production or in non-erythrocytic tissues, was determined by employing two isotopes of iron for measurements. One isotope was given as previously described in the form of non-viable erythrocytes, and the second isotope was injected intravenously to tag the plasma transferrin iron pool (Finch, Bothwell and Donohue, 1958). The distribution of radioiron from the plasma between new erythrocytes and other body tissues at 12 days was determined for this second isotope. From data of the activity of both isotopes injected and the amount appearing in circulating erythrocytes at 12 days, the following calculations could be made:

$$\text{Percentage erythrocyte iron utilized for haemoglobin production} = \frac{A_e^1 \times 100}{A_i^1} \tag{IV}$$

$$\text{Percentage plasma iron localized in non-erythrocytic tissues} = \frac{A_i^2 - A_e^2}{A_i^2} \times 100 \tag{V}$$

$$\text{Percentage erythrocyte iron released into plasma} = \frac{A_e^1 \times A_i^2}{A_i^1 \times A_e^2} \times 100 \tag{VI}$$

$$\text{Percentage erythrocyte iron localized in non-erythrocytic tissues} = (V) \times (VI) \quad (VII)$$

$$\text{Percentage erythrocyte iron remaining in reticulo-endothelial cells} = 100 - (VI) \quad (VIII)$$

In the above equations A_i^1 refers to the amount of radioactivity injected as non-viable erythrocytes and A_i^2 to the amount of radioactivity injected intravenously bound to plasma transferrin. A_e^1 refers to the amount of radioactivity derived from broken down erythrocytes which is incorporated in the circulating erythrocyte mass at 12 days and A_e^2 to the amount of radioactivity derived from the plasma-labelling isotope in the erythrocyte mass at 12 days. In determining these figures, the erythrocyte mass was calculated from an assumed blood volume of 65 ml. per kg. multiplied by the peripheral haematocrit value and by 0.9 (Donohue, Motulsky, Giblett, Pirzio-Biroli, Viranuvatti and Finch, 1955).

In addition to variations in the load of non-viable cells, several experiments were performed in man to clarify factors involved in cell disappearance and subsequent iron distribution. Sequential infusion of two loads of non-viable cells, each tagged with a separate isotope of iron, was carried out to determine the effect of a prior load on the clearance and on the distribution of the iron released. An attempt was made to block iron release from the reticulo-endothelial cells by intravenous injection of saccharated iron (200 mg.) 2 hours before the infusion of non-viable cells, and by the oral administration of 1 g. of exsiccated ferrous sulphate (368 mg. iron) at a similar time. The mobilization of radioiron over a period of 140 days was compared in normal subjects and subjects bled 500 ml. of whole blood every 2 weeks to a total of 3000 ml.

In certain experiments an attempt was made to localize the site of uptake of non-viable cells by surface counting of various areas of the body for radioiron content. A 5 cm. iodide crystal shielded with 76 mm. of lead was employed. The aperture measured 5 cm. and there was collimation of 7 cm. While activity was recorded over sternum, sacrum, thigh, liver and spleen at approximate intervals, only data concerning liver and spleen activity will be reported.

B. Animal Studies

Studies in animals were performed on New Zealand white male rabbits weighing 2.2–3.5 kg. Erythrocytes were tagged either *in vivo* with radioiron, or *in vitro* by room temperature incubation for 1 hour with $Na_2{}^{51}CrO_4$ in a concentration of less than 1 μg. per ml. of erythrocytes. Cells were rendered non-viable by storage at 4° C. in citrate for 24–28 days, by incubation at 50° C. for 45 minutes, by incubation with 0.05 M-Na_2HAsO_4 at 20° C. for 14 hours, or by the intraperitoneal injection of acetylphenylhydrazine dissolved in 95 per cent alcohol into a donor animal in a dosage of 40 mg. per kg., 12 hours before the removal of erythrocytes. In these studies the amount of erythrocytes transfused varied from 0.3 to 6.0 ml. per kg. and the amount of radioactivity varied from 0.5 to 10.0 μc ^{51}Cr. Organ localization of cells damaged by heat, arsenic and phenylhydrazine was determined by sacrifice of the animal 48 hours after injection of the damaged tagged cells. The organs removed, including liver, spleen and lungs, were digested in nitric acid and an aliquot was counted for radioactivity.

To test the effects of very high loads of non-viable cells, the clearance of phenylhydrazine-damaged cells was followed: (*a*) directly in rabbits given acetylphenylhydrazine, and (*b*) in a

recipient rabbit subjected to exchange transfusion with cells removed from donor animals 12 hours after injection of phenylhydrazine. In the latter experiment, the heparinized donor blood was infused into the marginal ear vein while an equal amount was removed by femoral artery cannula, until twice the animal's estimated blood volume had been exchanged.

Plasma iron determinations were performed by the method of Bothwell and Mallett (1955); iron-binding capacity was determined using the measured method of Rath and Finch (1948) and plasma haemoglobin was determined by the method of Flink and Watson (1942). Plasma iron turnover was calculated as described previously (Bothwell, Hurtado, Donohue and Finch, 1957). Radioiron and radiochromium were obtained from Oak Ridge National Laboratory with specific activities approximating to 2–3 mc per mg. for ^{59}Fe, 2.5–3.5 mc per mg. for ^{55}Fe and 35–45 mc per mg. for ^{51}Cr. The two isotopes of iron were differentially counted following wet-ashing and electroplating, using a helium-filled Geiger tube for ^{59}Fe and an argon-filled tube with a beryllium filter for ^{55}Fe (Peacock, Evans, Irvine, Good, Kip, Weiss and Gibson, 1946). When used separately, ^{59}Fe and ^{51}Cr were counted in a well-type scintillation counter.

RESULTS

A. Amount of Intravascular Haemolysis from Non-Viable Cells

After injection into the circulation, non-viable erythrocytes rapidly disappeared. A fraction of cell breakdown was reflected in intravascular liberation of haemoglobin. In seven subjects followed with frequent determinations of plasma haemoglobin, maximal levels were found within the first 15–30 minutes after injection. The free haemoglobin represented approximately 12 per cent (range 6–18 per cent) of the erythrocyte haemoglobin injected (Table I).

TABLE I

MAXIMUM PLASMA HAEMOGLOBIN REACHED FOLLOWING INJECTION OF NON-VIABLE ERYTHROCYTES

Subject	*Estimated plasma volume (ml.)*	*Amount Hb injected (mg.)*	*Max. plasma Hb level (mg./100 ml.)*	*Max. Hb in circulation (mg.)*	*Proportion of injected Hb in plasma (%)*
Pe	2490	7800	58	1445	18.5
Ma	2240	7860	39	875	11.1
Le	3230	46,400	92	2850	6.1
Wi	3300	11,200	40	1320	11.8
Ha	3400	11,200	39	1290	11.5
Mo	2672	22,300	102	2725	12.2
Fe	3400	22,300	112	3220	14.4

B. Clearance of Non-Viable Cells

The disappearance of tagged cells down to 10 per cent of the initial concentration appeared to be exponential (Fig. 1).

The disappearance of erythrocyte iron loads from 0.15 to 0.95 mg. iron per kg., expressed as half-time of circulating activity, varied between 33 and 150 minutes (Table II). A significant correlation ($r = .9917$, $t = 10.25$) between the load and the $T_{\frac{1}{2}}$ in 17 normal subjects (Fig. 2a) was found in the range of loads employed. The relation between the initial rate of clearance (K_1) and load, as illustrated in Fig. 2b, has a correlation coefficient of .8072 ($t = 4.18$).

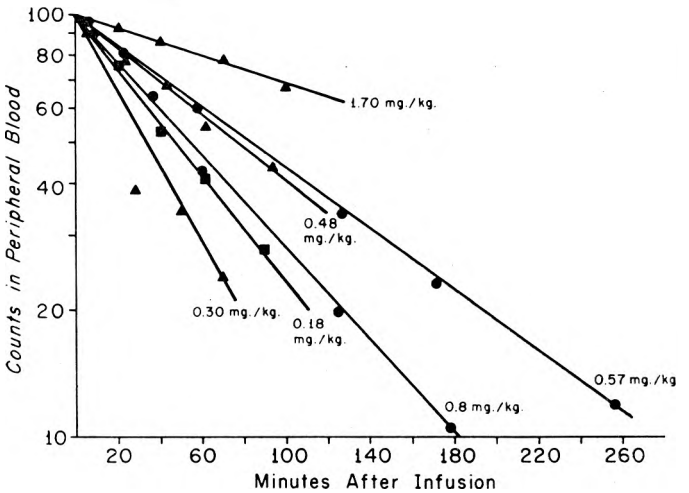


FIG. 1. The disappearance of erythrocyte radioactivity from the peripheral blood after infusion of tagged cells. In each instance the cell load is indicated as erythrocyte iron per kg. body weight of the recipient. The clearance appeared to be exponential over the period of observation.

TABLE II

CLEARANCE OF TAGGED NON-VIABLE ERYTHROCYTES FROM CIRCULATION

Condition of study	*Iron load (mg./kg.)*	$T_{\frac{1}{2}}$ *(min.)*	*Initial clearance (mg./kg./hr)*	*Transfusion group**	*Condition of study*	*Iron load (mg./kg.)*	$T_{\frac{1}{2}}$ *(min.)*	*Initial clearance (mg./kg./hr)*	*Transfusion group**
Normal	0.15	40	0.16	A	Oral iron load	0.36	21	0.72	J
,,	0.18	48	0.15	B	,,	0.40	19	0.88	K
,,	0.25	57	0.18	C	Intravenous iron load	0.17	43	0.16	B
,,	0.25	112	0.09	D	,,	0.26	67	0.15	B
,,	0.28	33	0.35	A	60 min. after 50 ml. erythrocytes	0.27	94	0.12	D
,,	0.29	150	0.08	D	,,	0.32	78	0.17	C
,,	0.30	48	0.26	C	120 min. after 50 ml. erythrocytes	0.23	104	0.05	D
,,	0.30	34	0.37	A	,,	0.28	82	0.14	C
,,	0.40	83	0.20	E	Iron deficiency	0.24	35	0.29	L
,,	0.47	55	0.35	F	,,	0.39	59	0.28	L
,,	0.48	75	0.27	F	,,	0.58	54	0.45	M
,,	0.53	44	0.51	F	,,	0.58	60	0.40	M
,,	0.56	86	0.27	F	Aplastic anaemia	0.66	129	0.21	N
,,	0.57	81	0.29	G	Carcinoma	0.46	24	0.80	L
,,	0.80	54	0.62	G	Haemolytic anaemia; arthritis	0.46	40	0.48	O
,,	0.94	69	0.48	H	Blood stored 3 weeks	0.46	100	0.19	E
,,	0.95	83	0.57	H	Blood stored 2 weeks	0.41	460	0.04	E
,,	1.70	204	0.35	B	Blood stored 1 week	0.43	∞	...	E
Mean	0.52	...	0.31 ± 0.16 (1 SD)						

* All subjects in any one transfusion group received cells from the same batch.

It thus appears that in this load range, the fractional rate of cell clearance decreases in proportion to the load of cells administered. The initial rate of clearance is even more significantly related to the cell load, as illustrated in Fig. 2b.

To observe the effect on clearance of higher cell loads, experiments were continued in rabbits. Cell loads in excess of 2.0 mg. per kg. resulted in relatively smaller changes in $T_{\frac{1}{2}}$ and fractional clearance (Table III). Considerably larger loads were produced in rabbits by

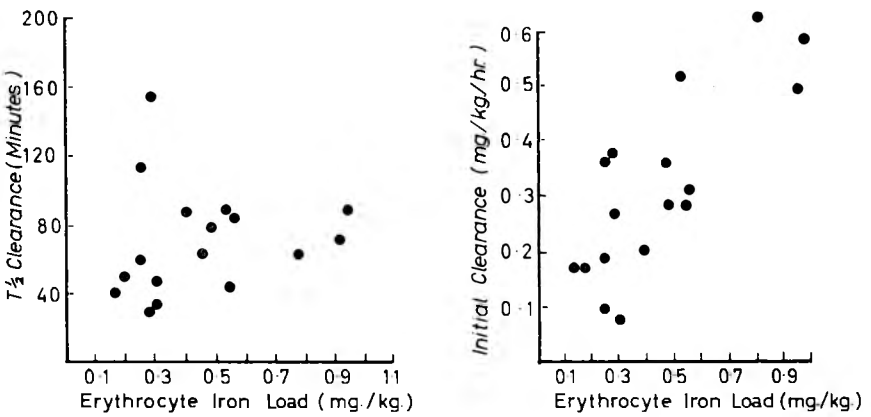


FIG. 2. The left-hand chart (Fig. 2a) represents the relation between the load of cells administered, expressed as erythrocyte iron per kg. body weight, and the half-time of disappearance of radioactivity from the circulation.

The right-hand chart (Fig. 2b) shows the relation between the cell load and the rate of initial clearance (K_1). There appears to be a significant correlation between the cell load and the rate of clearance.

TABLE III

CLEARANCE OF NON-VIABLE ERYTHROCYTES IN RABBITS

Load of non-viable cells (mg./kg.)	*Initial clearance (mg./kg./hr)*		
	Arsenic-treated cells	*Phenylhydrazine-treated cells*	*Cells incubated at 50° C.*
0.3	0.08	0.08	0.06
1.5	0.21	0.20	0.13
3.0	0.19	0.21	0.13
6.0	0.20	0.40	0.255

the administration of 40 mg. per kg. of acetylphenylhydrazine. This renders all circulating erythrocytes non-viable within 12 hours, as demonstrated by the disappearance of these cells within minutes after their infusion in small amounts to normal recipients. In rabbits treated with phenylhydrazine, the time for disappearance of one-half of the cell mass was 48 hours. To exclude any influence on clearance of the action of phenylhydrazine on other body tissues, a normal rabbit was perfused with two blood volumes of whole blood from animals given phenylhydrazine. This animal showed a similar initial clearance rate, amounting to about 0.5 mg. per kg. per hour.

The possibility that the type of cell damage might influence both the rate of clearance and the kinetics of cell removal was considered. Therefore, clearance rates were studied in animals after cell damage by phenylhydrazine *in vivo*, by cell heating and by arsenic *in vitro*. Generally similar rates of clearance were found with these various types of cell damage (Table III).

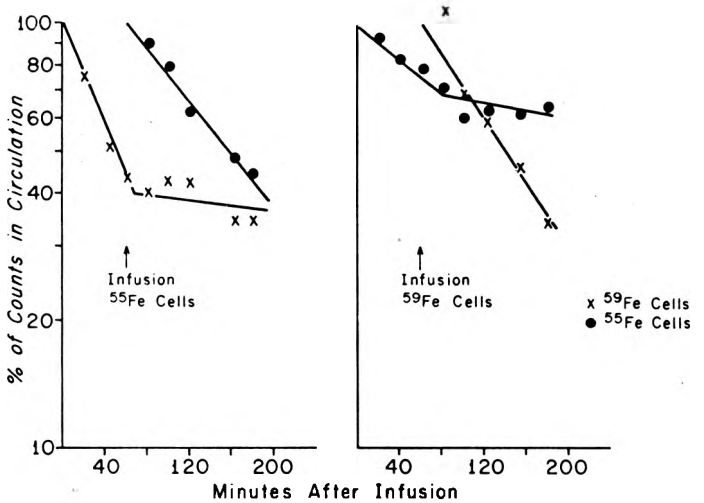


FIG. 3. The effect on the clearance of one population of cells by the infusion of a second population one hour later. There is some difference in the original clearance of cells from different donors, perhaps related to variability in the extent of cell damage. However, the most conspicuous finding is the marked slowing of the original cell clearance following the second infusion.

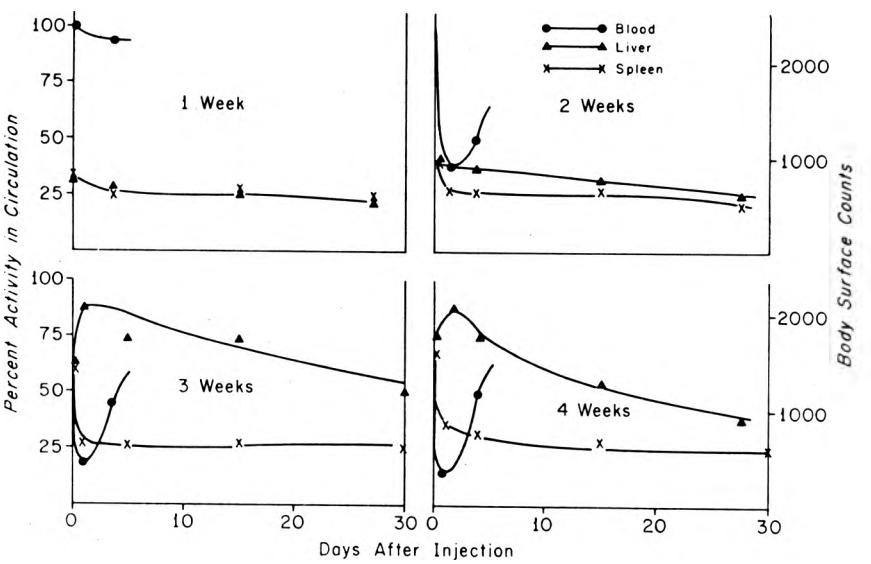


FIG. 4. Tissue localization of erythrocytes subjected to varying degrees of *in-vitro* storage damage. The activity in circulating blood is contrasted to the surface activity over the spleen and liver. Both the liver and spleen showed accumulation of radioactivity as tagged cells disappeared from the circulation, but liver activity fell slowly in contrast to the rapid release of activity from the splenic area.

To investigate the factors further influencing the clearance of non-viable cells, the effect of a second injection of cells shortly after the first injection was studied in man (Fig. 3). A marked slowing in disappearance of the remaining cells of the first cell population was observed after the second infusion was begun.

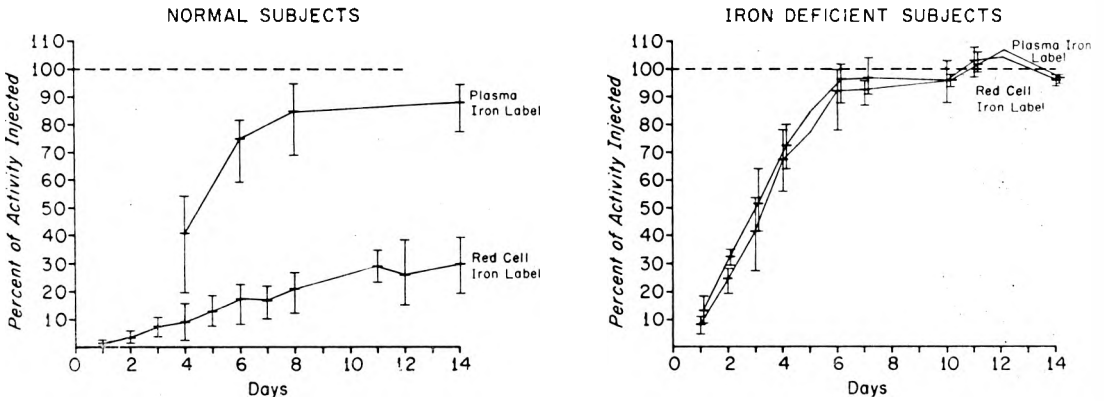


FIG. 5. Erythrocyte utilization of radioiron injected as non-viable erythrocytes (0.5 mg. iron per kg.) and as transferrin-bound iron compared in three normal and four iron-deficient subjects. At 14 days, more than 50 per cent of the radio-iron remains within the reticulo-endothelial system in normal subjects while 95 per cent appears in the circulation in iron deficiency.

TABLE IV

PRIOR IRON LOADING OF PLASMA TRANSFERRIN

Condition of iron load	*Erythrocyte iron load (mg./kg.)*	*Before infusion*		*At infusion of cells*		*2 hours after infusion*		*Initial clearance (mg./kg./hr.)*	*Radioiron in circulation at 12 days (%)*
		Serum iron	*Iron-binding capacity*	*Serum iron*	*Iron-binding capacity*	*Serum iron*	*Iron-binding capacity*		
		(μg./100 ml.)		(μg./100 ml.)		(μg./100 ml.)			
1.0 g. ferrous sulphate 2 hours before infusion	0.36	61	200	170	150	200	0	0.72	50
1.0 g. ferrous sulphate 2 hours before infusion	0.40	107	200	186	50	230	0	0.88	40
200 mg. intravenous iron 2 hours before infusion	0.17	73	100	130	150	...	...	0.16	40
200 mg. intravenous iron 2 hours before infusion	0.26	107	...	229	...	...	...	0.15	33

C. Tissue Localization

Coincidental with the disappearance of ^{59}Fe-tagged erythrocytes from circulation, there was an increase in surface counting over the liver and spleen. In order to determine whether the degree of storage damage affected tissue distribution, the tissue localization of cells stored for periods of 1–4 weeks was studied (Fig. 4). Increasing amounts were deposited in the liver and spleen as storage was prolonged. It was observed that radioactivity in the splenic area decreased again within a few hours of its accumulation, while that in the liver remained at an elevated level for many days.

D. Release of Erythrocyte Iron from the Reticulo-Endothelial Cell

The processing of haemoglobin iron from the erythrocyte occurred rapidly. In subjects

given the smaller loads (0.01 mg. of erythrocyte iron per kg.), radioiron was detectable in the plasma bound to transferrin within 30 minutes after injection of the cells and rose to 18–22 per cent of the radioiron injected at 2 hours.

The amount of radioiron derived from that injected which passed from the reticulo-endothelial cell through the marrow to the circulating erythrocyte mass, was determined in 19 subjects given erythrocyte iron in amounts from 0.1 to 1.0 mg. per kg. An average of 42 per cent (range 19–69 per cent) of the injected erythrocyte radioiron was found in circulating erythrocytes 12 days after injection. Within this dosage range there was no apparent relation between the degree of erythrocyte uptake and the amount of erythrocytes administered. However, in three subjects given 0.01 mg. per kg. the utilization was 85 per cent (range 81–88 per cent) at 12 days.

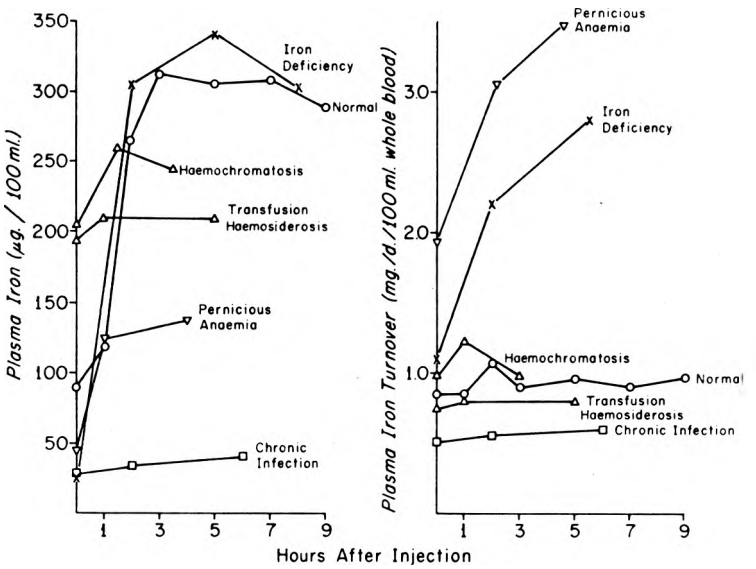


FIG. 6. The effect of non-viable erythrocyte infusion (0.5 mg. iron per kg.) on the plasma iron concentration and plasma iron turnover. The plasma iron turnover is significantly increased only in those subjects who showed evidence of a relative or absolute deficiency in iron supply to the marrow.

A portion of radioiron not appearing in circulating erythrocytes is retained in reticulo-endothelial tissue and the remainder is distributed to non-erythroid tissue following its release into the plasma. A graphic representation of such studies in normal and iron-deficient subjects is shown in Fig. 5. Since, in normal subjects, the distribution from plasma was 85 per cent to erythrocytes and 15 per cent to tissues at 12 days, the approximate distribution of a non-viable erythrocyte iron load of 0.1 to 1.0 mg. per kg. at 2 weeks would be: erythrocytes 40 per cent, other tissues 10 per cent and reticulo-endothelial tissue 50 per cent. In further studies (Table IV) an attempt was made to alter the retention of erythrocyte radioiron by reticulo-endothelial tissue in normal subjects by iron loading of the plasma. In four experiments the erythrocyte utilization at 12 days was 33–50 per cent of the administered dose.

The effect of certain pathological states on the handling of iron by the reticulo-endothelial tissue in man was also studied (Fig. 6). In two iron-deficient subjects the transfusion of non-viable cells was followed by an increase in both plasma iron and plasma iron turnover, and

the utilization of erythrocyte iron at 2 weeks was almost complete. In two patients with pernicious anaemia responding to therapy, a similar response was observed. In patients with infection, idiopathic haemochromatosis or aregenerative anaemia with transfusion haemosiderosis, there was no increase in either plasma iron or plasma iron turnover and the proportion appearing in the circulating erythrocyte mass was greatly reduced.

E. Turnover of Storage Iron

The change in erythrocyte activity levels of seven subjects over a period of 100–140 days after the injection of tagged non-viable erythrocytes is shown in Fig. 7. A total of 3000 ml. of blood was withdrawn by repeated phlebotomies from four of these subjects over a period of 12 weeks. Virtually all of the radioactivity given as non-viable cells was found in the circulation or removed by bleeding by the end of the study in these four subjects (Fig. 7), but only two-thirds was found in circulation in the remaining three subjects, who were not bled.

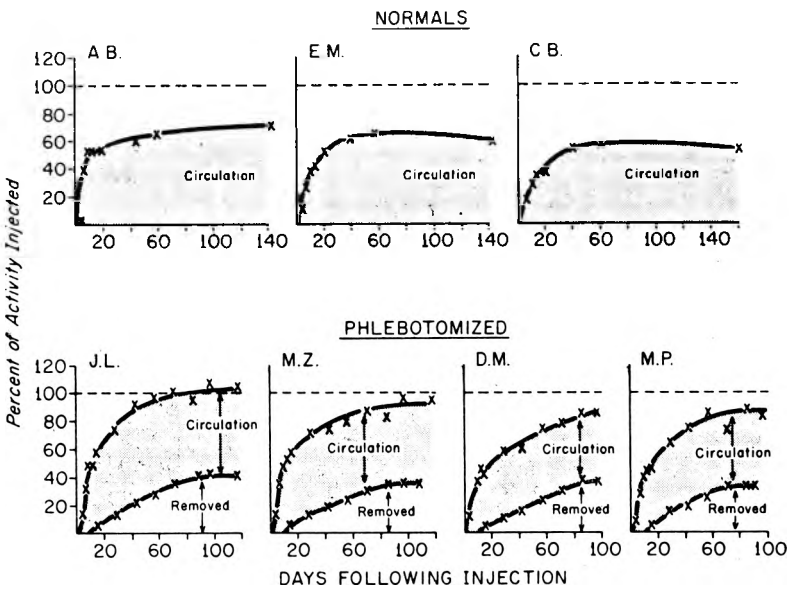


FIG. 7. Total amount of circulating erythrocyte radioiron during 140 days following the injection of non-viable erythrocytes (0.5 mg. iron per kg.) into three normal subjects (upper curves) and four phlebotomized subjects (lower curves). Approximately one-third fails to appear in the circulation of the unbled subjects, but virtually all of the radioiron initially retained in the reticulo-endothelial system of the phlebotomized subjects has been mobilized.

DISCUSSION

In this investigation one segment of erythrocyte iron turnover has been selected for study, namely the removal of erythrocytes from the circulation and the re-circuiting of their iron to the erythroid marrow. Erythrocytes were damaged sufficiently to result in their rapid disappearance from the circulation, but not sufficiently to lead to extensive intravascular haemolysis. On the basis of the amount of plasma haemoglobin found after transfusion and its maximal turnover rate in normal subjects of about 10 mg. of haemoglobin per minute (Garby and Noyes, 1959), it is estimated that no more than 20 per cent of the injected cells were disposed of in this fashion.

Non-viable human erythrocytes disappeared from the circulation of the recipient in a manner consistent with a simple first order reaction. Curves of similar contour have been obtained by Miescher (1957) in experiments on the transfusion of non-viable compatible animal cells and by others in studies of the clearance of colloidal or particulate material by the reticulo-endothelial system (Benacerraf, Biozzi, Halpern and Stiffel, 1957). The complexity of erythrocyte and reticulo-endothelial cell interaction, including the effects of the degree of erythrocyte damage and prior erythrocyte phagocytosis and the incidence of collision between erythrocytes and phagocytes, cannot be resolved by the present study. Of interest in this respect, however, were the clearances of two cell populations administered sequentially. Cells of the second transfusion were preferentially removed (Fig. 3). This might be interpreted to mean that there was a spectrum of cell damage in each population of cells and that the most severely damaged cells were removed first. The loads administered, 0.5 mg. iron per kg., would seem far less than would be required to saturate the clearance mechanism. Similar sparing effects have been observed between two species of colloidal particles administered in sequence (Dobson, 1957).

The ample capacity of the reticulo-endothelial system to remove non-viable cells is demonstrated by the data summarized in Table II. Rates of erythrocyte destruction of 20–40 times the normal were found. These studies in man do not define the maximal capacity for cell disposal. The phenylhydrazine studies in rabbits, however, demonstrate that there is a limit to the amount of erythrocytes which may be removed per unit time. After exposure to the drug, all erythrocytes are non-viable, and disappear from the circulation within minutes when transfused in small amounts to recipient animals. In the animal given an injection of phenylhydrazine, on the other hand, or in animals exchange-transfused with phenylhydrazine-damaged cells, approximately one-quarter of the erythrocyte mass is disposed of in one 24-hour period. The time required for disposal of the damaged cell population is enough to permit the animal to survive through production of new erythrocytes. Only when cell damage is sufficient to produce extensive intravascular haemolysis is the anaemia likely to prove fatal.

After initial phagocytosis, the erythrocyte iron is split from the haemoglobin molecule, part of it is retained within the reticulo-endothelial cell and part is returned to the plasma transferrin for distribution within the body. In normal subjects with approximately physiological loads of haemoglobin, most of the iron is promptly returned to the marrow. With an average load of 0.3 mg. erythrocyte iron per kg., however, nearly half the iron is retained at 12 days in the reticulo-endothelial cell. This distribution does not appear to be appreciably affected by variations in dosage between 0.15 and 0.95 mg. per kg. This calculation of reticulo-endothelial cell retention is indirectly made, and cannot exclude the possibility of transfer of iron directly from the reticulo-endothelial cell to another tissue cell without passage through the plasma. While Bessis and Breton-Gorius (1957) have suggested that this does indeed occur in the marrow, it seems unlikely from kinetic data that any large portion of erythrocyte iron uptake bypasses the plasma transport system.

Elevation of plasma iron by ingestion of iron or by the intravenous injection of saccharated iron oxide has no conspicuous effect on the total amount of iron released from the reticulo-endothelial system. It would appear that the level of the plasma iron has no more effect on the total amount of iron released than it does on the transport of iron across the intestinal mucosa.

Increased release of iron following infusion of non-viable erythrocytes was observed in patients with low plasma iron levels when this was associated with iron deficiency or increased marrow requirements. It is therefore necessary to postulate a regulation of iron release by

the reticulo-endothelial cell which is integrated with the iron needs of the erythroid marrow. The manner in which the marrow requirements are impressed on the reticulo-endothelial system is not known. A special situation is observed in chronic infection where a decreased plasma iron is associated with decreased iron release. This block in iron release in infection has already been well described in experimental animals (Freireich, Miller, Emerson and Ross, 1957).

There were differences in the handling of iron between different organs, as measured by *in vivo* counting. Thus, the spleen rapidly lost radioactivity initially localized in that area, whereas the activity within the liver remained for many days. This is interpreted to mean that the haemoglobin iron is rapidly released by the spleen for re-circuiting to the marrow, while liver iron is held in more slowly moving stores. This has also been suggested by the studies of Jandl, Jones and Castle (1957). Long-term studies of the iron in stores indicated that over the 50 days following injection there was a gradual movement of radioiron from the tissues to the erythrocyte mass (Fig. 7). Thus at 12 days, three subjects were calculated to have 38, 40 and 52 per cent of the administered erythrocyte iron remaining in the reticulo-endothelial system, while at 60 days they were calculated to have 29, 33 and 37 per cent in this system. In four subjects phlebotomized in order to mobilize tissue iron, virtually none of the radioiron remained in the reticulo-endothelial system. (The specific activity of erythrocyte iron of these subjects from day 15 to day 60 appeared the same as that in unbled subjects, indicating that stores initially mobilized were uniformly labelled with radioiron. By the 80th day the erythrocyte specific activity of the bled subjects was 20 per cent below that of the unbled subjects, indicating a contribution of unlabelled iron either from intestinal absorption or from less highly labelled stores.) A fraction of storage radioiron is slowly released, as indicated by a gradual rise over 60 days in the calculated erythrocyte activity in the three normal subjects. Approximately 45 per cent of the iron passing through the reticulo-endothelial system from these cell loads appears to be retained within this pool, of which perhaps one-quarter is returned to the marrow in the subsequent 6 weeks.

SUMMARY

The disappearance of non-viable erythrocytes from the circulation has been studied in human subjects. The loss of erythrocyte radioiron appeared to be a first order reaction. With injections of 0.1 to 1.0 mg. of erythrocyte iron, there was an average clearance of 61 per cent per hour or between 20 and 40 times the normal rate of erythrocyte destruction.

While small amounts of iron (i.e., 0.01 mg. per kg.) were almost completely utilized for new haemoglobin synthesis, loads of 0.1 to 1.0 mg. per kg. resulted in the retention of approximately half of the iron by the reticulo-endothelial tissue at 2 weeks. The degree of retention was not affected by the level of plasma iron, but did appear to be influenced by the iron requirements of the erythroid marrow. There also appeared to be a difference in the behaviour of the spleen and liver in processing the iron from non-viable cells; the spleen released its iron more rapidly to the plasma than did the liver. While a small additional portion of iron appeared in the erythrocytes over the following six weeks, the majority of the iron deposited in tissues remained there. This agrees with the concept of the relative stability of normal body iron stores.

ACKNOWLEDGMENT

The authors wish to thank Miss Betty Loden for valuable technical assistance.

REFERENCES

BENACERRAF, B., BIOZZI, G., HALPERN, B. N. and STIFFEL, C. (1957). 'Physiology of the phagocytosis of particles by R.E.S.' *in* Halpern, B. N. (1957), p. 51.

BESSIS, M. and BRETON-GORIUS, J. (1957). 'Le cycle du fer dans l'organisme révélé par le microscope électronique.' *Path. et Biol. (Paris)*, **19**, 829.

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 MALLETT, B. (1955). 'The determination of iron in plasma or serum.' *Biochem. J.*, **59**, 599.

DOBSON, E. L. (1957). 'Factors controlling phagocytosis.' *in* Halpern, B. N. (1957), p. 80.

DONOHUE, D. M., MOTULSKY, A. G., GIBLETT, E. R., PIRZIO-BIROLI, G., VIRANUVATTI, V. and FINCH, C. A. (1955). 'The use of chromium as a red-cell tag.' *Brit. J. Haemat.*, **1**, 249.

FINCH, C. A. (1959). 'Body iron exchange in man.' *J. clin. Invest.*, **38**, 392.

FINCH, C. A., BOTHWELL, T. H. and DONOHUE, D. M. (1958). 'Ferrokinetics.' *Proc. 6th Congr. int. Soc. Hemat.*, p. 259. Grune and Stratton, New York.

FLINK, E. B. and WATSON, C. J. (1942). 'A method for the quantitative determination of hemoglobin and related heme pigments in feces, urine and blood plasma.' *J. biol. Chem.*, **146**, 171.

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.

GARBY, L. and NOYES, W. D. (1959). 'Studies on hemoglobin metabolism. I. The kinetic properties of the plasma hemoglobin pool in normal man.' To be published.

HALPERN, B. N. (ed.) (1957). *Physiopathology of the Reticulo-Endothelial System.* Thomas, Springfield, Illinois.

JANDL, J. H., JONES, A. R. and CASTLE, W. B. (1957). 'The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms.' *J. clin. Invest.*, **36**, 1248.

MIESCHER, P. (1957). 'The role of the reticulo-endothelial system in hematoclasia.' *in* Halpern, B. N. (1957), p. 147.

PEACOCK, W. C., EVANS, R. D., IRVINE, J. W., JUNR., GOOD, W. M., KIP, A. F., WEISS, S. and GIBSON, J. G. 2nd. (1946). 'The use of two radioactive isotopes of iron in tracer studies of erythrocytes.' *J. clin. Invest.*, **25**, 605.

RATH, C. E. and FINCH, C. A. (1948). 'Sternal marrow hemosiderin.' *J. Lab. clin. Med.*, **33**, 81.

S. Afr. J. med. Sci. (1960) **25**, 141-147.

IRON KINETICS DURING ACUTE EPISODES OF SOUTH AFRICAN PORPHYRIA

by

T. H. BOTHWELL*, S. KRAMER, P. JACOBS and H. D. BARNES

Department of Medicine, University of Witwatersrand Medical School, Johannesburg, and South African Institute for Medical Research, Johannesburg.

[RECEIVED 20TH OCTOBER, 1960]

The type of porphyria found most commonly in white South Africans is an inherited metabolic error with variable clinical manifestations [Dean and Barnes, 1959]. In some subjects the disease remains latent; in others acute abdominal episodes occur often accompanied by neurological complications; while in a proportion cutaneous lesions due to photosensitivity are the striking feature. In addition, both abdominal episodes and skin lesions may occur in the same individual [Dean and Barnes, 1959]. Biochemically the disease is characterised by the presence of persistently raised levels of coproporphyrin and protoporphyrin in the stools and during acute attacks increased amounts of δ-amino-laevulic acid and porphobilinogen are found in the urine [Dean and Barnes, 1959]. The clinical features and biochemical findings in the urine during acute attacks are therefore similar to those found in acute intermittent porphyria [Waldenstrom, 1957]. However, the two conditions can be distinguished from each other since skin lesions are never seen in subjects with acute intermittent porphyria or in their relatives and urinary levels of δ-amino-laevulic acid and porphobilinogen are usually high during remissions. On the other hand South African porphyria appears to be the same condition described as mixed porphyria by Schmid *et al.* [1954], and as hereditary coproprotoporphyria by Waldenstrom [1957].

Although acute intermittent porphyria and the porphyria found in white South Africans appear to be distinct entities they are both thought to represent disturbances in the hepatic synthesis of porphyrins since it has been shown that increased amounts of porphyrins and/or their precursors are found in the liver in both conditions [Schmid *et al.*, 1954]. They have thus been classified as forms of hepatic porphyria as distinct from the rare erythropoietic variety in which the erythroid marrow shows increased amounts of porphyrins [Schmid *et al.*, 1954].

These various findings suggest that there is no interference with the synthesis of haemoglobin in hepatic porphyria and this is supported by the clinical observation that anaemia is not a feature in the South African cases [Murray, quoted by Barnes, 1951]. However, it remains possible that such a defect might exist during acute episodes but be of a sufficiently transitory nature to be undetectable by conventional techniques. Alternatively, it is possible that the formation of other iron porphyrin complexes might be impaired. A study was therefore undertaken to find out whether acute episodes of hepatic porphyria are ever associated with any detectable alterations in iron metabolism.

MATERIAL AND METHODS

Clinical Material. Twelve adult white South Africans were studied during acute abdominal episodes of porphyria. Six had a previous history of skin photosensitivity. The diagnosis in each was established by examining stools for copro- and proto-

*In receipt of a grant from the Council for Scientific and Industrial Research, Pretoria.

porphyrin by the method of Holti *et al.* [1958] and by examining the urine for δ-amino-laevulic acid and porphobilinogen by the method Mauzerall and Granick [1956].

As there was often a delay before the correct diagnosis was made the subjects were usually investigated at a time when the attack had already been present for some days. Six of the patients recovered and 4 died within 10 days of the commencement of radioiron studies.

Iron Methods. Plasma iron levels were estimated by a modification of the method of Bothwell and Mallett [1955] in which sulphonated bathophenanthroline [Trinder, 1956] was used as the colour reagent. Plasma iron turnovers were carried out as previously described [Bothwell *et al.*, 1957] using Fe59 in doses of approximately 5μc (specific activity 2-10μc/μg). The initial line of clearance of iron from the plasma was also used for the estimation of the plasma volume and the total blood volume was then calculated from this figure and from the packed cell volume corrected for trapped plasma [Chaplin and Mollison, 1952] and for the body/venous haematocrit ratio [Chaplin *et al.*, 1953]. In some subjects blood sampling was continued for a number of days after the injection of radioiron and the % utilisation of radioiron for red cell production was calculated from the following formula:

$$\% \text{ Red cell utilisation} = \frac{\text{Radioactivity in 1 ml. blood} \times \text{Total blood volume}}{\text{Total administered radioactivity}} \times 100$$

RESULTS

Porphyrin analyses. The stool and urine analyses at the time of study in each patient are shown in Table I.

TABLE I
Stool and urine analyses for porphyrin.

Case No.	Urine		Faeces	
	δ-Amino laevulic acid (mg./l.)	Porphobilinogen (mg./l.)	Coproporphyrin (μg./g. dry wt.)	Protoporphyrin (μg./g. dry wt.)
2	247	126	—	—
3	288	123	—	—
4	111	146	178	105
5	127	29	—	—
6	41	10	625	793
7	138	81	625	805
8	31	30	105	590
9	40	41	667	750
	44	36	—	—
10	—	Moderate excess	428	307
11	80	63	—	—
Normal	4	1	0—15	0—45

In cases 1 and 12 quantitative analyses were not carried out but screening tests on the urine showed large amounts of porphyrin and porphobilinogen.

Plasma iron levels. The plasma iron level was estimated in each of the 12 patients when they were initially seen and in 9 of these a number of further estimations were done at various times during their stay in hospital. In 7 subjects the level was initially above the upper limit of normal (150 μg./100 ml.) and in 2 others it rose subsequently

(Fig. 1). In the last 3 patients the plasma iron level was normal when first seen and no further tests were done. Two of these subjects died from respiratory failure shortly afterwards.

Correlation of plasma iron levels with liver "function" tests. Since liver cell necrosis is associated with raised plasma iron levels [Reissman and Dietrich, 1956] a comparison

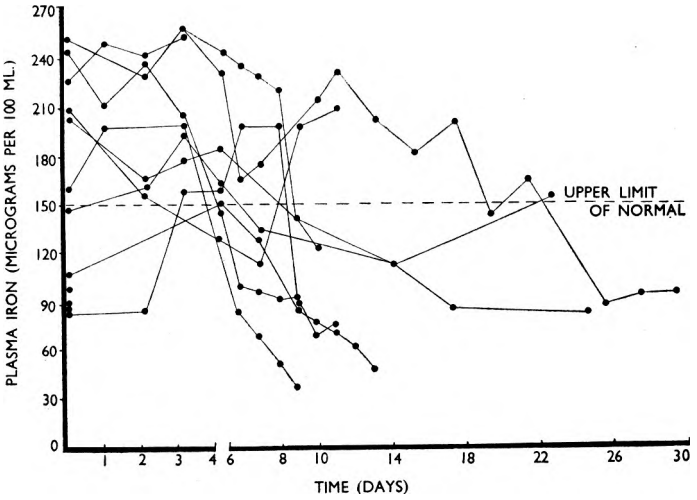


Fig. 1. Changes in plasma iron levels during acute episodes of porphyria.

TABLE II
Correlation between liver "function" tests and plasma iron levels.

Case No.	Plasma Iron (μg. %)	Transaminases		Thymol turb-idity (units)	Thymol flocc-ulation	Alk. P-ase (KA units)	ChE (%)	Total bili-rubin (mg. %)	Serum proteins	
		GOT (units)	GPT (units)						Alb. (g. %)	Glob. (g. %)
1	112	158	230	4·5	+++	5·9	100	1·0	3·0	3·9
2	255	230	—	1·0	Neg.	5·2	100	0·6	4·0	3·9
3	162	133	—	1·0	Neg.	9·8	100	1·6	4·4	3·4
	68	65	—	1·0	Neg.	7·9	64	0·6	2·5	2·3
4	150	218	—	5·5	+++	8·8	69	0·6	2·8	4·0
10	212	150	210	1·0	Neg.	9·1	97	1·2	4·3	3·4
	72	110	210	—	—	—	—	—	—	—
11	207	80	65	2·5	Neg.	8·8	77	0·6	2·9	2·5
12	152	115	180	—	—	—	—	—	—	—
Normal values	70-150	35-100	10-95	0-2	Neg.	4-13	80-120	0-1·2	4·0-5·7	1·3-3·0

Normal values. These are the normal ranges by the method used.
GOT, GPT — Glutamic-oxaloacetic and glutamic-pyruvic transaminases respectively.
Alk. P-ase — Alkaline phosphatase in King-Armstrong units.
ChE — Cholinesterase. The 100% value is 0·728 △ pH/hr.

was made between the plasma iron level and the results of a series of liver "function" tests in 7 subjects (Table II). It can be seen that there was, in general, no evidence of gross liver dysfunction and no obvious correlation between these changes and the level of plasma iron.

Plasma iron turnover. Plasma iron turnovers were carried out on all 12 subjects (Table III). Although there was a wide scatter the mean plasma iron level was higher than normal (160 μg./100 ml., S.D. ± 66) and the mean time taken for radioiron to reach half concentration was longer than normal (140 minutes, S.D. ± 81). The mean plasma iron turnover/100 ml. whole blood/day was, however, normal (0·69 mg., S.D. ± 0·17). Thus, there was no significant increase in the amount of iron passing through the plasma in spite of the raised plasma iron levels.

The plasma iron turnover was repeated in two subjects with initially high plasma iron values at a time when they were in clinical remission and the plasma irons were normal. In both instances there was some increase but the changes were not striking (Table III).

TABLE III
Plasma iron turnover and red cell utilisation data.

Case No.	Age	Sex	PCV (cc.)	Reticulocytes (%)	Plasma iron (μg. %)	$T\frac{1}{2}$ (min.)	Turnover	Utilisation
1	20	F	36	1·6	108	100	0·69	—
2	30	M	57	0·1	255	296	0·37	49
3	46	F	36	1·6	87	68	0·81	44
4	27	F	49	0·4	150	100	0·76	49
5	22	F	50	0·1	89	48	0·93	62
6	56	F	45	—	88	90	0·53	40
7	35	F	49	—	228	128	0·91	27
8	29	M	48	—	155	117	0·69	—
9	28	M	43	—	248	285	0·50	—
			43	—	110	72	0·86	—
10	23	M	42	0·5	210	200	0·61	—
			42	0·2	73	60	0·70	—
11	40	M	32	1·4	207	161	0·87	38
12	41	F	38	1·4	96	91	0·65	—
Average normal values (and range)			45 (42—50)	1·2 (0·2—2·0)	110 (70—150)	90 (60—120)	0·6 (0·4—0·8)	80 (70—90)

$T\frac{1}{2}$ — Time in minutes for radioiron to reach half concentration.
Turnover — Plasma iron turnover (mg./day/100 ml. whole blood).
Utilisation — Per cent red cell utilisation on 10th day, except in case 5 which gives the utilisation at 4 days, the patient dying shortly afterwards.

Utilisation of radioiron for red cell production. The % red cell utilisation was measured in 7 subjects (Fig. 2). In 6 it was reduced with a mean value at 10 days of 41% (S.D. ± 8%). The remaining patient, who appeared clinically to be going into remission, had a red cell utilisation of 62% by the 4th day. However, on the 6th day she suddenly deteriorated and died in respiratory failure.

The diminished red cell utilisation noted in these subjects suggested a depression in erythropoetic activity. Ancillary evidence in favour of this was obtained in the only subject who was followed in detail during an attack which lasted several weeks. This patient, who had a persistently raised plasma iron level and a low reticulocyte count, showed a drop in haemoglobin level of about 4 g./100 ml. over a period of 3 weeks

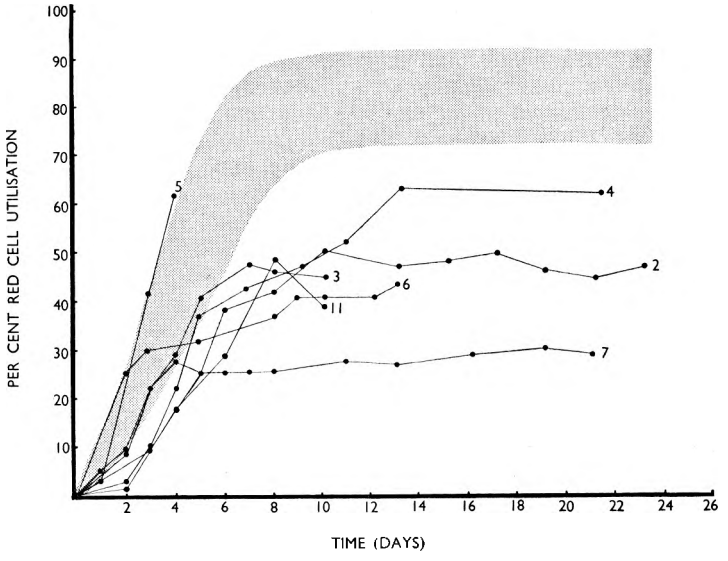


Fig. 2. The red cell utilisation of radioiron in 7 subjects. (The shaded area represents the range of normal utilisation.)

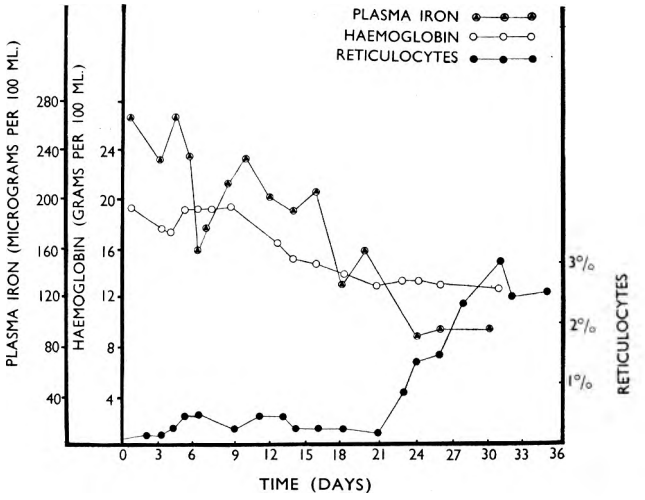


Fig. 3. Serial changes in the levels of hemoglobin, plasma iron and reticulocytes during an acute attack of porphyria (Case 2).

(Fig. 3). Recovery was associated with a fall in the plasma iron level and a rise in the reticulocyte count.

DISCUSSION

The most striking finding in the present study was the common occurrence of raised plasma iron levels during acute abdominal episodes of porphyria. There appeared to

be a definite correlation between the course of the episode and the level of plasma iron. When symptoms were marked the plasma iron was usually high and with recovery it returned to normal levels. Although the reasons for these changes may be complex some insight into the various possibilities can be obtained by considering the factors which are known to influence the plasma iron level.

Most of the iron entering the plasma is derived from the breakdown of red cells in the reticulo-endothelial system and the major portion leaving the plasma is taken up by the bone marrow for red cell production. In addition stores, tissues and gut also contribute to the iron passing through the plasma but the amounts are usually small. A rise in plasma iron level is therefore almost always the result of an imbalance between the rate at which iron is being fed into the plasma from reticulo-endothelial cells and the rate at which it is removed by the developing red cell mass. There are, however, certain situations in which other factors may be of importance. In states of iron overload transfer of iron between stores increases and the plasma iron rises [Finch and Finch, 1955], while with hepatocellular necrosis iron is released into the plasma from damaged liver cells [Reissman and Dietrich, 1956]. With these exceptions changes in plasma iron levels indicate some alteration in the function of the reticulo-endothelial system or the erythroid marrow. Raised levels can therefore occur (a) when the rate of red cell destruction is increased acutely over a short period, (b) when there is decreased blood formation, and (c) when there is a defect in the incorporation of iron into haem [Bothwell *et al.*, 1957].

Since hepato-cellular necrosis can give rise to a raised plasma iron level, liver "function" tests were carried out in 7 subjects. Although the majority showed certain alterations these did not correlate well with the plasma iron levels. Since the concentrations of glutamic-oxaloacetic and glutamic-pyruvic transaminases are sensitive indicators of hepatocellular necrosis special attention was paid to these measurements. The highest levels obtained were only about twice normal which is several times lower than the values reported in infective hepatitis [Wróblewski, 1959]. When these results are taken in conjunction with previous observations of only minimal histological changes in the majority of subjects dying during acute attacks [Goldberg, 1959] it would appear unlikely that the high plasma iron levels are the result of liver cell necrosis.

Two other possible reasons for the raised plasma iron levels were considered. It was thought that they might either be due to a general depression in erythropoietic activity or to some interference with the incorporation of iron into haem. If either of these changes were present they would be reflected in the amount of iron passing through the plasma and in the percentage used for red cell production. Plasma iron turnovers and the percentage red cell utilisation of injected radioiron were therefore measured since the first is a moderately accurate measure of total erythropoietic activity and the second an index of effective red cell production [Giblett *et al.*, 1956]. Although the results for plasma iron turnover were within the normal range these findings are not incompatible with marrow hypofunction since the measurement has been found to be relatively insensitive when erythropoietic activity is *less* than normal [Bothwell *et al.*, 1957]. More noteworthy was the fact that the turnover was not increased in 4 subjects with haematocrits below 40%. Such a finding is strongly suggestive of a relative hypofunction of the erythroid marrow since the plasma iron turnover is normally increased 2 to 3 times the normal in the presence of anaemia [Bothwell *et al.*, 1957]. Added evidence to support the presence of marrow hypofunction was the diminished utilisation of the radioiron for red cell production in 6 of 7 subjects.

The question remains as to whether these various findings are indicative of a defect in the incorporation of iron into haemoglobin or whether they only reflect some less specific depression in overall erythropoietic activity. Although it might be argued that the fundamental disturbance in pyrrole pigment metabolism leads to a lack of available protoporphyrin for combination with iron there are several reasons why this is unlikely. Firstly, it has not been possible to demonstrate any abnormal porphyrins in the marrows of subjects recovering from acute attacks [Schmid *et al.*, 1954]. In the second place, defects in haem synthesis do not affect the actual uptake of iron by red cell precursors and the abnormal metabolic pathways of iron actually lead to a plasma iron turnover greater than normal [Bothwell *et al.*, 1957; Crosby, 1959]. Finally, the observation that the reticulocyte count was reduced in a subject during an acute attack and rose with recovery is more in keeping with a temporary depression in overall erythropoietic activity. On balance, therefore, the pattern of iron kinetics demonstrated in the present study is most likely the result of a hypofunction of the erythroid marrow. Whether this is a direct result of the disturbance in porphyrin synthesis or only a non-specific manifestation of the widespread metabolic upset present in the acute attack is, however, not clear.

SUMMARY

Iron kinetics were studied in 12 adult white subjects during acute abdominal episodes of hepatic porphyria. The plasma iron level was usually raised, the plasma iron turnover was within normal limits and the percentage utilisation of injected radioiron for red cell production was reduced. Although the interpretation of these findings is difficult they suggest that there may be temporary depression in erythropoietic activity during acute attacks.

REFERENCES

BARNES, H. D. (1951). Further South African cases of porphyrinuria. *S. Afr. J. Clin. Sci.*, **2**, 117-169.

BOTHWELL, T. H. and MALLETT, B. (1955). The determination of iron in plasma or serum. *Biochem. J.*, **59**, 599-602.

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**, 409-427.

CHAPLIN, H., Jr. and MOLLISON, P. L. (1952). Correction for trapped plasma in the red cell column of the hematocrit. *Blood*, **7**, 1227-1238.

CHAPLIN, H., Jr., MOLLISON, P. L. and VETTER, H. (1953). The body/venous hematocrit ratio; its constancy over a wide hematocrit range. *J. Clin. Invest.*, **32**, 1309-1316.

CROSBY, W. (1959). Evidence of recycling of siderocyte iron. *J. Clin. Invest.*, **38**, 997.

DEAN, G. and BARNES, H. D. (1959). Porphyria in Sweden and South Africa. *S. Afr. Med. J.*, **33**, 246-253.

FINCH, S. C. and FINCH, C. A. (1955). Idiopathic hemochromatosis, an iron storage disease. A. Iron metabolism in hemochromatosis. *Medicine*, **34**, 381-430.

GIBLETT, E. R., COLEMAN, D. H., PIRZIO-BIROLI, G., DONOHUE, D. M., MOTULSKY, A. G. and FINCH, C. A. (1956). Erythrokinetics. Quantitative measurements of red cell production and destruction in normal subjects and patients with anemia. *Blood*, **11**, 291-309.

GOLDBERG, A. (1959). Acute intermittent porphyria. *Quart. J. Med.*, **28**, 183-209.

HOLTI, G., RIMINGTON, C., TATE, B. C. and THOMAS, G. (1958). An investigation of porphyria cutanea tarda. *Quart. J. Med.*, **27**, 1-18.

MAUZERALL, D. and GRANICK, S. (1956). Occurrence and determination of amino-levulinic acid and porphobilinogen in urine. *J. Biol. Chem.*, **219**, 435-446.

REISSMAN, K. R. and DIETRICH, M. R. (1956). On the presence of ferritin in the peripheral blood of patients with hepatocellular disease. *J. Clin. Invest.*, **35**, 588-595.

SCHMID, R., SCHWARTZ, S. and WATSON, C. J. (1954). Porphyrin content of bone marrow and liver in various forms of porphyria. *Arch. Int. Med.*, **93**, 167-190.

TRINDER, P. (1956). The improved determination of serum iron. *J. Clin. Path.*, **9**, 170-172.

WALDENSTRÖM, J. (1957). The porphyrias as inborn errors of metabolism. *Amer. J. Med.*, **22**, 758-773.

WROBLEWSKI, F. (1959). The clinical significance of transaminase activities of serum. *Amer. J. Med.*, **27**, 911-923.

Journal of Clinical Investigation
Vol. 42, No. 7, 1963

THE QUANTITATIVE ESTIMATION OF TOTAL IRON STORES IN HUMAN BONE MARROW

By EDWARD GALE, JOHN TORRANCE, AND THOMAS BOTHWELL *

(From the Department of Medicine, University of Witwatersrand Medical School, Johannesburg, South Africa)

(Submitted for publication December 3, 1962; accepted March 7, 1963)

In normal adult males, about 25% of the body iron content is in storage depots (1). This iron exists in two forms: as a diffuse soluble fraction called ferritin, in which the molecules are dispersed, and as insoluble aggregates of hemosiderin, which can be visualized by conventional microscopy (2). Although the liver is regarded as the chief storage organ, chemical analyses suggest that it normally contains up to 300 mg (3–5), which is only between one-quarter and one-third of what can be mobilized from total stores when healthy young males are repeatedly phlebotomized (6). Although little is known of the quantities present in other organs, hemosiderin can be seen in the reticuloendothelial cells of the bone marrow (7) and spleen (8), and there is some chemical evidence to indicate that significant amounts of storage iron may be present in skeletal muscles (9, 10).

The present study was undertaken to find out how much iron is normally stored in the reticuloendothelial cells of the bone marrow and to define the extent to which these stores can expand when iron overload is present. In addition, a comparison was made between the concentrations of iron present in the bone marrows and livers of subjects with varying quantities of storage iron.

MATERIALS AND METHODS

Clinical material. The total iron stores present in bone marrow were estimated in 61 adult subjects undergoing thoracotomy. Forty-one were white and the remainder were Bantu. The individual diagnoses are shown in Table I.

The chemical concentrations of storage iron were estimated in the livers and bone marrows of adult Bantu and white subjects dying in the hospital. The Bantu specimens (58 males and 44 females) were obtained from Baragwanath Hospital, Johannesburg, and the white specimens (25 males and 28 females) from the General Hospital, Johannesburg.

The use of radioiron as a marrow label. When a tracer quantity of radioiron is injected intravenously, most of it is taken up by the red-cell precursors of the bone marrow (11). This iron is subsequently released over the next few days as part of the hemoglobin of new red cells, and the percentage present in circulation at 10 to 14 days has been used as a measure of the fraction initially taken up by the bone marrow (12). If a specimen of bone marrow is therefore removed between 18 and 24 hours after the injection of the radioiron (i.e., at a time when marrow activity is maximal, and circulating activity is negligible), it is possible to use the Fe^{59} as a label for relating values in the sample to the total marrow. This principle, which has previously been employed for estimating the total number of cell precursors (13–15), was used in the present study as a means of calculating the total amount of storage iron present in the bone marrow. To do this, we must assume that the reticulum cells containing storage iron are fairly evenly dispersed throughout the erythroid marrow.

Isotopic techniques. Subjects undergoing thoracotomy were given between 5 and 10 μc high specific activity Fe^{59} citrate intravenously between 18 and 24 hours before surgery. A weighed sample of this solution was set aside as a standard. Blood samples were collected over the next few hours, and plasma volumes were then calculated from the line of clearance of radioiron as described previously (16). The total blood volume was obtained from the plasma volume and packed cell volume corrected for the body: venous hematocrit ratio at an altitude of 5,900 feet (17). With this approach, the mean figure for blood volume in the group was 68.2 ml per kg (SD, 10.7).

The rib removed at operation was squeezed dry, and the marrow so obtained was weighed and counted for radioactivity in a well-type counter. The amount of storage iron present was then measured chemically (see Chemical methods).

Blood samples were collected from all the subjects 10 to 14 days after the injection of radioiron. The total percentage of injected radioactivity in circulation was then calculated by using the estimation of blood volume obtained from radioiron data on the first day of the study. The mean figure for the group was 83% (SD, 10.7). On

* This work was supported by grant AM04912–02 from the National Institutes of Health and by a grant from the Council for Scientific and Industrial Research, South Africa.

the assumption that the radioiron present in circulation at this time was in the marrow 18 to 24 hours after the original injection, it was then possible to obtain a measure of the total iron stores present in bone marrow. Marrow iron stores = (fraction of injected Fe^{59} in circulation at 10 to 14 days × counts injected/counts in sample) × nonheme iron in marrow sample.

Chemical methods. The quantity of nonheme iron present in specimens of marrow was estimated by a modification of the method of Brückmann and Zondek (18). Four ml saturated sodium pyrophosphate was added to the marrow and sufficient 20% trichloroacetic acid to give a final volume of 10 ml. (In this calculation the specific gravity of the marrow was assumed to be 1.0). After thorough mixing, the tube was heated in a water bath and was maintained at a temperature of 80° C for 10 minutes. During this time the solution was stirred frequently. After cooling, the mixture was filtered and an iron estimation was carried out on a sample of the filtrate by use of the thioglycolic acid method (19).

The validity of the whole procedure as a means of separating heme from nonheme iron was established in preliminary studies with solutions of hemoglobin and ferritin and suspensions of hemosiderin granules. Ferritin was prepared by the method of Mazur and Shorr (20), and hemosiderin by the method of Shoden and Sturgeon (21). Only 5% (range, 3 to 8%) of heme iron was present in the filtrate. Calculations based on the quantities of hemoglobin present in marrow samples indicated that the error that could be introduced by hemoglobin iron was not more than 10 μg per g marrow. The mean recovery of ferritin iron in concentrations varying between 100 and 2,000 μg per ml was 104%

TABLE I

Clinical diagnoses in 61 patients subjected to thoracotomy

Diagnosis	White		Bantu	
	M	F	M	F
Mitral stenosis	7	9	0	0
Intrathoracic neoplasms	8	0	1	0
Pulmonary tuberculosis	3	0	8	3
Pulmonary sepsis	0	4	6	2
Hiatus hernia	6	4	0	0
Total	24	17	15	5

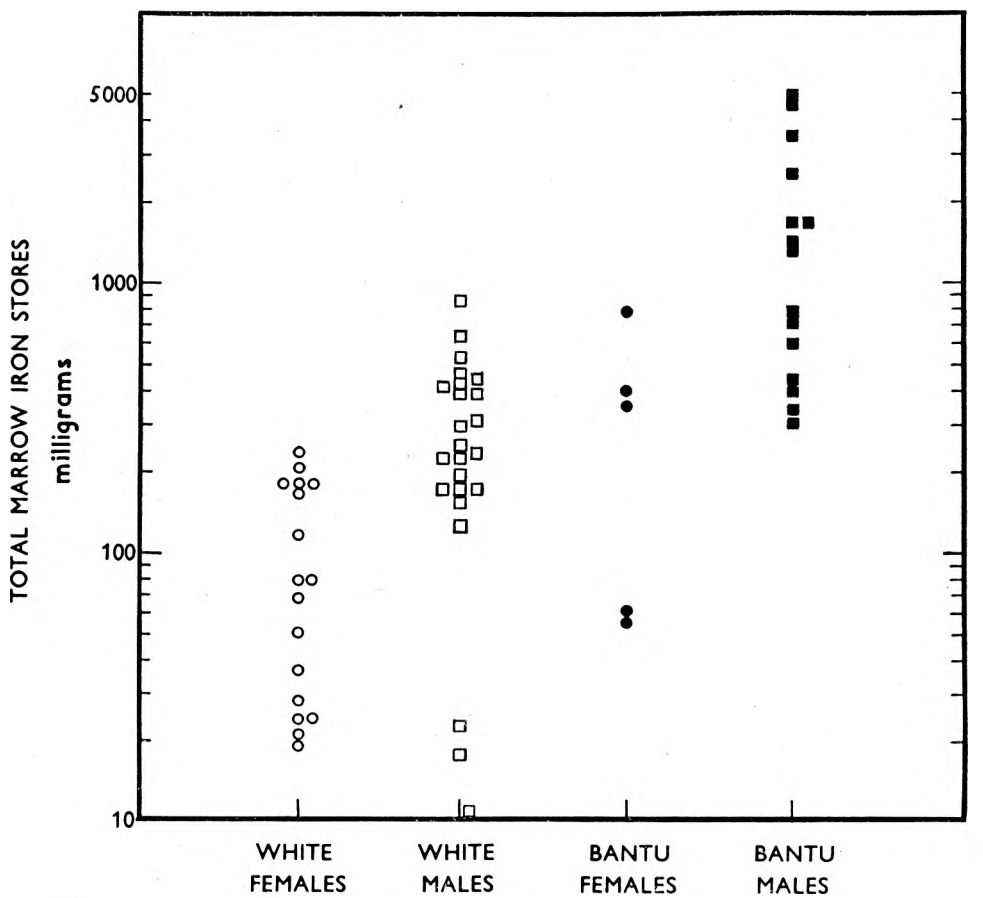


FIG. 1. CALCULATED VALUES FOR TOTAL MARROW IRON STORES IN 61 HUMAN SUBJECTS.

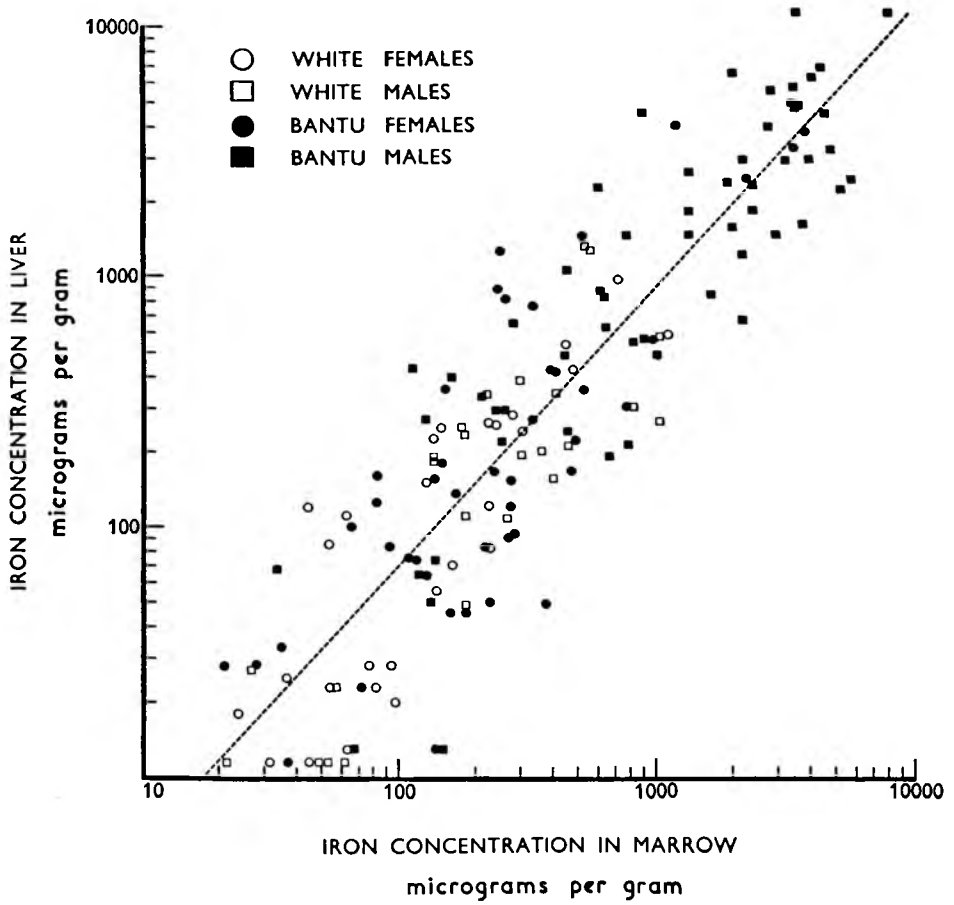


FIG. 2. CORRELATION BETWEEN STORAGE IRON CONCENTRATIONS IN THE MARROWS AND LIVERS OF 155 NECROPSY SUBJECTS. The regression line is indicated ($r = +0.88$, $p < .001$).

(range 100 to 106%), whereas that of hemosiderin iron in concentrations varying between 70 and 10,000 μg per ml was 103% (range 97 to 111%).

In necropsy studies, specimens of rib and samples of liver were collected. Marrow was obtained from the ribs by squeezing them dry, and the concentrations of nonheme iron were estimated with the method described in the previous paragraphs. As the validity of results obtained for the concentrations of storage iron in marrow depended on there being a moderately constant relationship between storage iron and the weight of marrow samples, some preliminary studies were done in which the concentrations of storage iron were estimated in samples of marrow obtained from different sites. Specimens were taken from two sites in subjects with concentrations of marrow storage iron varying between 15 to 4,476 μg per g. The average difference from the mean of each pair of observations was 5.9% (range 0 to 23%) in 16 cases. It was felt that this degree of agreement was sufficiently close for meaningful information to be obtained from single samples.

The concentration of total iron present in each formalin-treated sample of liver was estimated as described previously (8). In addition, the concentration of heme iron was estimated on a separate sample of liver, and the concentration of storage iron was then obtained by subtracting this result from the figure for total iron. This proved somewhat difficult as there are no simple, described techniques for the quantitative estimation of hemoglobin iron in formalin-treated tissues. The method eventually devised was as follows. Approximately 1 g formalin-treated liver was blotted and weighed. The sample was then cut into slices and placed in a 50-ml volumetric flask. One-half ml of the detergent sodium alkyl sulfate [1] was added, and the flask was made up to volume with 1 N sodium hydroxide. The addition of the detergent ensured a clear final filtrate. The flask was placed in an oven at 56° C for 18 hours. After cooling, the solution was filtered, and the optical density of the alkali-heme was determined by using an Evelyn colorimeter with a 540 filter. The concentration of hemoglobin iron present was then read from a calibration curve constructed with hemoglobin solutions of known iron con-

[1] Marketed as Teepol by the Shell Chemical Co., London, England.

tent which had been treated in the same way. With this technique, reproducible calibration curves were obtained on solutions of hemoglobin that had been stored in formalin for as long as 3 months.

In an attempt to establish the specificity of the method, estimations of hemoglobin iron were carried out on deeply jaundiced livers obtained from rats in which the bile duct had been tied several days previously. The results were no higher than in normal rats. In addition, the fact that concentrations of heme iron were in the same range as those obtained by other methods on fresh liver specimens (9, 22) further supports the validity of the present technique.

Histological methods. In the majority of studies, samples of marrow specimens were squashed onto slides that were then stained for iron by Dry's method (23). The arbitrary histological gradings of storage iron in marrow particles were as follows: grade 0—no visible iron under oil immersion (magnification × 720); grade 1+—small iron particles just visible in reticulum cells under oil immersion (magnification × 720); grade 2+—small, sparsely distributed iron particles usually visible under low power (magnification × 80); grade 3+—numerous small particles present in reticulum cells throughout the marrow particles; grade 4+—larger particles throughout the marow with tendency to aggregate into clumps; grade 5+—dense, large clumps of iron throughout the marrow; and grade 6+—very large deposits of iron, both intra- and extracellular, which obscure cellular detail in the marrow particles.

RESULTS

Total iron stores in the marrow (Figure 1). The mean value in 17 white females was 99 mg (range 19 to 237 mg), and in 24 white males, 288 mg (range 10 to 833 mg). Only 3 of the males had values below 100 mg, and 2 of these subjects gave a history of previous gastrointestinal hemorrhage. The 3 males with figures above 500 mg were all suffering from bronchial carcinoma.

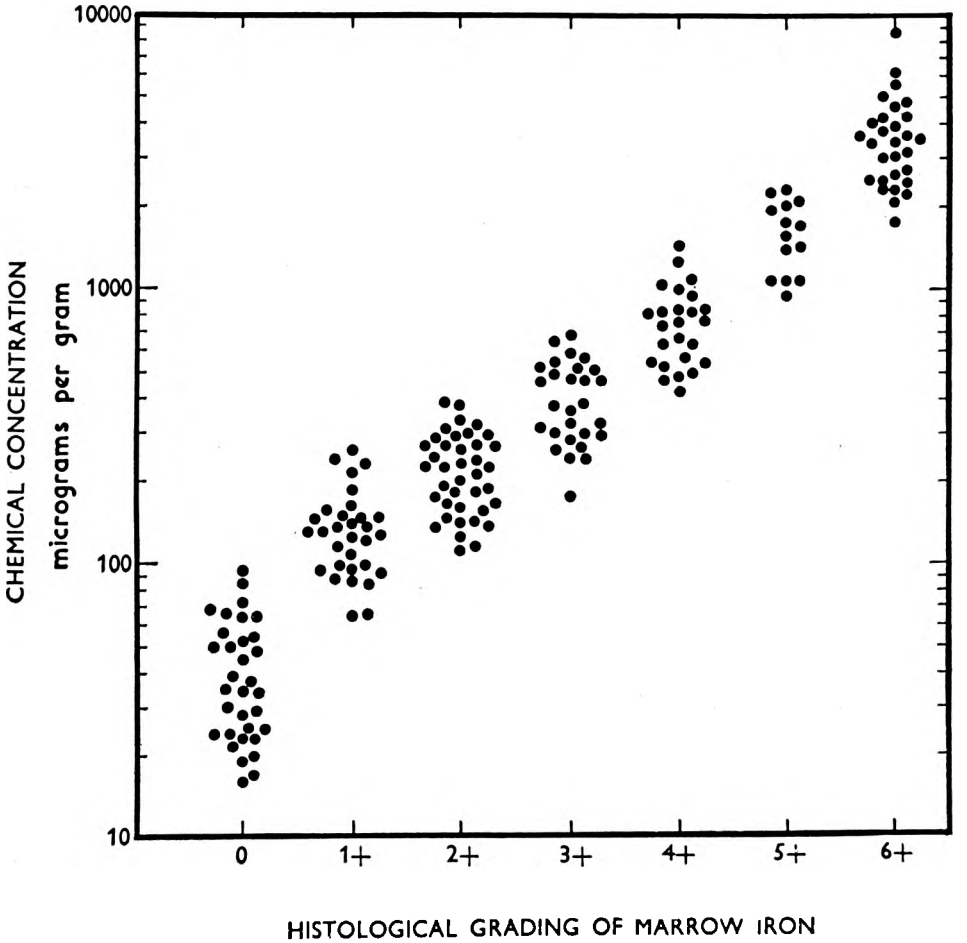


FIG. 3. RANGE OF CHEMICAL CONCENTRATIONS OF STORAGE IRON IN THE MARROWS OF 199 SUBJECTS IN WHOM THE EXTENT OF RETICULOENDOTHELIAL STORES WAS ASSESSED HISTOLOGICALLY.

Only 5 Bantu females were studied. Results 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).

Correlation between concentrations of storage iron in marrow and liver (Figure 2). The results in 155 subjects show that the concentrations of storage iron present in the two organs were approximately the same over a very wide range.

Correlation between chemical and histological assessment of storage iron in the marrow. The results in 199 subjects are shown in Figure 3. Although there was some degree of overlap, moderately good agreement was obtained between the histological gradings and the chemical concentrations of storage iron in the marrow. The mean figures for each grade were as follows: 0 = 43 μg per g (SD, 23); 1+ = 130 μg per g (SD, 50); 2+ = 223 μg per g (SD, 75); 3+ = 406 μg per g (SD, 131); 4+ = 762 μg per g (SD, 247); 5+ = 1,618 μg per g (SD, 464); and 6+ = 3,681 μg per g (SD, 1,400).

DISCUSSION

Estimates of the amounts of iron present in stores have been made in different ways. When normal subjects are repeatedly phlebotomized, the red-cell deficit is corrected by mobilizing iron from stores. Calculations from such data indicate that the total stores in healthy young males vary between 1,000 and 1,500 mg (6). Although data derived from an isotopic dilution technique have given a mean figure of only 600 mg in man (24), these discrepancies are not surprising, since the response to repeated phlebotomies is a measure of the total mobilizable stores, whereas the isotopic technique is only a measure of miscible stores.

The major sites of storage iron in the body have been demonstrated by using both histological and chemical methods. The liver normally contains up to 300 mg, and significant quantities are present in the reticuloendothelial cells of the bone marrow (7, 25) and spleen (3, 8). In addition, there is some chemical evidence to suggest that significant amounts of storage iron may be present in skeletal muscles (9, 10). There is, however, little quantitative information on the total amounts of iron present in organs other than the liver and spleen.

In the present study, an attempt was made to estimate the total amount of storage iron in the bone marrow. This was done by adapting an isotopic dilution technique which has been previously used for gauging the number of cell precursors in bone marrow (13–15). The data obtained with this technique indicate a mean figure for marrow storage iron of 288 mg in white males and 99 mg in white females. Some of the individual values must certainly have been modified by the diseases from which the subjects were suffering. For example, the highest figures in males were obtained in subjects with cancer, while the lowest results were in subjects with hiatus hernia and a history of previous gastrointestinal hemorrhage. In spite of these qualifications, it is felt that the results obtained are probably a fair reflection of the findings in the general population, as the mean values in the 7 males and 9 females suffering from uncomplicated mitral stenosis, with no signs of rheumatic activity, were very similar to those in the whole group, i.e., 256 mg and 70 mg, respectively. All these subjects had hemoglobin levels above 13 g per 100 ml, and only two, both of whom were females, had ever donated blood (2 and 5 pints, respectively). The well-marked sex difference, which is probably a reflection of the increased losses of iron via menstruation and pregnancies in females, has also been noted previously in histological assessments of bone-marrow iron (26) and in chemical estimations of liver iron concentrations (9, 27).

Figures for storage iron in Bantu subjects were a good deal higher than in white subjects. The mean figure of 1,629 mg in Bantu males was approximately 6 times the value in the white subjects, and the highest figure obtained was 17 times greater. Although the majority of these subjects were suffering from sepsis, the relative hemosiderosis that is so often a feature in chronic infection would not be expected to raise reticuloendothelial stores to more than about twice normal (28), and the very high values obtained therefore represent an absolute increase in the body iron content. This fits in with several previous studies that have demonstrated a high incidence of varying degrees of iron overload in the adult Bantu population of Southern Africa (8, 22,

29–31). The excess iron in these subjects is largely derived from the containers used for the preparation of alcoholic beverages (32). In most individuals, the iron is almost exclusively stored in the liver and in the reticuloendothelial system of the body (8, 29).

In an extension of the initial investigation, specimens of bone marrow and liver were obtained at necropsy from white and Bantu subjects in order to compare the concentrations of storage iron present in the two organs. It was of considerable interest to find that the concentrations of iron were very similar in subjects with depleted stores, normal stores, and all degrees of iron overload. Although it is not possible to translate the necropsy data into accurate figures for the absolute amounts of storage iron present in bone marrow and liver, some idea of the relative capacities of the two organs can be obtained from the isotopic data. Calculations from these results gave a mean figure for the total marrow weight of about 1,200 g, which is in the same range as the normal liver weight. This suggests that the liver and marrow have a capacity to store iron that is of the same order. Although the relationship probably holds true when iron stores are normal or moderately increased, there is usually significant hepatomegaly when gross degrees of iron overload are present (3, 5), and the liver's capacity is therefore increased. In quantitative terms, the highest marrow iron concentration obtained in the autopsy study was 8,647 μg per g. If it is presumed that the total marrow weight in this subject was 1,200 g, then the total marrow iron stores must have been approximately 10 g. This is about half the amount usually present in the liver in idiopathic hemochromatosis (3, 5).

One final point is worthy of comment. In the past, the histological assessment of hemosiderin in the reticulum cells of marrow particles has been widely used as possibly the best method for assessing the status of iron stores in an individual (7, 26, 33, 34). In the present investigation, a comparison was made between histological gradings and chemical estimations of storage iron in the marrow. The good agreement obtained over a wide range of iron concentrations further confirms the validity of the histological assessment of marrow particles as a useful means of gauging the size of reticuloendothelial stores.

SUMMARY

Total bone-marrow iron stores were estimated in 61 human subjects by use of an isotopic dilution technique. 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.

Chemical analyses of iron concentrations in the marrows and livers of 199 white and Bantu necropsy subjects revealed a close correlation over a wide range of iron concentrations.

There was moderately good agreement between histological assessments of bone-marrow iron stores and chemical estimations of iron concentrations.

ACKNOWLEDGMENTS

The authors are indebted to Mr. L. Fatti, Mr. P. Marchand, Mr. D. Fuller, Mr. E. Joubert, Mr. G. Katz, and Mr. I. Opeskin for access to patients under their care and to Professor B. J. P. Becker and Dr. C. Isaacson, who kindly supplied the necropsy material. Thanks are also due to Mr. A. D. Joffe for help with the statistical analyses.

REFERENCES

1. Granick, S. Iron metabolism in animals and plants *in* Trace Elements. New York, Academic Press, 1958, p. 367.
2. Granick, S. Ferritin: its properties and significance for iron metabolism. Chem. Rev. 1946, **38**, 379.
3. Sheldon, J. H. Haemochromatosis. London, Oxford University Press, 1935, pp. 205, 208.
4. Gross, H., M. Sandberg, and O. M. Holly. Changes in copper and iron retention in chronic diseases accompanied by secondary anemia. II. Changes in liver, spleen and stomach. Amer. J. med. Sci. 1942, **204**, 201.
5. Butt, E. M., R. E. Nusbaum, T. C. Gilmour, and S. L. Didio. Trace metal patterns in diseased states. I. Hemochromatosis and refractory anemia. Amer. J. clin. Path. 1956, **26**, 225.
6. Haskins, D., A. R. Stevens, Jr., S. Finch, and C. A. Finch. Iron metabolism. Iron stores in man as measured by phlebotomy. J. clin. Invest. 1952, **31**, 543.
7. Rath, C. E., and C. A. Finch. Sternal marrow hemosiderin. A method for the determination of available iron stores in man. J. Lab. clin. Med. 1948, **33**, 81.
8. Bothwell, T. H., and B. A. Bradlow. Siderosis in the Bantu. A combined histopathological and chemical study. Arch. Path. 1960, **70**, 279.
9. Roth, O., B. Jasiński, and H. v. Bidder. Das Gewebeeisen beim Menschen bei normalen und patho-

logischen Zuständen. Helv. med. Acta 1951, **18**, 159.

10. Mazur, A., and E. Shorr. A quantitative immunochemical study of ferritin and its relation to the hepatic vasodepressor material. J. biol. Chem. 1950, **182**, 607.
11. Huff, R. L., P. J. Elmlinger, J. F. Garcia, J. M. Oda, M. C. Cockrell, and J. H. Lawrence. Ferrokinetics in normal persons and in patients having various erythropoietic disorders. J. clin. Invest. 1951, **30**, 1512.
12. Huff, R. L., T. G. Hennessy, R. E. Austin, J. F. Garcia, B. M. Roberts, and J. H. Lawrence. Plasma and red cell iron turnover in normal subjects and in patients having various hematopoietic disorders. J. clin. Invest. 1950, **29**, 1041.
13. Suit, H. D. A technique for estimating the bone marrow cellularity *in vivo* using ^{59}Fe. J. clin. Path. 1957, **10**, 267.
14. Donohue, D. M., B. W. Gabrio, and C. A. Finch. Quantitative measurement of hemopoietic cells of the marrow. J. clin. Invest. 1958, **37**, 1564.
15. Donohue, D. M., R. H. Reiff, M. L. Hanson, Y. Betson, and C. A. Finch. Quantitative measurement of the erythrocytic and granulocytic cells of the marrow and blood. J. clin. Invest. 1958, **37**, 1571.
16. Bothwell, T. H., S. Callender, B. Mallett, and L. J. Witts. The study of erythropoiesis using tracer quantities of radioactive iron. Brit. J. Haemat. 1956, **2**, 1.
17. Metz, J., N. W. Levin, and D. Hart. Effect of altitude on the body/venous haematocrit ratio. Nature (Lond.) 1962, **194**, 483.
18. Brückmann, G., and S. G. Zondek. Iron, copper and manganese in human organs at various ages. Biochem. J. 1939, **33**, 1845.
19. McCance, R. A., E. M. Widdowson, and L. R. B. Shackleton. The nutritive value of fruits, vegetables and nuts *in* Medical Research Council Special Report. London, His Majesty's Stationery Office, 1936, p. 21.
20. Mazur, A., and E. Shorr. Hepatorenal factors in circulatory homeostasis. IX. The identification of the hepatic vasodepressor substance, VDM, with ferritin. J. biol. Chem. 1948, **176**, 771.
21. Shoden, A., and P. Sturgeon. Hemosiderin. I. A physicochemical study. Acta haemat. (Basel) 1960, **23**, 376.
22. Gillman, J., J. Mandelstam, and T. Gillman. 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. 1945, **10**, 109.
23. Dry, D. S. Improved methods for the demonstration of mitochondria, glycogen, fat and iron in animal cells. S. Afr. J. Sci. 1945, **41**, 298.
24. Finch, C. A. Body iron exchange in man. J. clin. Invest. 1959, **38**, 392.
25. Kerr, L. M. H. A method for the determination of non-haem iron in bone marrow. Biochem. J. 1957, **67**, 627.
26. Stevens, A. R., Jr., D. H. Coleman, and C. A. Finch. Iron metabolism: clinical evaluation of iron stores. Ann. intern. Med. 1953, **38**, 199.
27. Bothwell, T. H. Unpublished data.
28. Finch, S. C., and C. A. Finch. Idiopathic hemochromatosis, an iron storage disease. A. Iron metabolism in hemochromatosis. Medicine (Baltimore) 1955, **34**, 381.
29. Higginson, J., T. Gerritsen, and A. R. P. Walker. Siderosis in the Bantu of Southern Africa. Amer. J. Path. 1953, **29**, 779.
30. Wainwright, J. Siderosis in the African. S. Afr. J. Lab. clin. Med. 1957, **3**, 1.
31. Bothwell, T. H., and C. Isaacson. Siderosis in the Bantu. A comparison of incidence in males and females. Brit. med. J. 1962, **1**, 522.
32. Walker, A. R. P., and U. B. Arvidsson. Iron "overload" in the South African Bantu. Trans. roy Soc. trop. Med. Hyg. 1953, **47**, 536.
33. Davidson, W. M., and R. F. Jennison. The relationship between iron storage and anaemia. J. clin. Path. 1952, **5**, 281.
34. Beutler, E., W. Drennan, and M. Block. The bone marrow and liver in iron-deficiency anemia. A histopathologic study of sections with special reference to the stainable iron content. J. Lab. clin. Med. 1954, **43**, 427.

IRON METABOLISM IN SCURVY WITH SPECIAL REFERENCE TO ERYTHROPOIESIS

BY

T. H. BOTHWELL, B. A. BRADLOW, P. JACOBS, K. KEELEY, S. KRAMER, H. SEFTEL and S. ZAIL

Reprinted from British Journal of Haematology, Vol. 10, No. 1, January 1964

BLACKWELL SCIENTIFIC PUBLICATIONS
OXFORD

Brit. J. Haemat., 1964, 10, 50.

Iron Metabolism in Scurvy with Special Reference to Erythropoiesis*

T. H. Bothwell, B. A. Bradlow, P. Jacobs, K. Keeley, S. Kramer, H. Seftel and S. Zail

Departments of Medicine and Chemical Pathology, University of Witwatersrand Medical School,† and Baragwanath Hospital, Johannesburg

A number of observations suggest that ascorbic acid may be involved in several phases of iron transport. Iron is probably absorbed in the ferrous state (Moore, Dubach, Minnich and Roberts, 1944) and there is evidence that ascorbic acid facilitates the process (Moore, 1955). After passing through mucosal cells, iron combines with a specific protein, transferrin, which carries it to receptor tissues, such as the bone marrow and storage depots. The iron attached to transferrin is in the ferric state (Surgenor, Koechlin and Strong, 1949). The iron in haem is in the ferrous form and Goldberg (1959) has shown that ascorbic acid facilitates the formation of this complex. There is also evidence to indicate that ascorbic acid may be concerned in the combination of iron with apoferritin to form ferritin (Fineberg, Kasbekar and Loewus, 1959; Mazur, Carleton and Carlsen, 1961) and it has been shown that a number of reducing agents, including ascorbic acid, are able to release iron fom ferritin (Mazur, Baez and Shorr, 1955).

Since these various observations suggest that ascorbic acid may play an important role in iron transport, a series of studies was carried out in scorbutic subjects to delineate the possible relationships. Special interest centred on the absorption of iron, its transport through the plasma and its incorporation into haemoglobin. In addition, it was possible to make certain observations on the nature of the anaemia present in scorbutic patients.

MATERIAL AND METHODS

CLINICAL MATERIAL

Twenty-five adult Bantu patients admitted to Baragwanath Hospital suffering from scurvy were studied. The diagnosis in each was made according to the strict criteria established in this hospital by Grusin and Kincaid-Smith (1954). Further confirmation was provided by the dramatic cessation of bleeding and by the return of haemoglobin levels to normal on treatment with ascorbic acid alone.

METHODS

Routine haematological studies, including haemoglobin, packed cell volume (PCV) and reticulocyte count, were carried out on admission. Plasma iron was estimated in the majority by a modification of the method of Bothwell and Mallett (1955) and the unsaturated iron-binding capacity was also measured in some subjects by the method of Bothwell, Jacobs and Kamener (1959). Bone-marrow smears were examined in over half the patients.

Iron-absorption studies were carried out in several subjects after the oral administration of a dose of 5 mg. of ferrous sulphate labelled with 15 μc. of ^{59}Fe chloride. The percentage ab-

* This work was supported in part by a grant (AM-04912-02) from the National Institutes of Health, U.S.A., and in part by a grant from the Council for Scientific and Industrial Research, South Africa.

† Postal address: Hospital Street, Johannesburg, S. Africa.

sorption was calculated from the red-cell radioactivity 8 days after administration of ^{59}Fe. (Preliminary studies indicated that between 80 and 100 per cent of the ^{59}Fe introduced into the plasma was present in red cells at this stage in recently treated subjects.)

The absorption of ^{59}Fe was also measured in scorbutic guinea-pigs. Scurvy was produced by a diet of bran (45 per cent), oats (30 per cent) and dried skimmed milk; the milk was heated to 110° C. for 2 hours to ensure destruction of all ascorbic acid. Supplements of cod liver oil were given twice weekly. Control animals received the same diet except for the addition of ascorbic acid to the drinking water. In one study 50 µg. of ^{59}Fe in the form of ferric chloride was fed to the fasting animals 25 days after the commencement of the diet; at this stage two of the test animals had already died from scurvy and anaemia was present in most of the remainder. In the second study a dose of 25 µg. of iron was used and absorption was measured 21 days after the start of the ascorbic-acid-free diet. In both investigations ascorbic acid was fed to the test animals 12 hours after giving ^{59}Fe, and they were maintained on a normal diet thereafter. The percentage absorption was measured 8 days after the administration of the ^{59}Fe chloride by placing the animals in a counter composed of a ring of Geiger-Müller tubes (Veall and Vetter, 1952).

Plasma iron turnovers were carried out as described previously (Bothwell, Hurtado, Donohue and Finch, 1957). The percentage of injected ^{59}Fe which subsequently reappeared in circulating red cells was measured in several subjects. In these calculations a blood volume of 70 ml. per kg. was assumed.

The uptake of ^{59}Fe by bone marrow in vitro was measured on several specimens. Aliquots of marrow were incubated for varying periods in a mixture containing Eagle's medium (Eagle, 1959) and plasma labelled with ^{59}Fe. After incubation at 37° C., the cells were washed with 0.9 per cent saline and the radioactivity was measured in a scintillation counter. The cells were then haemolysed with water and after centrifugation at 3000 rev./min. further analyses were carried out on the supernatants. Haemin was crystallized by the method of Labbe and Nishida (1957). In order to correct for the incomplete recoveries obtained with this technique a solution of pure haemoglobin labelled with ^{55}Fe was added to the ^{59}Fe haemolysates at the beginning of the procedure and the washed haemin crystals were finally digested, electroplated on to copper discs and counted for ^{55}Fe and ^{59}Fe using methods previously described (Bothwell *et al.*, 1957). In this way it was possible to calculate the percentage of ^{59}Fe-haem in the original haemolysate. Ferritin was identified using an antiserum to human ferritin prepared in rabbits; the immunochemical procedures were slight modifications of those described by Reissmann and Dietrich (1956).

RESULTS

Haematological Data

The mean values obtained on 25 patients before treatment were as follows: haemoglobin, 9.7 g. per 100 ml. (range 6.7–13.3); PCV, 29.2 per cent (range 20.0–39.0); MCHC, 33.1 per cent (range 27.6–38.0); plasma iron, 60 µg. per 100 ml. (range 20–112) and total iron-binding capacity (measured in 14 subjects), 247 µg. per 100 ml. (range 167–353).

On examination of peripheral blood smears the red cells were found to be normocytic and normochromic in most subjects, but seven patients showed mild or moderate hypochromia. The reticulocyte count on admission was variable, with figures ranging from 0.2 to 12.6 per cent (mean 4.2 per cent). Bone-marrow smears were examined in 14 subjects: erythroid hyperplasia of normoblastic type was present to varying degrees in all the marrows examined, and moderate to marked reticulo-endothelial siderosis was a constant finding.

The frequency of iron overload was confirmed by carrying out liver biopsies on 12 subjects after recovery. Siderosis was regarded as severe in 11 of them, using the criteria of Bothwell and Isaacson (1962).

Absorption of ^{59}Fe

The absorption of a 5 mg. dose of ferrous sulphate was measured in eight patients: in the four subjects tested before treatment the mean absorption was 4.1 per cent (range 1.5–8.0), as compared with a figure of 8.0 per cent (range 2.0–16.0) in four subjects studied shortly after beginning treatment. All these figures are within the range found in apparently normal Bantu males (Bothwell, unpublished).

Since these inconclusive results were probably complicated by the coexistence of iron overload in the patients studied, it was decided to investigate the problem further in scorbutic guinea-pigs. The results in two studies are shown in Table I. In both experiments the presence

TABLE I

ABSORPTION OF IRON IN SCORBUTIC AND CONTROL GUINEA-PIGS

Dose of iron (μg.)	*Type and No. of animals*	*Hb* (*g./*100 *ml.*)	*Reticulocytes* (%)	*Absorption* (%)
50	Scorbutic (6)	11.3 (9.4–14.6)	21.3 (12.0–40.1)	11.8 (5.1–20.4)
	Controls (4)	16.8 (16.3–17.7)	1.4 (0.8–1.7)	1.8 (1.1–1.5)
25	Scorbutic (11)	14.7 (12.1–16.2)	0.9 (0.2–2.0)	14.7 (4.1–31.1)
	Controls (9)	15.4 (13.1–17.8)	0.6 (0.2–1.8)	4.3 (0.7–8.7)

All values are given as means with ranges in parentheses.

of scurvy was associated with an increase in the absorption of iron. This increase did not appear to be related to the presence of anaemia, as it was nearly as marked in the group of animals studied before significant anaemia had developed.

Ferrokinetic Measurements

Plasma iron turnovers were carried out on 15 patients before treatment (Table II): the mean value was 2.6 times normal. In all except two subjects (Nos. 13 and 15) there was moderately good agreement between values for plasma iron turnover and the reticulocyte count. These results suggested that erythropoietic activity was effective in terms of the delivery of viable red cells to the circulation. This point was investigated further by studying the red-cell utilization of ^{59}Fe in three subjects who were maintained on a scorbutic diet for several days. During this period the subjects showed no clinical evidence of remission and there was no significant rise in haemoglobin, reticulocyte count or plasma iron. The percentage of ^{59}Fe used for red-cell production was within the normal range in all three patients (Fig. 1).

In a further three patients, serial plasma iron turnovers were carried out before and after treatment with ascorbic acid, to find out whether this was associated with any change in total

TABLE II

HAEMATOLOGICAL AND FERROKINETIC DATA IN 15 UNTREATED PATIENTS WITH SCURVY

Subject No.	*Sex*	*PCV* (%)	*Reticulocytes* (%)	*'Corrected'* reticulocytes* (%)	*Plasma iron* (μg./100ml.)	*Total iron-binding capacity* (μg./100ml.)	*^{59}Fe-$T_{\frac{1}{2}}$* (*min.*)	*Plasma iron turnover* (*mg./day/ 100 ml. whole blood*)	*Production index†*	
									Reticulocytes (%)	*Plasma iron turnover* (%)
1	M	37	3.0	2.3	53	235	32	1.04	230	170
2	F	30	2.1	1.3	39	223	21	1.30	130	213
3	M	23	4.0	1.9	49	174	26	1.46	190	239
4	M	32	4.5	3.0	59	290	38	1.06	300	174
5	M	25	11.4	5.9	75	300	18	3.13	590	513
6	M	19	12.0	4.7	20	242	14	1.16	470	190
7	M	26	4.6	2.5	70	319	46	1.13	250	185
8	M	36	2.4	1.8	40	240	18	1.42	180	233
9	M	27	3.0	1.7	72	205	24	2.19	170	359
10	M	20	10.2	4.2	66	256	24	2.20	420	361
11	M	30	2.6	1.6	43	...	21	1.43	160	234
12	M	25	5.0	2.6	68	167	27	1.89	260	310
13	M	30	0.2	0.12	71	269	47	1.06	12	174
14	M	38	2.2	1.7	112	353	36	1.93	170	316
15	M	35	0.2	0.15	96	181	42	1.49	15	244
Mean		29	4.5	2.4	62	247	29	1.59	236	261

* 'Corrected' reticulocytes = % reticulocytes $\times \frac{\text{Patient's PCV}}{\text{Normal PCV (48 per cent)}}$

† In these rough estimates of erythropoietic activity a 'corrected' reticulocyte value of 1.0 per cent and a plasma iron turnover of 0.61 mg. per 100 ml. whole blood per day were each taken as 100 per cent and individual figures were related to these normal values.

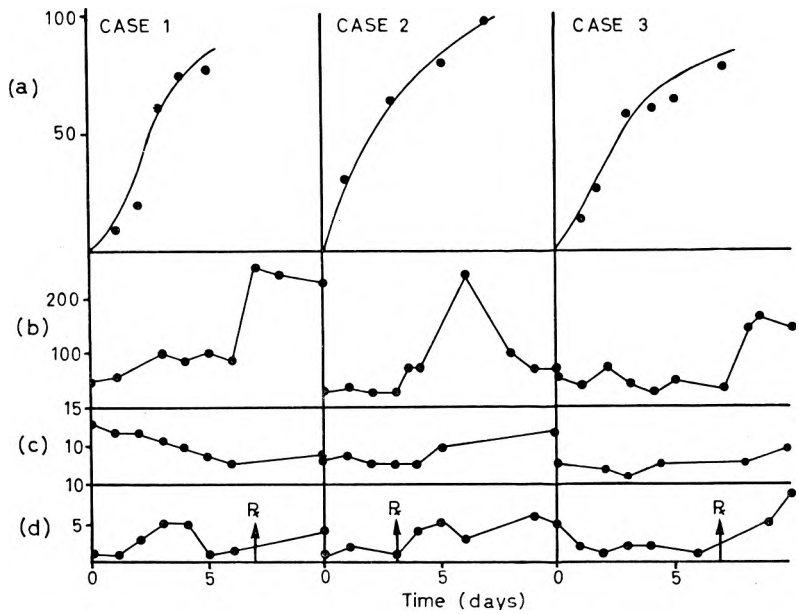


FIG. 1. (a) Percentage of injected ^{59}Fe appearing in red cells in three untreated patients with scurvy. The serial changes in (b) plasma iron (μg. per 100 ml.), (c) haemoglobin (g. per 100 ml.), and (d) reticulocytes (per cent) are also shown before and after the beginning of treatment (R) with 1.0 g. of ascorbic acid daily.

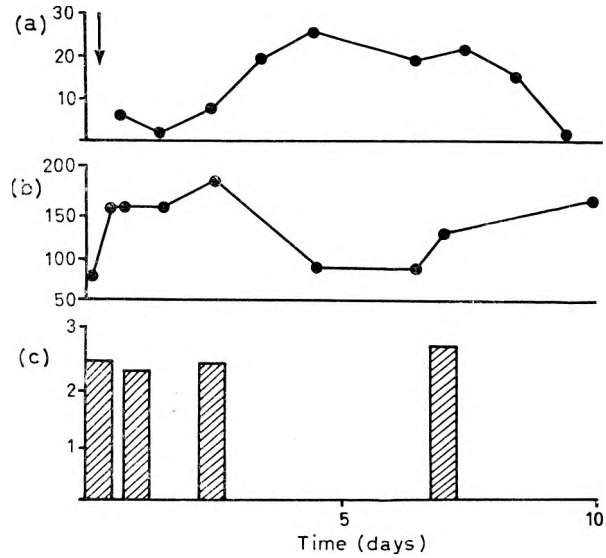


FIG. 2. Serial plasma iron turnover measurements in a patient with scurvy before and after treatment. (a) Reticulocytes (per cent); (b) Plasma iron (μg. per 100 ml.); (c) Plasma iron turnover (mg. per day per 100 ml. whole blood). The intravenous dose (arrow) of ascorbic acid (500 mg. of vitamin C) was followed by 1.0 g. daily by mouth.

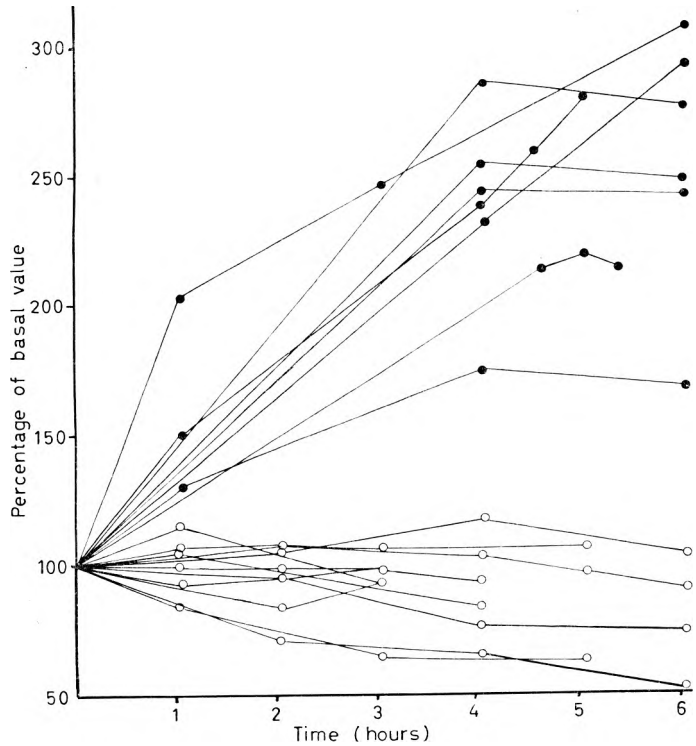


FIG. 3. Effect of ascorbic acid (500 mg. intravenously) on the plasma iron in patients with scurvy (closed circles) and in a control group (open circles).

erythropoietic function. In one there was a rise in plasma iron turnover from 1.2 to 2.0 mg. per 100 ml. whole blood per day within several hours of administering ascorbic acid and this turnover rate was maintained over the next few days. In the other two patients there was no change in plasma iron turnover after treatment: the findings in one of these two patients are illustrated in Fig. 2.

One point of interest was the rise in the level of plasma iron after giving ascorbic acid. This phenomenon is apparent in Figs. 1 and 2, and was studied in more detail in eight other patients with scurvy. The prompt and dramatic rise, which reached a maximum between 4 and 6 hours, was not observed in a control group comprising four normal subjects, three

TABLE III

UPTAKE OF ^{59}Fe BY SCORBUTIC MARROW CELLS

Time of incubation (hr.)	*Total uptake (counts/sec.)*	
	Without ascorbic acid	*With ascorbic acid*
1	1330	1430
2	2100	2330
4	3190	3200

TABLE IV

DISTRIBUTION OF ^{59}Fe IN SCORBUTIC MARROW CELLS

	Time (hr.)	*Radioactivity in fractions (% of total)*			
		Haemin	*Ferritin*	*Deposit*	*Total*
Scorbutic marrow without ascorbic acid	1	81.4	7.9	18.5	107.8
	2	71.5	8.3	16.8	96.6
	4	83.7	11.7	13.6	109.0
Scorbutic marrow with ascorbic acid	1	77.4	7.0	18.7	103.1
	2	66.4	7.6	17.5	91.5
	4	72.2	9.8	17.2	103.2

patients with cancer, two with chronic infection and one with iron overload, or in a patient with scurvy who had already been adequately treated (Fig. 3). *In-vitro* testing revealed that the rise was not due to any local effect of ascorbic acid on scorbutic red cells and the most likely explanation for its occurrence seemed to be an increased release of iron from reticulo-endothelial cells.

Marrow Uptake of ^{59}Fe

The fact that the major portion of injected ^{59}Fe reappeared within new red cells in untreated patients ruled out any major interference with haemoglobin synthesis in scurvy. As it remained possible, however, that more subtle alterations might exist, several *in-vitro* studies were done in which aliquots of marrow from untreated patients with scurvy were incubated with ^{59}Fe-labelled scorbutic plasma.

In the first experiment the effect of adding ascorbic acid in a final molarity of 1×10^{-4} was

studied. This molarity has been found to be optimal for the incorporation of iron into haem *in vitro* (Goldberg, 1959). The absolute uptakes are shown in Table III. After haemolysing the washed cells the percentage of radioactivity present in haemin and ferritin fractions was then measured. It can be seen (Table IV) that the addition of ascorbic acid did not affect the percentage of haemin formed.

In further experiments on other scorbutic marrows ascorbic acid was used in molarities varying between 1×10^{-5} and 5×10^{-4}, but essentially similar results were obtained. Marrows were also incubated for periods up to 21 hours but again there was no difference.

DISCUSSION

These studies indicate that there is no interference with iron absorption in scurvy. Although the percentage absorption of a small dose of iron was found to be relatively low in four patients tested, this was also true of those who had already been treated. It therefore seems likely that the absorption of iron in scorbutic Bantu is modified by the severe degree of iron overload which is present in the majority. This is supported by the two studies in scorbutic guinea-pigs, since these showed that the absorption rates were actually greater than in control animals. Although this enhanced absorption rate was probably the result of increased erythroid marrow activity, it is somewhat surprising that it was noted in one group of animals at a time when there had been no real change in haemoglobin and reticulocyte levels. Whatever the explanation, it is clear that the increase in the absorption of food iron, which has been noted in normal and iron-deficient subjects when ascorbic acid is added (Moore, 1955), must be due to local effects within the lumen and not to any change in cellular behaviour.

Ferrokinetic measurements in patients with scurvy revealed a moderately consistent pattern, with an erythropoietic rate, as judged from plasma iron turnover data, of between 2 and 3 times normal. In addition, the utilization of ^{59}Fe for red-cell production was normal in the three subjects in which it was tested. These findings suggest that erythropoiesis is effective in scurvy and further support for this is provided by the fact that the reticulocyte count was usually raised to the same degree as the plasma iron turnover. It is clear, however, that this is not always the case, since there are several reports of megaloblastic erythropoiesis in scurvy (McMillan and Inglis, 1944; Bronte-Stewart, 1953; Brown, 1955). In the past this has been ascribed to a disturbance in folic-acid metabolism secondary to the ascorbic-acid deficiency, but there is recent evidence to indicate that diets with a low ascorbic-acid content are also deficient in folic acid and that if such a diet is supplemented with ascorbic acid alone, frank megaloblastic anaemia may develop (Zalusky and Herbert, 1961). Although the folic-acid status of the subjects in the present study was not assessed, there is evidence that Bantu with scurvy invariably show some biochemical evidence of folic-acid deficiency (J. Metz, personal communication). It is of interest that two of the patients in the present study had disproportionately low reticulocyte counts and it is possible that these particular patients had a more severe degree of folic-acid deficiency. The bone marrow, however, was judged as being normoblastic in each of them.

The plasma iron was lower than normal in scorbutic subjects, despite the fact that they had enlarged iron stores. The prompt rise in plasma iron that occurred when ascorbic acid was given seemed to be due to an increased release of iron into the plasma, presumably from reticulo-endothelial stores. The fact that a similar rise was not found in normal subjects or in patients with infection, neoplasia or uncomplicated iron overload, suggests that there must be some alteration in the way in which iron is released from reticulo-endothelial cells

into the plasma in scurvy. At the same time it is clear that the low plasma iron levels found in untreated scorbutic subjects are sufficient to supply the needs of the marrow, since there was little or no change in plasma iron turnover when the plasma iron had been raised by treatment with ascorbic acid.

Although the present investigation was designed primarily to study iron metabolism in scurvy, the results obtained give some insight into the pathogenesis of anaemia in this condition. In the subjects studied, erythroid marrow activity was in the range which would be expected as a result of acute anaemia (Bothwell *et al.*, 1957) and both *in-vivo* and *in-vitro* studies suggested that haemoglobin formation was normal. The anaemia present in these subjects must therefore have been the result of a degree of haemolysis and/or haemorrhage greater than the marrow's capacity to respond. Although the survival time of transfused red cells has been found to be less than normal in scorbutic patients (Merskey, 1953), interpretation of data is difficult since similar results would be expected with continuing haemorrhage into tissues. Red cells from three of the subjects in the present study, when labelled with ^{51}Cr according to the method of Mollison and Veall (1955), survived normally in compatible recipients. This suggests that there is no intrinsic red-cell defect, but even this evidence is not conclusive, since it is theoretically possible that any defect in the cells might be corrected by the entry of ascorbic acid from normal plasma. Even if some degree of haemolysis does exist in patients with scurvy, however, it is important to stress that the striking clinical manifestations are due to haemorrhage into tissues and this would seem to be the dominant, if not the only, factor of importance in the production of anaemia.

One last point relates to the significance of the siderosis which was a major feature in the Bantu subjects studied. This association, which has also been observed by other workers (Schulz and Swanepoel, 1962) seemed to be more than fortuitous since the degrees of iron overload present were greater than would be expected in a hospital population of comparable age structure (Bothwell and Isaacson, 1962). It has been suggested that persons with siderosis living on a borderline diet may be precipitated into clinical scurvy as a result of irreversible oxidation of available ascorbic acid by the excessive deposits of ferric iron. Although there is no concrete evidence to support this contention, two observations are at least compatible with it. In one recent investigation attempts to saturate two siderotic scorbutic subjects with oral ascorbic acid were unsuccessful and in one of them urine excretion was less than normal, even after repeated parenteral injections (Schulz and Swanepoel, 1962). In the present investigation two groups of guinea-pigs, one with normal and the other with increased iron stores (50 mg./kg.), were placed on a scorbutic diet and followed to death. The average survival time of the normal animals was 28 days (range 24–31) as opposed to 22 days (range 19–24) in the siderotic group. Although both these studies suggest that excessive iron stores influence the metabolism of ascorbic acid they do not indicate the mechanisms involved.

SUMMARY

The results of ferrokinetic measurements, carried out in adult Bantu with scurvy and in scorbutic guinea-pigs, indicate that there is no major impairment in iron transport when ascorbic-acid deficiency is present.

Iron absorption is greater than normal in ascorbic-acid deficiency, probably in response to an increased erythropoietic activity of between 2 and 3 times normal. This activity appears to be effective in terms of the delivery of viable red cells to the circulation and *in-vitro* studies suggest that haemoglobin synthesis within the marrow is normal.

Although the plasma iron is lower than normal in scurvy, this does not limit the erythropoietic response, since the prompt rise which occurs when ascorbic acid is given does not lead to any marked change in the rate of blood formation.

The fact that Bantu subjects with scurvy almost invariably have moderate to severe degrees of iron overload suggests that excessive iron deposits may modify the metabolism of ascorbic acid in the body.

REFERENCES

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. A comparison of incidence in males and females.' *Brit. med. J.*, **i**, 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.

BRONTE-STEWART, B. (1953). 'The anaemia of adult scurvy.' *Quart. J. Med.*, N.S. **22**, 309.

BROWN, A. (1955) 'Megaloblastic anaemia associated with adult scurvy: report of a case which responded to synthetic ascorbic acid alone.' *Brit. J. Haemat.*, **1**, 345.

EAGLE, H. (1959). 'Amino acid metabolism in mammalian cell cultures.' *Science*, **130**, 432.

FINEBERG, R. A., KASBEKAR, D. K. and LOEWUS, M. W. (1959). 'Physiologic agent for the uptake of ferric iron by apoferritin.' *Fed. Proc.*, **18**, 225.

GOLDBERG, A. (1959). 'The enzymic formation of haem by the incorporation of iron into protoporphyrin; importance of ascorbic acid, ergothioneine and glutathione.' *Brit. J. Haemat.*, **5**, 150.

GRUSIN, H. and KINCAID-SMITH, P. S. (1954). 'Scurvy in adult Africans; clinical, haematological, and pathological study.' *Amer. J. clin. Nutr.*, **2**, 323.

LABBE, R. F. and NISHIDA, G. (1957). 'A new method of hemin isolation.' *Biochim. biophys. Acta (Amst.)*, **26**, 437.

MCMILLAN, R. B. and INGLIS, J. C. (1944). 'Scurvy: a survey of fifty-three cases.' *Brit. med. J.*, **ii**, 233.

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.

MERSKEY, C. (1953). 'Survival of transfused red cells in scurvy.' *Brit. med. J.*, **ii**, 1353.

MOLLISON, P. L. and VEALL, N. (1955). 'The use of the isotope ^{51}Cr as a label for red cells.' *Brit. J. Haemat.*, **1**, 62.

MOORE, C. V. (1955). 'The importance of nutritional factors in the pathogenesis of iron-deficiency anemia.' *Amer. J. clin. Nutr.*, **3**, 3.

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.

REISSMANN, K. R. and DIETRICH, M. R. (1956). 'On the presence of ferritin in the peripheral blood of patients with hepatocellular disease.' *J. clin. Invest.*, **35**, 588.

SCHULZ, E. J. and SWANEPOEL, H. (1962). 'Scorbutic pseudoscleroderma, an aspect of Bantu siderosis.' *S. Afr. med. J.*, **36**, 367.

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.

VEALL, N. and VETTER, H. (1952). 'An apparatus for the rapid estimation of tracer quantities of radioactive isotopes in excreta.' *Brit. J. Radiol.*, N.S. **25**, 85.

ZALUSKY, R. and HERBERT, V. (1961). 'Megaloblastic anemia in scurvy with response to 50 microgm. of folic acid daily.' *New Engl. J. Med.*, **265**, 1033.

Journal of Clinical Investigation
Vol. 43, No. 4, 1964

Studies on the Formation of Ferritin in Red Cell Precursors *

SOLAM S. ZAIL, ROBERT W. CHARLTON, JOHN D. TORRANCE, AND THOMAS H. BOTHWELL

(From the Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa)

The mechanisms involved in the uptake and utilization of iron by red cell precursors have been studied by a number of investigators. Initially iron is released from the plasma protein, transferrin, onto the cell membrane and passes rapidly into the cell interior (1, 2). The results of several studies indicate that it is then taken up by a nonhemoglobin protein before being incorporated into hemoglobin (2–4). The nature of the precursor complex is, however, still not clear. In a series of *in vitro* studies on human and dog marrow suspensions, Greenough, Peters, and Thomas (4) found that it could be distinguished from ferritin but had properties in common with globin. They therefore suggested that the iron combines with a protein, which is possibly a part of the globin molecule, and that this complex (sideroglobin) then unites with protoporphyrin to form hemoglobin. Although such a concept could well account for one pathway taken by iron within the red cell precursor, it does not elucidate the significance of the appreciable quantities of ferritin that can be seen in erythroid precursors by electron microscopy (5). Theoretically it is possible that a proportion of the iron entering the cell is temporarily stored in ferritin before being used for hemoglobin synthesis, and recent evidence has been presented in support of this thesis (6). Alternatively, the ferritin iron may represent a surplus that is excreted when the cell has matured.

The present studies were undertaken to try to elucidate the possible role of ferritin in hemoglobin synthesis and to define the relationship, if any, between it and the hemoglobin precursor (Fraction 1) described by Greenough and coworkers (4).

* Submitted for publication August 22, 1963; accepted December 4, 1963.

This work was supported by a grant (AM 04912-02) from the National Institutes of Health, Bethesda, Md., and by grants from the Council for Scientific and Industrial Research and the Atomic Energy Board, South Africa.

Methods

Marrow was obtained either from the ribs of subjects undergoing thoracotomy or by aspiration from the posterior iliac crest. All the marrows were obtained from hematologically normal subjects, except for two specimens that were obtained from patients with proven iron-deficiency anemia. Venous blood containing between 15 and 20% reticulocytes was obtained from two patients with pernicious anemia in early remission, and from one subject with idiopathic autoimmune hemolytic anemia. Reticulocyte-rich blood was also obtained from rats 3 to 4 days after the parenteral administration of an ethanolic solution of phenylhydrazine (30 mg per kg). All these samples were treated the same as the marrow. In addition, *in vivo* studies were carried out in one normal, splenectomized human subject and in both normal and splenectomized rats.

Specimens of marrow were suspended in balanced salt solution (B.S.S.) (7) containing 200 U penicillin, 100 μg streptomycin, and 100 μg neomycin per ml, and were processed and used within 1 hour after removal from the patient. Marrow obtained from ribs was shaken vigorously and then syringed repeatedly through a 20-gauge Luer lock needle until adequate dispersion of the cells was obtained. In certain studies splenic suspensions were also prepared in this way. The marrow was washed once in cold B.S.S. to remove the supernatant fat and was then suspended in B.S.S. containing 10 to 30% autologous serum.

$Fe^{59}Cl_3$ (SA, 20 to 30 μc per μg) bound to autologous serum was added to the marrow suspension at the start of the incubation. The amount of $Fe^{59}Cl_3$ added was always less than the binding capacity of the serum present. Serum iron levels were estimated by a modification of the method of Bothwell and Mallett (8) and iron binding capacities by the method of Bothwell, Jacobs, and Kamener (9). Equal samples of marrow suspension were incubated at 37° C in air in 25-ml Erlenmeyer flasks placed on top of a slowly revolving drum inclined at 10° to the horizontal. Samples of marrow were removed at varying times, and the reaction was stopped by the addition of 0.5% potassium cyanide in saline and rapid cooling to 0° C. In some experiments, further Fe^{59} labeling of the marrow was stopped after 5 to 10 minutes'

incubation by cooling the marrow rapidly to 4° C, washing it free of external radioactivity, and reincubating it in unlabeled incubation medium. After incubation the marrow cells were washed 3 times with saline and were then lysed by the addition of 4 vol of distilled water. The samples were then shaken vigorously and kept at 4° C for 30 minutes. The lysate was centrifuged for 20 minutes at $1,500 \times g$. The resulting clear red supernatant solution was siphoned off and kept. The precipitate ("stromal fraction") was washed twice in 10 vol of cold distilled water and centrifuged at $1,500 \times g$ for 20 minutes.

Isolation of Fraction 1 and main hemoglobin. A sample of the supernatant solution was chromatographed on Amberlite CG-50 resin, Type II,[1] prepared according to the method of Hirs, Moore, and Stein (10) as modified by Greenough and co-workers (4). The fraction eluting with the solvent front (Fraction 1) was separated with the starting buffer (0.0067 M sodium phosphate with 0.0033 M KCN at pH 7.0), and all other components (main hemoglobin) were eluted from the column by changing directly to the limiting buffer (0.5 M NaCl dissolved in the starting buffer). Recoveries of radioactivity were between 85 and 95%. To ascertain whether the main hemoglobin fraction represented an accurate measure of hemin synthesis, the percentages of counts of Fe^{59} incorporated into main hemoglobin and into hemin in a sample of supernatant solution were compared. Hemin was crystallized by the method of Labbe and Nishida (11). To correct for the incomplete recoveries obtained with this technique, a solution of pure hemoglobin, labeled with Fe^{55}, was added to the Fe^{59} supernatant solution at the beginning of the procedure, and the washed hemin crystals were finally digested, electroplated onto copper discs, and counted for Fe^{55} and Fe^{59} with methods previously described (12). In this way it was possible to calculate the percentage of Fe^{59}-labeled hemin in the original supernatant solution. With this approach the mean percentage of Fe^{59} incorporated into hemin was 83.4% (range, 77% to 90%) of the value for main hemoglobin.

Isolation of ferritin. Human liver ferritin was prepared from siderotic Bantu livers by the method of Mazur and Shorr (13). An antiserum was prepared by bleeding rabbits immunized with samples of alum-precipitated human ferritin by a modification of the method of Proom (14). (One ml of the pooled antiserum precipitated approximately 1 mg ferritin). In studies on rats an antiserum to purified horse spleen ferritin was used, since a cross-immunity has been shown between these two species (15).

To 1 vol of the solution to be tested was added an equal volume of 0.2 M sodium ethylenediaminetetraacetic acid (EDTA) buffered to pH 7.0. One ml antiserum and 0.2 ml carrier ferritin solution containing approximately 500 μg ferritin were then added, and the mixture was incubated for 1 hour at 37° C. The ferritin-antiferritin complex, which appeared as a visible precipitate, was washed 3 times with distilled water after centrifugation at $1,500 \times g$ for 10 minutes. The Fe^{59} activity of the precipitate was determined.

In this method carrier ferritin was added to the solution for two reasons: *1)* Precipitation could be verified by chemical methods, and *2)* it allowed for the measurement of minute amounts of labeled ferritin. The theoretical possibility that free Fe^{59} or some other labeled small molecule in the supernatant solution might attach to the carrier ferritin and be precipitated with the ferritin-antiferritin complex was avoided by the addition of EDTA to the solution, since preliminary studies showed that it prevented nonspecific attachment of free Fe^{59} to carrier ferritin. In a further experiment, antiserum was added to a marrow hemolysate containing EDTA and ferritin labeled physiologically with Fe^{59}. Under these circumstances 96% of the radioactivity was precipitated. The specificity of the antiserum was confirmed by the demonstration that when the antiserum was incubated with solutions of hemoglobin and transferrin labeled with Fe^{59} no activity was precipitated. In addition, negative results were also obtained with Fe^{59}-labeled main hemoglobin, obtained after chromatography of marrow supernatant solutions.

Ferritin was also identified using the Ouchterlony agar gel technique as described by Bodman (16). Antiferritin serum was placed in the center well while human liver ferritin, labeled *in vitro* with Fe^{59}, and marrow supernatant solutions were placed in the peripheral wells. After the bands of precipitation had formed, the gels were washed for 3 days with frequent changes of distilled water. They were dried until paper-thin and were then placed in apposition with X-ray films for several days to obtain radioautographs.

Results

Demonstration of physiologically labeled ferritin in marrow suspensions at various times of incubation. The incorporation of Fe^{59} into the "stromal fraction," Fraction 1, ferritin, and main hemoglobin was followed over several hours in six experiments. Since the major changes in the pattern of incorporation of Fe^{59} into the various fractions occurred in the first hour of incubation, most samples were analyzed during this period. To permit comparison between results obtained in the different experiments, the amounts of radioactivity present in the various fractions at different times were expressed as percentages of the total cellular uptake at 1 hour.

Radioiron was rapidly taken up by the marrow suspension. After 5 minutes between 23 and 37% was already present in cells, and this rose to between 71 and 79% by 30 minutes. Approximately half of this uptake was present in the

[1] Manufactured by the Rohm & Haas Co., Philadelphia, Pa.

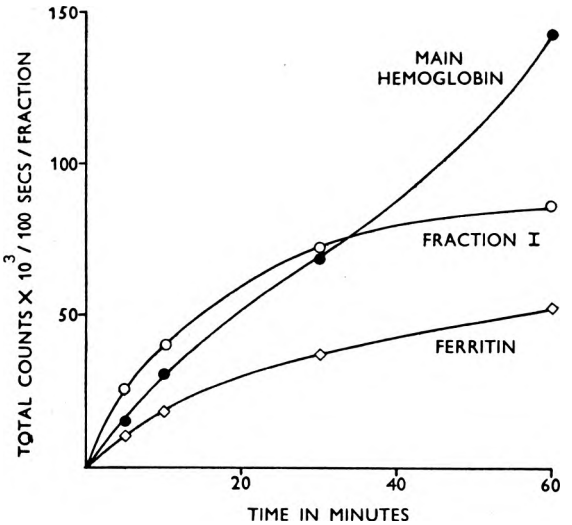


FIG. 1. INCORPORATION OF Fe^{59} INTO FRACTION 1, MAIN HEMOGLOBIN, AND FERRITIN. Analysis of samples of human rib marrow culture at various times up to 1 hour.

"stromal fraction." Between 5 and 30 minutes the mean percentage present in Fraction 1 rose from 5.5 (range, 3.7 to 9.9%) to 16.8 (range, 12.7 to 22.0%). The rate of increase over the next 30 minutes was much slower, the mean figure at 1 hour being 18.5% (range, 15 to 28%). Between 2.0 and 4.6% of the Fe^{59} was incorporated into ferritin by 10 minutes, and thereafter the radioactivity in ferritin increased in linear fashion up to 1 hour, when between 8.3 and 16.4% was present in this fraction. It was noteworthy that the amount of radioactivity in ferritin was always less than in Fraction 1. Only small amounts of Fe^{59} (between 2.0 and 5.9%) were incorporated into main hemoglobin in the first 10 minutes, but by 60 minutes between 19.0 and 36% was present in this compound. The pattern of changes occurring in one of the experiments is shown in Figure 1.

Between 1 and 12 hours there were no marked changes in the distribution of Fe^{59} between the various fractions. The rate of uptake of radioactivity by the cells was not as rapid as over the first hour, the figures after 4 hours ranging between 1½ and 3 times the 1-hour uptakes. At this stage between 17.6 and 32.5% of the activity taken up was in Fraction 1, from 10.2 to 23.0% in ferritin, and between 22 and 46% in main hemoglobin.

Site of formation of ferritin. As marrow is a heterogeneous tissue it was necessary to determine whether the ferritin isolated in the marrow supernatant solution had been labeled in red cell precursors or in reticuloendothelial cells. Since preliminary studies indicated that bone marrow suspensions obtained from the femurs of rats metabolized iron in similar fashion to human bone marrow suspensions, three *in vitro* studies were done in which the uptake of Fe^{59} from labeled autologous plasma by marrow suspensions was compared with that of splenic suspensions prepared in the same way (see Methods). After 3 hours' incubation the samples were washed 3 times with normal saline and were then counted for radioactivity. The total uptakes by the splenic suspensions were less than 10% of those obtained with equivalent wet weights of marrow. After homogenization with 4 vol of water, samples of the supernatant solution from the splenic suspensions were set up with antiferritin serum. It was found that 0, 2, and 14% of the activity taken up by splenic cells was precipitated as ferritin. The comparable figures obtained with the marrow suspensions were 10, 23, and 16%, respectively. When these figures were related to the total uptakes of the two tissues, the spleen's capacity to incorporate Fe^{59} into ferritin was between 0 and 10% of a comparable weight of marrow. Although it is possible that reticuloendothelial cells in the marrow may behave differently from those in the spleen, this seems unlikely, and the results obtained therefore suggest that the labeling of ferritin observed in marrow cultures was occurring predominantly in red cell precursors and not in reticuloendothelial cells.

Demonstration of physiologically labeled ferritin in Fraction 1. Addition of antiferritin serum to Fraction 1 resulted in the precipitation of a significant number of counts as ferritin. When a solution of Fraction 1 was placed opposite antiferritin serum on an agar gel diffusion plate no visible band resulted, but a radioautograph of the washed plate showed a line of radioactivity opposite Fraction 1 that coalesced with the lines of radioactivity corresponding to the bands of labeled pure ferritin (Figure 2). A similar band was noted when a sample of the whole supernatant solution was treated in the same way. These results therefore confirmed the presence of

ferritin in both the supernatant solution and Fraction 1.

When the percentage of counts incorporated into ferritin at various time intervals was measured directly on the supernatant solution, the results were in close agreement with those obtained when the same estimation was carried out on Fraction 1 (Table I). These findings indicate that all the ferritin present in the marrow supernatant solution was in Fraction 1. In addition, the percentage of radioactive ferritin present in Fraction 1 increased with time (Table II).

Effects of stopping labeling on different fractions. Four experiments were performed in which the marrow was incubated with Fe^{59}-labeled plasma for a period of 5 to 10 minutes, and further labeling of the marrow with Fe^{59} was then stopped. In this way it was possible to follow the fate of a "cohort" of radioiron at various time intervals. Figure 3 illustrates the pattern obtained in one experiment. The radioactivity in Fraction 1 and the "stromal fraction" fell with time up to 6 hours, whereas the activity in hemoglobin rose rapidly at first and then reached a plateau. In contrast, the radioactivity in ferritin remained the same throughout the time of incubation. When the counts present in Fraction 1 were corrected for the ferritin present, the nonferritin fraction fell rapidly to a very low level. During this period there was a reciprocal increase in the activity present in main hemoglobin.

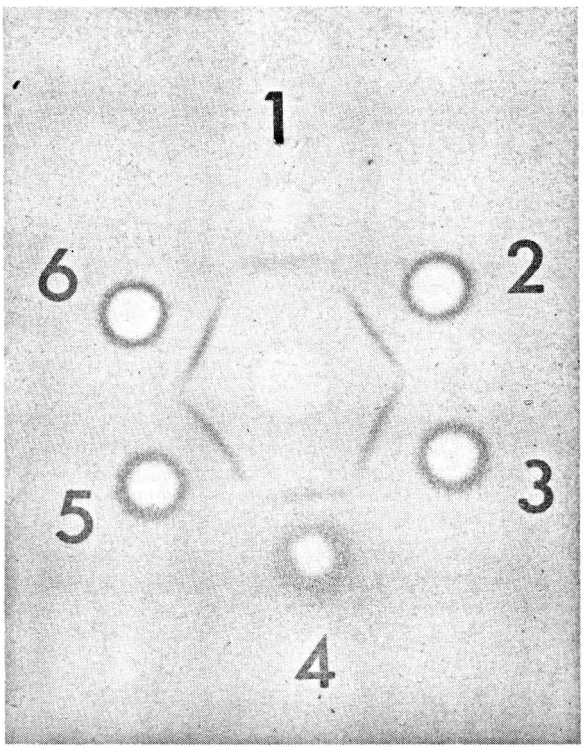


FIG. 2. RADIOAUTOGRAPH OF AGAR-GEL DIFFUSION PLATE. Center well, human liver ferritin antiserum; 1, Fe^{59}-labeled Fraction 1; 4, Fe^{59}-labeled marrow supernatant solution; 2, 3, 5, and 6, Fe^{59}-labeled human liver ferritin. Although no visible precipitate could be seen opposite the wells containing Fraction 1 and the marrow supernatant solution, bands of radioactivity coalescing with the liver ferritin bands are demonstrated.

TABLE I

A comparison of radioactivity in ferritin (expressed as a percentage of total marrow uptake) determined in marrow supernatant solution and in Fraction 1 after various periods of incubation in vitro

Exp't no.	Minutes			Hours				
	5	10	30	1	2	3	4	6
I. Supernatant solution	9.7	7.0	8.3		7.9		16.8	
Fraction 1	7.4	8.0	8.9		13.5		15.3	
II. Supernatant solution	9.9	8.4	9.8	9.4		15.1		
Fraction 1	8.4	9.2	9.9	10.0		16.1		
III. Supernatant solution	7.2	9.9	10.1	10.3	11.0			
Fraction 1	4.2	6.6	8.3	8.3	10.2			
IV. Supernatant solution		10.5	11.4	11.5	11.8		11.3	
Fraction 1		8.5	10.5	8.4	10.5		10.7	
V. Supernatant solution	11.1		9.2	11.2			11.2	8.9
Fraction 1	10.0		9.6	10.2			8.1	8.9

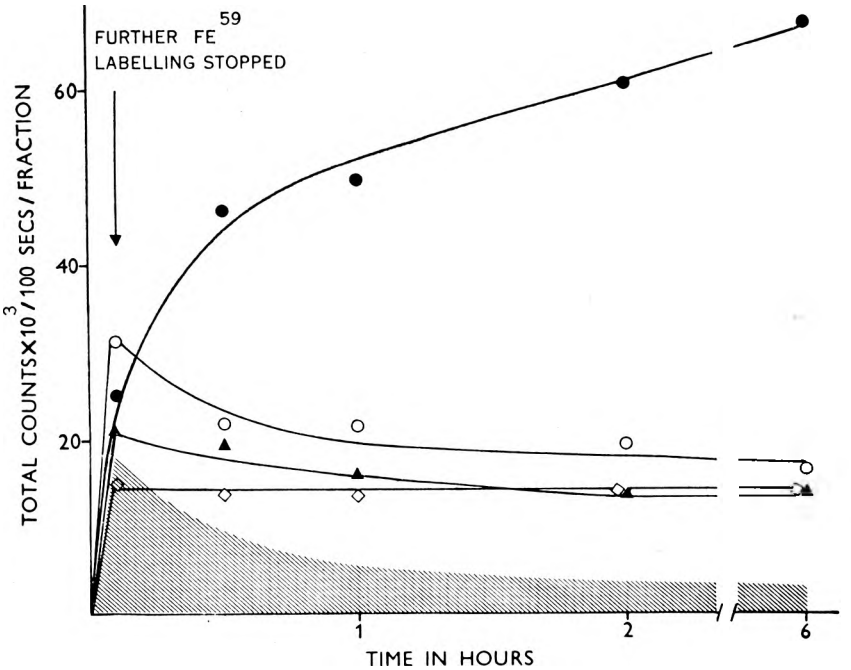


FIG. 3. INCORPORATION OF Fe^{59} INTO "STROMAL FRACTION," FRACTION 1, MAIN HEMOGLOBIN, AND FERRITIN. Analysis of samples of human rib marrow culture at various times up to 6 hours. Further labeling with Fe^{59} was stopped after 5 minutes by washing the cells and resuspending them in unlabeled medium. Closed triangles, "stromal fraction"; open circles, Fraction 1; closed circles, main hemoglobin; open diamonds, ferritin; hatched area, hemoglobin precursor, i.e., Fraction 1 minus ferritin.

In the three other experiments the results were very similar. The percentages of radioactivity precipitable as ferritin when labeling was stopped were 9.9, 5.1, and 6.5 as compared with figures of 9.9, 6.5, and 8.9, respectively, at 6 hours. During this period the nonferritin activity in Fraction 1 fell from 9.6 to 2.7, from 11.1 to 2.2, and from 11.3 to 6.1. The increase in main hemoglobin was from 26.3 to 64.6%, from 19.8 to 44.3%, and from 37.6 to 67.3% in the three experiments. It thus appears that the portion of Fraction 1 not precipitable by antiferritin serum is the active intermediate for iron incorporation into hemoglobin (hemoglobin precursor). Since in control experiments in which labeling was not stopped the activity in hemoglobin continued to rise to between 12 and 25 times the figure at 10 minutes, these data also suggest that ferritin does not act as an active intermediate for the incorporation of iron into hemoglobin in marrow cultures over 6 hours.

TABLE II

The percentage radioactivity precipitable as ferritin in Fraction 1 after various periods of incubation of marrow in vitro (labeling with Fe^{59} not stopped)

Exp't no.	Minutes			Hours				
	5	10	30	1	2	3	4	6
I	43	51	51	59	69		74	
II	41	46	51	61		67		
III	30	43	49	50	58			
IV	31	33	55	58		71		78

Kinetics of Fe^{59} incorporation into ferritin in marrow cells in vivo. Since it was not possible to show any drop in ferritin activity during the incubation of marrow *in vitro,* further studies were carried out *in vivo* in rats to find out whether labeled ferritin was still present in the reticulocytes entering the circulation. Since it seemed possible that the ferritin might be removed by the spleen, studies were done in both normal and splenectomized animals. Twelve splenectomized rats and 12 controls were given an intravenous injection of Fe^{59}-labeled plasma,

and batches were killed at intervals thereafter. Four hours after injection the pooled marrows from three splenectomized and three control animals contained 9.6 and 12.0%, respectively, of the radioactivity in the ferritin fraction. At 24 hours the corresponding figures were 1.8 and 2.2%. However, it was not possible to show radioactive ferritin in the circulating red cells of the remaining animals at any time up to 4 days after the administration of the radioiron. In an extension of the study, plasma (labeled with 25 μc Fe^{59}) was injected into a normal splenectomized human subject, and blood samples were collected at daily intervals for 7 days thereafter. At no time was labeled ferritin demonstrated. These findings suggest that the ferritin present in red cell precursors is either ultimately used for hemoglobin synthesis or is extruded before the cells leave the marrow.

Iron uptake by reticulocyte suspensions. Reticulocyte suspensions, obtained from the blood of two patients with pernicious anemia in early remission, were incubated with labeled plasma for 4 hours. In both cases hemoglobin and Fraction 1 formation was normal. No label was detectable in ferritin in one experiment, but small and possibly significant amounts of Fe^{59} were incorporated into ferritin in the other. In a third experiment, a reticulocyte suspension from a patient with idiopathic autoimmune hemolytic anemia was incubated for 1 hour in labeled plasma. At this time 10.3% of the activity taken up by the reticulocytes was in ferritin. Several samples of the suspension were then reincubated in unlabeled medium after the cells had been washed 4 times in cold saline. After a further 3 hours' incubation 8.5% of the radioactivity was still present in the ferritin fraction.

Two similar experiments were carried out on reticulocyte suspensions obtained from rats that had been given phenylhydrazine 3 to 4 days previously. After 1 hour's incubation 2.0 and 0.9%, respectively, of the Fe^{59} were present in ferritin. After 3 further hours of incubation in unlabeled

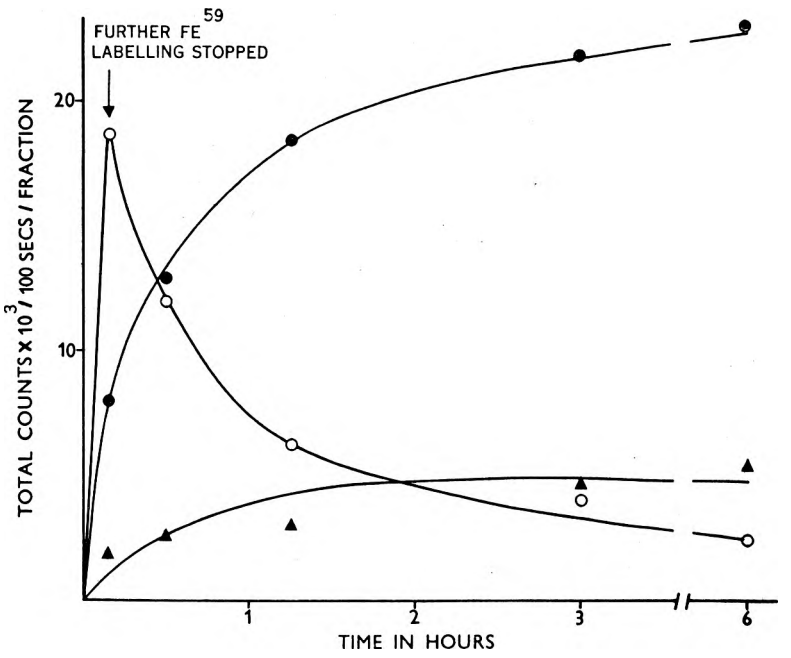


FIG. 4. IRON-DEFICIENT HUMAN ILIAC CREST MARROW CULTURE. INCORPORATION OF Fe^{59} INTO "STROMAL FRACTION," FRACTION 1, AND MAIN HEMOGLOBIN. Analysis of samples at various times up to 6 hours. Further labeling was stopped after 8 minutes by washing the cells and resuspending them in unlabeled medium. Closed triangles, "stromal fraction"; open circles, Fraction 1; closed circles, main hemoglobin. No ferritin could be identified at any stage.

substrate the comparable figures were 3.2 and 0.6%.

Iron uptake by iron-deficient marrows. Marrow from a case of iron deficiency was incubated with labeled plasma, and samples were taken at progressive times of incubation up to 4 hours. The incorporation of Fe^{59} into hemoglobin and Fraction 1 was similar to that observed in normal marrows, but no labeling of ferritin could be detected.

In an experiment using another severely iron-deficient marrow in which further labeling with Fe^{59} was stopped after 8 minutes' incubation (Figure 4), the activity in Fraction 1 fell to much lower levels relative to the total uptake than in normal marrows. This can be ascribed to the fact that it contained only the hemoglobin precursor, since no labeled ferritin was demonstrable at any time.

Effect of lead on the incorporation of Fe^{59} into ferritin, Fraction 1, and main hemoglobin. In five experiments 10^{-4} M lead acetate was added to the marrow suspension at the start of incubation. There was little effect upon total iron uptake, but radioactivity in the stromal fraction increased by a mean of 95% (range, 40 to 154%) compared with the control tubes. Both Fraction 1 and ferritin activity were greater by mean percentages of 38 (range, 12 to 97) and 40 (range, 12 to 90), respectively. The effect upon Fe^{59} in hemoglobin precursor (i.e., Fraction 1 minus ferritin) was variable, ranging from a decrease of 46% to increases of 2, 34, 36, and 125%. Incorporation of radioactivity into main hemoglobin was inhibited by from 38 to 80% (mean, 55%). The results in one experiment are illustrated in Figure 5.

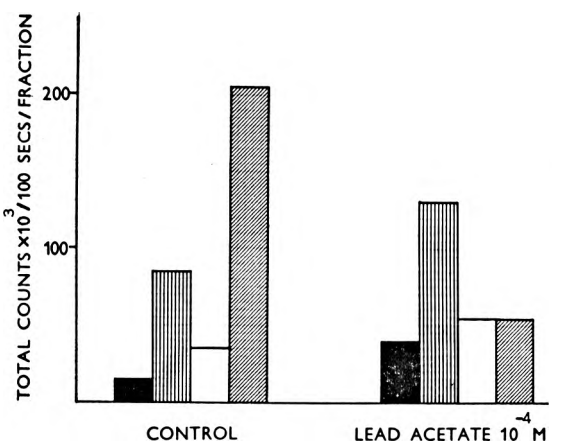


FIG. 5. EFFECT OF 10^{-4} M LEAD ACETATE ON HUMAN RIB MARROW CULTURE. INCORPORATION OF Fe^{59} INTO "STROMAL FRACTION," HEMOGLOBIN PRECURSOR, MAIN HEMOGLOBIN, AND FERRITIN AFTER 4 HOURS. Black, "stromal fraction"; vertical lines, ferritin; unshaded, hemoglobin precursor, i.e., Fraction 1 minus ferritin; diagonal lines, main hemoglobin. There is diminished radioactivity in hemoglobin and increases in "stromal fraction," ferritin, and hemoglobin precursor in the lead-poisoned culture as compared with the control.

Effect of varying saturations of transferrin on ferritin and main hemoglobin synthesis. A comparison was made between the uptake of iron into ferritin and main hemoglobin, respectively, at various saturations of transferrin. At high saturations the amount of iron incorporated into main hemoglobin did not increase, whereas incorporation into ferritin continued to rise progressively. The results in one experiment are shown in Figure 6. In five similar experiments the general pattern was confirmed, with a mean drop in the ratio of the Fe^{59} main hemoglobin to Fe^{59} ferritin from 4.1 at saturations between 30 and 40% to 2.8 at saturations between 80 and 100%.

Discussion

Greenough and his collaborators have shown that iron in red cell precursors passes through a nonhemoglobin iron-protein complex (Fraction 1) before being incorporated into hemoglobin (4). Although these workers were not able to identify radioactive ferritin in Fraction 1, the present studies indicate that it contains an appreciable amount and that the proportion present rises progressively with time. There are two possible reasons for these discrepant findings. In the first place experiments done in the current study revealed that approximately 50% of the radioactive ferritin in Fraction 1 is removed by the high-speed centrifugation (100,000 × *g* for 30 minutes) to which Greenough and co-workers subjected the marrow lysate before chromatography, and secondly there were differences in the immunochemical techniques used in the two studies. As a result, the kinetic patterns for Fraction 1 reported by Greenough and co-workers correspond more closely to those obtained for the hemoglobin precursor (Fraction 1 minus ferritin) in the present investigation. The current findings

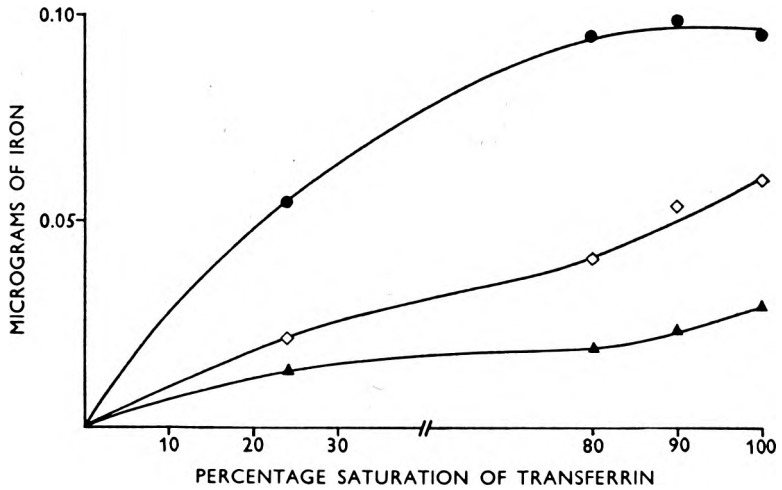


FIG. 6. RELATIONSHIP BETWEEN TRANSFERRIN SATURATION AND THE INCORPORATION OF IRON INTO "STROMAL FRACTION," FERRITIN, AND MAIN HEMOGLOBIN IN HUMAN MARROW CULTURE. After the addition of 10 μc Fe^{59} to 20 ml plasma, unlabeled iron as ferrous ammonium sulfate was added to equal samples in amounts calculated to produce the desired saturation of the transferrin. These samples were then dialyzed against running iron-free water for 16 hours before use. Closed triangles, "stromal fraction"; open diamonds, ferritin; closed circles, main hemoglobin. At high saturations of transferrin more iron was incorporated into ferritin.

lend further support to the suggestion (4) that this substance is an important precursor of hemoglobin. In experiments where labeling was allowed to continue for several hours radioactivity appeared in this fraction at an earlier stage than in hemoglobin and then leveled off. In addition, in experiments where further uptake of Fe^{59} was stopped after 5 minutes' incubation, the radioactivity present in the fraction dropped while that in hemoglobin rose reciprocally. The fact that the fraction only fell to a limited extent is compatible with the suggestion that it was being fed from a large stromal pool, or alternatively, it is possible that some other active precursor substance was present.

Significant amounts of iron taken up by marrow cultures were rapidly incorporated into ferritin. However, since ferritin is visible on electron microscopy both in red cell precursors and in reticuloendothelial cells of the marrow (5) it was important to establish the cellular location of the labeled ferritin in the various experiments. In an attempt to resolve this problem the uptake of plasma-bound radioiron and its incorporation into ferritin were compared in bone marrow and splenic suspensions. Since only negligible amounts of radioiron were incorporated into ferritin in the splenic suspensions, it seemed likely that the reticuloendothelial cells of the bone marrow were not responsible for the degree of ferritin labeling observed in marrow cultures. These findings are in agreement with the results of other workers (17–19) who have shown in several different ways that reticuloendothelial cells have an extremely limited capacity to take up iron bound to transferrin. Although it therefore seems probable that the incorporation of radioiron into ferritin observed in the present study was taking place predominantly in developing red cell precursors, there is still some doubt as to its significance. Recently Mazur and Carleton (6) have suggested on the basis of both *in vivo* and *in vitro* studies that most of the iron derived from plasma is incorporated into ferritin before its incorporation into heme. They based this conclusion on several observations. When rats were given Fe^{59} intravenously, the activity in marrow ferritin dropped between 5 and 12 hours while the activity in hemoglobin was still rising. This relationship was not shown in animals in which radioiron uptake had been stimulated by phenylhydrazine or depressed by endotoxin. Under

these circumstances the rise and fall of radioactivity in ferritin and hemoglobin fractions ran parallel. In further experiments Mazur and Carleton incubated reticulocytes *in vitro* with Fe^{59}-labeled plasma. Further labeling was then stopped after 20 minutes. At this time the specific activity of the ferritin iron was much lower than that of the iron in heme. However, since ferritin contains approximately 2,400 atoms of iron per molecule as compared with only 4 in heme, they calculated that each ferritin molecule had accumulated many more atoms of iron than each heme molecule. They therefore interpreted these observations as indicating a precursor relationship between ferritin iron and heme iron. At the same time these authors pointed out that when incubation of reticulocytes was continued for 120 minutes after further labeling had been stopped, the calculated loss of Fe^{59} per micromole of ferritin was many times greater than the gain by hemoglobin. These results suggested that the major portion of the labeled iron in ferritin might eventually leave the cell. These interpretations depend on the assumption that Fe^{59} taken up by ferritin is uniformly dispersed in a large iron pool. It is, however, doubtful whether such an assumption is justifiable, since it has been shown that plasma-bound Fe^{59} is incorporated to a greater extent into the low density, iron-poor molecules of hepatic ferritin than into the high density ferritin molecules of high iron content (15). This lack of homogeneity of hepatic ferritin indicates the difficulties associated with any quantitative interpretation of the uptake and release of Fe^{59} by ferritin.

In the present study an attempt was made to elucidate the significance of the ferritin formed in red cell precursors by studying the incorporation of Fe^{59} into this fraction under various experimental conditions. At high saturations of transferrin more iron was taken up by red cell precursors, but this additional uptake was not incorporated into heme. These results are in agreement with those obtained by other workers (20). In the present investigation the bulk of the extra iron was found to be incorporated into ferritin. A similar build-up in ferritin activity was noted when hemoglobin synthesis was depressed by lead. In contrast, when marrow from an iron-deficient subject was incubated in radioactive plasma, there was a rapid movement of iron through the hemoglobin precursor into hemoglobin, but no ferritin was formed at any time. These observations suggest that iron is only incorporated into ferritin when more iron enters the cell than can be immediately utilized for hemoglobin synthesis. Since this storage form of iron could not be identified in circulating red cells, it is either utilized for the synthesis of heme at some later stage, or is removed from the red cell precursors. Although it seems entirely possible that ferritin iron is available for hemoglobin formation if required, no evidence in support of this thesis was obtained in the present investigation. In the experiments in which isotope labeling was stopped, the activity in ferritin rapidly reached a plateau, and there was no significant fall in activity for several hours afterwards. In contrast, there was a sharp decline in the activity present in the hemoglobin precursor while the activity in hemoglobin rose. In addition, in studies where labeling was allowed to continue for as long as 12 hours there was a steady build-up in ferritin over the whole period. Although these results in no way exclude the possibility that iron taken up and stored in ferritin is slowly released for hemoglobin synthesis, they afford no positive evidence that this occurs.

If ferritin iron is not utilized for heme synthesis, it must leave the red cell precursors, since labeled ferritin could not be identified in circulating erythrocytes. This could theoretically occur either within the bone marrow or after the reticulocyte enters the circulation. Since it has been established that the spleen has the capacity to remove hemosiderin granules from young red cells (21), *in vivo* experiments were carried out in splenectomized rats to find out whether labeled ferritin could be identified in circulation at any time after the intravenous administration of radioiron. Although appreciable quantities of radioactive ferritin were present in the marrows of these animals, none was found in the circulation. Although these observations appear incompatible with the demonstration that reticulocytes incubated *in vitro* can incorporate radioiron into ferritin, the explanation for this apparent discrepancy may lie in the limited capacity of the reticulocyte to take up iron from the plasma under normal circumstances (17). If Fe^{59} incorporated into fer-

ritin within red cell precursors is not utilized for heme synthesis, then it seems probable that it leaves the cell before it enters the circulation. This possibility would fit in well with previous electron microscopic findings in which it has been demonstrated that ferritin can move freely between the red cell precursors and the reticuloendothelial cells of the marrow (5). Although it was originally suggested by Bessis that the movement was in the opposite direction, several workers have questioned the validity of this interpretation (12, 22). If the present proposal is a valid one it might explain, in part at least, ferrokinetic data which indicate that some of the iron delivered to the marrow enters a labile pool which may feed it back into the plasma again (23). Although evidence has been produced to show that this pool is located on the stroma of red cell precursors, the present findings are compatible with another short circuit involving the movement of iron from plasma into ferritin in red cell precursors, and thence to reticuloendothelial cells that release the iron back into the plasma.

Summary

In vitro and *in vivo* studies were carried out in human subjects and in rats on the uptake and utilization of transferrin-bound iron by red cell precursors. Iron rapidly appeared in a fraction, isolated on column chromatography, which consisted of ferritin and an active hemoglobin precursor. Further experiments were then carried out to elucidate the significance of the ferritin component.

With increasing percentage saturation of transferrin more iron was incorporated into ferritin, while no ferritin labeling was detected when marrow from an iron-deficient subject was cultured. These observations suggest that the iron not immediately required for hemoglobin synthesis in red cell precursors is incorporated into ferritin. No evidence of the later utilization of this ferritin iron for the formation of hemoglobin was obtained, although this possibility could not be excluded. Since labeled ferritin could not be identified in circulating erythrocytes, ferritin iron not utilized for heme synthesis must leave the red cell precursors. The possibility that the spleen might remove ferritin from reticulocytes was investigated in splenectomized rats. Although labeled ferritin was demonstrated in the marrow of these animals after the intravenous injection of Fe^{59}, at no stage could it be detected in circulating red cells. It was concluded that iron incorporated into ferritin in red cell precursors may leave these cells before they enter the circulation. It is possible that this removal is effected by reticuloendothelial cells within the marrow.

Acknowledgments

We would like to thank Drs. H. C. Seftel, D. N. Fuller, and E. J. Joubert for their help.

References

1. Walsh, R. J., E. D. Thomas, S. K. Chow, R. G. Fluharty, and C. A. Finch. Iron metabolism. Heme synthesis *in vitro* by immature erythrocytes. Science 1949, **110**, 396.
2. Allen, D. W., and J. H. Jandl. Kinetics of intracellular iron in rabbit reticulocytes. Blood 1960, **15**, 71.
3. Faber, M., and I. Falbe-Hansen. Nonhæm iron in erythrocytes as a precursor for hæmoglobin. Nature (Lond.) 1959, **184**, 1043.
4. Greenough, W. B. III, T. Peters, Jr., and E. D. Thomas. An intracellular protein intermediate for hemoglobin formation. J. clin. Invest. 1962, **41**, 1116.
5. Bessis, M. C., and J. Breton-Gorius. Iron particles in normal erythroblasts and normal and pathological erythrocytes. J. biophys. biochem. Cytol. 1957, **3**, 503.
6. Mazur, A., and A. Carleton. Relation of ferritin iron to heme synthesis in marrow and reticulocytes. J. biol. Chem. 1963, **238**, 1817.
7. Hanks, J. H., and R. E. Wallace. Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. exp. Biol. (N. Y.) 1949, **71**, 196.
8. Bothwell, T. H., and B. Mallett. The determination of iron in plasma or serum. Biochem. J. 1955, **59**, 599.
9. Bothwell, T. H., P. Jacobs, and R. Kamener. The determination of unsaturated iron-binding capacity of serum using radioactive iron. S. Afr. J. med. Sci. 1959, **24**, 93.
10. Hirs, C. H. W., S. Moore, and W. H. Stein. A chromatographic investigation of pancreatic ribonuclease. J. biol. Chem. 1953, **200**, 493.
11. Labbe, R. F., and G. Nishida. A new method of hemin isolation. Biochim. biophys. Acta (Amst.) 1957, **26**, 437.
12. Bothwell, T. H., and C. A. Finch. Iron Metabolism. Boston, Little, Brown, 1962, pp. 31 and 225.

13. Mazur, A., and E. Shorr. Hepatorenal factors in circulating homeostasis. IX. The identification of the hepatic vasodepressor substance, VDM, with ferritin. J. biol. Chem. 1948, **176**, 771.
14. Proom, H. The preparation of precipitating sera for the identification of animal species. J. Path. Bact. 1943, **55**, 419.
15. Mazur, A., S. Green, and A. Carleton. Mechanism of plasma iron incorporation into hepatic ferritin. J. biol. Chem. 1960, **235**, 595.
16. Bodman, J. Agar gel, starch block, starch gel and sponge rubber electrophoresis *in* Chromatographic and Electrophoretic Techniques, I. Smith, Ed. New York, Interscience Publishers, 1960, vol. 2, p. 91.
17. Lajtha, L. G., and H. D. Suit. Uptake of radioactive iron (^{59}Fe) by nucleated red cells *in vitro*. Brit. J. Haemat. 1955, **1**, 55.
18. Austoni, M. E. Cytoautoradiography Fe^{59} uptake by bone marrow cells. I. Normal human erythroblasts and young erythrocytes. Proc. Soc. exp. Biol. (N. Y.) 1956, **92**, 6.
19. Hosain, F., G. Marsaglia, W. Noyes, and C. A. Finch. The nature of internal iron exchange in man. Trans. Ass. Amer. Phycns 1962, **75**, 59.
20. Najean, Y. Étude de l'influence du plasma sur l'utilisation globulaire du fer "in vitro." II. Étude physio-pathologique. Rev. franç. Étud. clin. biol. 1962, **7**, 605.
21. Crosby, W. H. Siderocytes and the spleen. Blood 1957, **12**, 165.
22. Lajtha, L. G. *In discussion of* Erythropoiesis, as seen with the electron microscope *in* The Kinetics of Cellular Proliferation, F. Stohlman, Ed. New York, Grune & Stratton, 1959, p. 29.
23. Pollycove, M., and R. Mortimer. The quantitative determination of iron kinetics and hemoglobin synthesis in human subjects. J. clin. Invest. 1961, **40**, 753.

THE USE OF RADIOISOTOPES IN THE STUDY OF IRON METABOLISM

T. H. Bothwell
Department of Medicine, Medical School, University of the Witwatersrand
Johannesburg
South Africa

Reprinted from
"RADIOISOTOPES IN TROPICAL MEDICINE"

INTERNATIONAL ATOMIC ENERGY AGENCY
VIENNA 1962

THE USE OF RADIOISOTOPES IN THE STUDY OF IRON METABOLISM

T. H. Bothwell
Department of Medicine, Medical School, University of the Witwatersrand
Johannesburg
South Africa

Abstract—Résumé—Аннотация—Resumen

The use of radioisotopes in the study of iron metabolism. Radioactive isotopes of iron have contributed considerably to present concepts of iron metabolism. Of the many facets which have been studied most attention has been directed to the absorption and excretion of the metal and to the kinetics of its transport within the body.

In the normal adult male only small amounts of iron are excreted and these losses are balanced by the amounts absorbed from the gut. Although it is apparent that variations in the rate of absorption are of importance in the maintenance of iron balance, there is still no clear understanding of how this is achieved. However several factors seem to be of importance. These include: (a) the amount and form of the iron presented to the gut; (b) the functional integrity of the intestinal mucosa; (c) the state of the body's iron stores, and (d) the activity of the erythroid marrow.

In the metabolism of iron within the body the plasma occupies a central role since all the iron being transported from one site to another passes through this compartment. Quantitative kinetic measurements using tracer amounts of Fe^{59} have shown that most of this iron is concerned with haemoglobin production and that erythropoietic activity is the major factor determining the amount of iron passing through the plasma. Using this approach it has been possible to define the patterns of red cell production in a variety of conditions. Since the techniques involved are simple ones they can easily be applied to the routine handling of haematologic problems.

Les radioisotopes dans l'étude du métabolisme du fer. Les radioisotopes du fer ont largement contribué à expliquer le métabolisme de ce métal. L'auteur a surtout étudié l'absorption et l'excrétion du fer, ainsi que la cinétique de son transport à l'intérieur de l'organisme.

L'adulte mâle normal n'excrète que de faibles quantités de fer; ces pertes sont compensées par les quantités absorbées de l'intestin. Il est manifeste que les variations du taux d'absorption influent sur le bilan du fer, mais le mécanisme de ce processus est encore mal connu. Toutefois, plusieurs facteurs semblent intervenir, notamment: *a*) les quantités de fer offertes à l'intestin ot la forme sous laquelle le métal se présente; *b*) l'intégrité de la fonction de la muqueuse intestinale; *c*) l'état des réserves de fer de l'organisme; *d*) l'activité de la moelle rouge.

Le plasma joue un rôle primordial dans le métabolisme du fer à l'intérieur de l'organisme, car c'est par lui qu'est transporté tout le fer qui passe d'un endroit à l'autre du corps. Il ressort de mesures cinétiques quantitatives faites à l'aide du fer-59 employé comme indicateur, que la plus grande partie du fer sert à produire de l'hémoglobine et que les quantités de fer transportées par le plasma dépendent surtout de l'activité érythropoiétique. Cette méthode a permis de définir les modes de production des globules rouges dans différentes conditions. Les techniques employées sont simples et se prêtent aisément à la solution courante des problèmes d'hématologie.

Использование изотопов в исследованиях метаболизма железа. Использование радиоактивных изотопов железа внесло значительный вклад в нынешнюю концепцию метаболизма железа. Из многих изученных фактов наибольшее внимание было уделено абсорбции и выделению этого металла и кинетике его перемещения внутри организма.

У нормального взрослого мужчины выделяется лишь небольшое количество железа, и эти потери уравновешиваются количествами, поглощаемыми из кишечника. Хотя очевидно, что различия в степени абсорбции имеют значение для сохранения равновесия железа в организме, все еще не ясно, каким образом это достигается. Ряд факторов, однако, представляется важным. В их число входят: a) количество и форма железа, присутствующего в кишечнике, b) функциональная целостность слизистой оболочки кишечника, c) состояние запасов железа в организме, и d) деятельность эритроидного мозга.

В метаболизме железа в организме плазма играет центральную роль, так как все железо, переносимое из одного участка в другой, проходит через эту преграду. Количественные кинетические замеры с использованием индикаторных количеств железа-59 показали, что большая часть этого железа связана с производством гемоглобина и что эритротворная деятельность является главным фактором, определяющим количество железа, проходящего через плазму. При этом подходе стало возможным определить формы производства красных кровяных телец в различных условиях. Поскольку связанные с этим методы являются простыми, они легко могут применяться при обычной разработке гематологических проблем.

Los radioisótopos en el estudio del metabolismo del hierro. Los isótopos radiactivos del hierro han contribuido notablemente a ampliar nuestros conocimientos sobre el metabolismo de ese elemento. El autor ha estudiado con particular atención los procesos de absorción y excreción del hierro, así como la cinética de su transporte en el interior del organismo.

El varón adulto normal sólo excreta pequeñas cantidades de hierro; esas pérdidas son compensadas por las cantidades absorbidas al nivel del intestino. Si bien es evidente que las variaciones del índice de absorción ejercen influencia sobre el balance del hierro, el mecanismo de este proceso sólo se conoce imperfectamente. Con todo, existen al parecer varios factores determinantes, entre ellos, los siguientes: a) la cantidad y la forma del hierro presente en el intestino; b) la integridad funcional de la mucosa intestinal; c) el estado de las reservas de hierro del organismo; d) la actividad de la médula roja.

El plasma desempeña un papel primordial en el metabolismo del hierro, puesto que asegura el transporte de todo el hierro que pasa de un punto a otro del organismo. Se ha demostrado mediante mediciones cinéticas cuantitativas, efectuadas con ^{59}Fe como indicador, que la mayor parte del hierro interviene en la producción de hemoglobina y que la actividad eritropoyética es el principal factor determinante de la cantidad de metal transportada por el plasma. Este procedimiento ha permitido determinar el esquema de formación de los hematíes en diferentes condiciones.

Las técnicas empleadas son sencillas y permiten resolver los problemas hematológicos corrientes.

Introduction

Using radioactive isotopes of iron it has been possible to approach the investigation of iron metabolism from a number of different angles. Such measurements can be divided into two large groups. In the first the exchange between the individual and his environment has been characterized by quantitating the amounts absorbed and excreted while, in the second, exchange between tissues within the body has been followed. In this way it has been possible to construct an overall scheme for iron metabolism and to define a number of ways in which it may become disturbed.

In studies using radioactive iron two isotopes have been used. Fe^{59} has a half-life of 45 d and decays with both beta- and gamma-ray emission while Fe^{55} has a half-life of 2.94 yr and decays with the emission of Mn characteristic X-rays. Of the two isotopes Fe^{59} has been more widely used since its gamma emissions are easy to count and, in addition, it has a relatively short half-life. The type of counting set-up used

is dependent on the size and nature of the samples to be analyzed. A well-type scintillation counter is ideal for blood and plasma samples but is not as suitable for samples larger than a few cc (e.g. faeces) since aliquots must then be taken. Under such circumstances it is preferable to use a counter large enough to take the whole sample as this eliminates the necessity for homogenization. A suitable counter for this type of work is one constructed from a ring of mached GM tubes [1]. A carton containing the sample is placed in the centre of the ring and the counts obtained are recorded on a suitable scaler. In some studies both Fe^{59} and Fe^{55} are used. When this is done the preparation and counting of samples is more complex as the samples must be digested and electroplated on to copper discs. The beta emissions of the Fe^{59} can then be counted with a helium-filled GM tube and the X-rays of the Fe^{55} with an argon-filled tube covered by a beryllium filter [2]. Although such techniques are laborious they are not particularly difficult and have several applications since there are a number of ways in which the two isotopes can be advantageously used in the same individual.

External iron exchange

Technical considerations

Iron Absorption. In early studies the percentage absorbed was estimated by determining the amount which subsequently appeared in the blood as circulating haemoglobin [3]. The method was a simple one since all that was required was an aliquot of blood collected 10—14 d after the administration of radioiron. The total amount in circulation was calculated by using a figure for blood volume estimated from body weight and it was then possible to calculate the percentage of the administered dose which was absorbed. In this method it was assumed that all the iron absorbed into the plasma was rapidly utilized for haemoglobin production. However, subsequent investigations have shown that this is not usually so and only in iron deficiency anaemia is 100% incorporated into red cells [4—6]. In normal subjects the figure is about 80% while in some anaemias it may be very low and the percentage of radioiron in circulation under these circumstances is therefore no reflection of the amount absorbed. In spite of these qualifications the method is still applicable to certain types of study and can be expected to give moderately accurate results in normal subjects and in patients with iron deficiency anaemia.

Another method for measuring iron absorption is one in which the amount of unabsorbed radioiron in faeces is measured [7]. The percentage absorption is then determined by the difference between intake and faecal loss. In this technique all faeces are collected until less than 0.5% of the administered dose is present in a 24 h specimen. This normally occurs between 5 and 7 d after the giving of the test dose. The chief disadvantages of the method lie in the prolonged faecal collections and in the very limited absorption normally found. Since the percentage absorption is usually less than 10%, a minor inaccuracy either in stool collections or in the estimation of their radioiron content may alter the calculated figure for absorption considerably. The method is, however, accurate enough for most purposes and has yielded a considerable amount of meaningful information.

In many ways the most attractive of current methods for measuring iron absorption is one in which two isotopes of iron are used [8]. With this technique it is possible to obtain absolute figures for iron absorption without resorting to stool collections or to blood volume estimations. The rationale of the method is as follows: when iron is absorbed from the bowel it passes into the plasma. From there the major

portion is directed towards the erythroid marrow and subsequently it appears in the circulation incorporated in haemoglobin. In addition, variable amounts go to other tissues. Now the blood is the most convenient iron compartment to sample but no absolute measure of absorption can be obtained unless corrections can be made for the fraction going to other sites. This can be done using two isotopes of iron. A known amount of Fe^{55} is given orally and a known amount of Fe^{59} bound to plasma is simultaneously injected intravenously. The subsequent distribution of the iron absorbed into the plasma from the gut has been shown to be the same as that given intravenously [8—9] so that if a blood sample is collected when circulating radioactivity has reached a stable level, the actual amount of absorption can be calculated:

$$\% \text{ Absorbed} = \frac{\text{Ratio } Fe^{55}/Fe^{59} \text{ in Red Cells}}{\text{Ratio } Fe^{55}/Fe^{59} \text{ Administered}} \times 100$$

Although the fact that samples must be digested and electroplated prior to counting is a disadvantage the method is the most accurate of current techniques and has been profitably applied to clinical studies. Both isotopes can also be used in another way. Interest sometimes centers not so much on the absolute amount of iron absorbed as on the relative difference between the absorption of iron administered in various ways. However, the individual variations in absorption are so large that it is difficult to compare results in different subjects without testing large numbers. This problem can be overcome when both test doses are given consecutively on 2 days to the same individual [9]. The subsequent levels of the two isotopes in a blood sample collected 2 weeks later will then give a measure of their relative absorption. This approach is especially valuable in assessing the efficiency of different therapeutic preparations of iron and the effects of such factors as reducing agents, blocking agents and food on the absorption of iron.

Iron Excretion. Two different approaches have been used in the measurement of iron excretion using radioactive iron. In the first, radioiron is injected intravenously and the amounts which subsequently appear in excreta, hair, nails and sweat are measured [10]. Such studies can be continued over long periods especially when Fe^{55}, with its long half-life is used. The amount of iron excreted is then calculated by relating the isotope excretion to the ratio of injected radioiron to total body iron. In the second method Fe^{55} is again injected intravenously and measurements are then made of the change in specific activity with time [11]. When this is done there is an initial drop in the level of radioactivity in circulating blood over the first year which has been interpreted as due to mixing of radioiron with non-erythrocytic iron. Thereafter the drop in circulating radioactivity occurs at a slow exponential rate which probably reflects the loss of iron from the body. Calculations from such data suggest that the normal male loses about 0.6 mg daily and the menstruating female 1.2 mg [11].

Pathophysiology of external iron exchange

Quantitative aspects of iron absorption and excretion have been well defined in a number of studies. During infancy and childhood there is a positive iron balance in which more is being absorbed into the body than is excreted. Under optimal conditions the infant absorbs about 0.5 mg daily and this probably rises a little in childhood and adolescence to between 0.5 and 1 mg daily [12]. These amounts more than balance the small quantities excreted with the result that there is a steady increase in the iron content of the body. In this way the needs of an expanding red cell mass

and an enlarging storage depot are satisfied so that by the time adulthood is reached 3—4 g are present in the body. During adult life the situation is somewhat different. In the male the absorption rate falls to about 0.6 mg daily which is about equal to the losses in excreta and from the desquamation of skin [13—15]. In the female, on the other hand, there are the added losses incurred by menstruation and pregnancies which raise the daily requirements to between 1 and 2 mg.

From these various observations it is apparent that the absorptive mechanism plays a key role in the maintenance of iron balance and isotopic studies have contributed considerably to the understanding of what factors influence mucosal behaviour. Iron is probably absorbed in the ferrous state by mucosal cells [16] and it has been shown that this occurs maximally in the duodenum with a decreasing gradient through the lower bowel [17]. Since iron in food is mostly in the ferric form, complexed with organic molecules, there must be mechanisms within the lumen of the upper bowel whereby the iron is first made ionizable and then is reduced to render it available for absorption. How this is accomplished is by no means certain. Although gastric hydrochloric acid was previously thought to play a role in the breakdown of the iron-protein complexes in food, subjects with achlorhydria do not show impaired absorption [18]. This does not imply that hydrochloric acid has no action but suggests that it is not a critical factor. Of more importance seem to be the composition of the diet and the relative proportions of different constituents. It has been shown that absorption is enhanced when large amounts of reducing agents, such as ascorbic acid, are added to the diet [13, 15] or when the phosphate content is low [19]. On the other hand absorption is reduced when an excess of phytate is added [20]. It is, however, still not clear to what extent absorption of food iron is influenced by the quantities of these substances present in the normal diet. Possibly there are local secretory factors of importance although as yet there is no experimental proof of their existence.

The quantity of iron absorbed at any one time does not, however, depend solely on the number of free ferrous ions presented to the mucosa of the upper bowel. The absorptive process is an active one, and the mucosal cells have the capacity of accepting or rejecting iron according to the body's needs. Although this ability is a more limited one than was formerly thought, there seems no doubt that there are internal regulatory factors that affect the behaviour of the mucosal cells. Two major influences appear to be important: the size of the iron stores and the rate of erythropoiesis [9]. When the body is depleted of iron or when red-cell production is increased, there is a rise in the absorption rate, whereas increased iron stores or decreased erythropoiesis leads to a depression of iron absorption. How these various stimuli exert their effects on the mucosal cells is not known.

Although the variations in absorption that have been described in different conditions are of considerable academic interest, it is important to realize that the amounts of iron that can be absorbed from food, even with a maximal stimulus, are fixed within a comparatively narrow range. A normal diet contains about 10 to 15 mg of iron, and in the healthy adult only about 5% of this is absorbed and even when gross iron deficiency anaemia is present absorption can only be increased to between 2 to 5 mg daily [13, 15]. When this is measured against the small, though significant, normal losses it is apparent that the balance between absorption and excretion is a delicate one that can easily become disturbed when abnormal losses of iron occur.

The relative unavailability of iron in foodstuffs for absorption is due to a number of factors, the most important of which is probably the tight binding of the iron into organic molecules. Thus simple iron salts, especially the ferrous ones, are ab-

sorbed at least twice as well as iron in food. When such salts are administered to healthy adults in doses up to 1 mg/kg about 5 to 15% is absorbed while in patients with iron deficiency anaemia 20 to 60% is retained [7, 9].

The isotopic investigations which have defined the various limits to iron absorption and excretion provide a useful backcloth against which to consider the clinical disorders that are due to derangements in external iron exchange. In essence these disorders can be divided into two major groups:—those in which there is too little iron and those in which there is too much iron in the body.

Iron deficiency

Iron deficiency can only develop in one of three ways. It is either due to (a) deficient intake or poor absorption, (b) excessive loss of iron from the body, or (c) a combination of both factors.

A *deficient intake of iron* is the most common cause of iron deficiency in the infant and growing child. As with the adult the infant normally absorbs less than 10% of the iron in food and it has been calculated that an intake of 1.0 to 1.5 mg/kg/d is necessary if requirements are to be satisfied [12]. These requirements are only just met when the health is good and the diet optimal and in the absence of such conditions iron deficiency can easily develop.

In the adult male a deficient intake is almost never a cause of iron deficiency, since losses are small and when the diet is inadequate extra iron can readily be mobilized from body stores. In the adult female on the other hand there are the added losses incurred by menstruation and pregnancies. Thus, to remain in iron balance the normal female must absorb greater quantities of iron from the diet but this is not easy to do since much of the iron in food is relatively unavailable. These difficulties are even more serious when the intake of iron-containing foods is less than normal and under these circumstances iron deficiency readily develops.

Poor absorption through the bowel wall is a definite though rare cause of iron deficiency. It is a feature of idiopathic steatorrhoea in which there is a general malabsorptive defect but is not present in steatorrhoea due to anatomic disease of the bowel wall or to pancreatic failure [21]. It has also been noted that patients who have undergone partial gastrectomy do not absorb iron from food well [15]. This is, however, almost certainly not due to any change in the behaviour of the bowel mucosa since the absorption of simple iron salts is normal [22]. It therefore seems probable that the defective absorption is due to the rapid transmission of the food bolus through that part of the upper bowel where conditions for absorption are optimal.

Although an inadequate intake of iron is sometimes the primary factor in the aetiology of iron deficiency it is more often only a secondary one. By far the commonest cause is *blood loss*. The degree of haemorrhage need not be great. For example, a loss of 5 to 10 cc of blood/d, which is equivalent to 2 to 5 mg of iron is sufficient to cause a negative iron balance and eventual iron deficiency. In the female the equilibrium is most commonly disturbed by excessive menstruation or repeated pregnancies while in the male the source of bleeding is usually in the bowel. Although these are the only important sources of loss in Western climates the possibility has been raised that excessive sweating may be a significant one in tropical climates. Results of chemical studies suggest that the concentration of iron in sweat may be as much as 6 mg/l most of it being derived from desquamated cells [23]. Although chemical methods have given misleading results in the past, these findings are of such potential importance that the problem deserves careful reappraisal using isotopic techniques.

Iron excess

Although iron deficiency is the most common disorder of iron metabolism there are three situations in which an excess of iron can accumulate in the body. The first is the condition of idiopathic haemochromatosis, a disease characterized by an alteration in the behaviour of the gut mucosa that leads to an excessive absorption of iron [24]. The second occurs when large amounts of iron are present in the diet [25] and the final one is found in patients with refractory anaemias who are given many blood transfusions [24].

In idiopathic haemochromatosis the deviation from the normal absorption rate is not a large one. Isotopic studies indicate that the majority of subjects with the disease only absorb about 3 to 4 mg daily but this is enough to create a positive iron balance and by middle age between 25 and 50 g are present in the body [24]. The same thing can happen when there are excessive quantities of iron in the diet. For example, the Bantu of Southern Africa consume a diet containing up to 100 mg daily [25] and the majority of adults show varying degrees of iron overload [26]. It is therefore apparent that there is no absolute block to iron absorption and that if enough is presented to the mucosal cells excessive amounts will be absorbed. The last situation associated with an excess of iron in the body is found in subjects with refractory anaemias maintained on repeated blood transfusions for long periods. Since each pint of administered blood contains 250 mg iron large amounts can be introduced by the intravenous route. Although this is the commonest cause of iron overload in subjects with refractory anaemias it is not the only one since there is evidence that a proportion develop iron overload even when little blood has been given. This is seen particularly in those with hypercellular marrows who have received prolonged oral medication with iron [27].

There seems little doubt that gross degrees of iron overload can eventually lead to pathologic changes and this is especially so when the deposits are predominantly in parenchymal cells [24]. Thus the liver, which contains the largest amounts becomes cirrhotic, pancreatic involvement leads to diabetes and myocardial deposits to heart failure.

Internal iron exchange

Over the past few years a considerable amount of work has been done on the transport of iron within the body. In this transport the plasma occupies a central role since all the iron being transferred from one part of the body to another passes through this compartment. If, therefore, the plasma iron is labelled with a tracer quantity of radioactive iron the normal transport mechanism can be studied [28].

Technical considerations

About 10 ml of the patient's plasma is incubated with 5—10 μc of high specific activity Fe^{59} for about 30 min. A weighed amount of the plasma is then injected intravenously and a small quantity is kept for the preparation of standards. Several blood samples are collected over the next 2 to 3 h and their radioactive content is measured. In addition a plasma iron estimation is also performed. The levels of radioactivity in plasma samples over the first few hours are then plotted on semi-logarithmic paper and normally give a straight line showing an exponential fall in activity. The amount of iron passing through the plasma per day can then be calculated using the following formula:

$$\text{Plasma Iron Turnover} = \frac{\text{Total Plasma Iron (mg)} \times \text{Natural Logarithm of } 2 \times 24}{\text{Time taken for Radioactivity to reach Half Concentration (h)}}$$

Normally the plasma iron turnover is between 30 and 35 mg/d which indicates that the small plasma iron pool is renewed about 12 times daily [28].

When plasma iron turnover measurements are carried out it is customary to continue blood sampling at daily intervals for about 2 weeks, in order to find out how much of the iron passing through the plasma is incorporated in new red cells [28]. Normally about 70 to 90% of the injected radioactivity re-appears in circulation which suggests that most of the iron being transported through the plasma is normally being directed to the bone marrow for haemoglobin synthesis.

Additional useful information can also be obtained if plasma iron turnover and red cell utilization data are coupled with *in-vivo* counting over various body organs [29]. This is usually done over bone marrow, liver and spleen. When the counting rates obtained at various times after the injection of radioiron are plotted graphically a qualitative measure is provided of the movement of iron in and out of these organs.

Interpretation of ferrokinetic measurements

Although tracer studies are relatively easy to perform there are a number of difficulties relating to their interpretation. This is due to the fact that at least three major variables might theoretically affect the plasma iron turnover. These include the rate of red cell destruction, the rate of red cell production and the state of the body's stores. Attempts have, therefore, been made to find out to what extent these different factors do, in fact, influence plasma iron turnover.

In assessing the effects of increased transfer between stores the best example is probably that of idiopathic haemochromatosis since the stores are enlarged manifold in this condition. In spite of this the plasma iron turnover only rises by about 50% which suggests that gross alterations in the size of body stores can affect it but to only a limited extent [30]. The second condition is one in which there is increased red cell destruction but no increase in red cell production. When such a situation is produced experimentally the plasma iron level rises but the turnover remains the same [30]. It is therefore evident that increased red cell destruction does not affect the amount of iron passing through the plasma. On the other hand there is a close correlation between the plasma iron turnover and the rate of erythropoiesis. After bleeding it rises to approximately twice normal and when the rate of erythropoiesis is depressed by X-irradiation, cytotoxic agents or hypertransfusion there is a drop to about 50% of normal [30].

All these various observations seem to indicate that the major regulating factor for the amount of iron passing through the plasma is the rate of erythropoiesis and the plasma iron turnover has therefore been extensively used as a simple and moderately accurate index of erythropoietic activity [6, 28, 30]. On the whole the correlation between such measurements and other indices of erythropoietic activity such as the reticulocyte count has been a good one. In acute haemolytic anaemias the turnover is 2 to 3 times normal while in haemolytic anaemias of long standing (e.g. congenital spherocytosis) it may increase to between 6 and 8 times normal. With marrow hypofunction results tend to be lower than normal but the results are not quantitatively meaningful since there is increased exchange between other tissues [30]. In these various conditions the red cell utilization patterns are also helpful. Normally about 80% of the injected radioactivity re-appears in red cells

within 10—14 d. In haemolytic anaemias the radioiron usually re-appears rapidly but stabilizes between 50 and 60% while in aplastic anaemia the red cell utilization is very low [6]. Although ferrokinetic measurements usually correlate well with other indices there is a group of conditions in which interesting discrepancies have been noted. It includes pernicious anaemia [31], Cooley's anaemia [32] and certain refractory normoblastic anaemias [33]. In these diseases the plasma iron turnover is very high which is consistent with the hypercellular erythroid marrows present. However, very little of the radioiron appears in new red cells and the reticulocyte counts are low. It has been suggested on this and other evidence that a good deal of the marrow activity is ineffective in terms of the delivery of viable red cells to circulation [34]. It seems probable that in these conditions a large number of red cell precursors are broken down *in situ* in the marrow or that red cells are delivered into circulation which only survive for a very short period.

Since the plasma iron turnover and red cell utilization data are so helpful in defining the degree and type of erythropoietic activity they have wide applications in clinical practice. However, it should be remembered that such measurements do not stand alone but must be correlated with morphologic and other criteria. When used in this way it is possible to gain insight into quantitative aspects of red cell production and to assess whether the marrow is effective or ineffective in terms of the delivery of viable red cells. In addition, calculations can be made which indicate whether the erythroid marrow is responding optimally to the stimulus it is receiving. From available data it would appear that the marrow should increase its output two to threefold with acute anaemia and more than sixfold with chronic anaemia. When figures less than this are obtained relative marrow hypofunction is present and it has been shown that this is a factor in the genesis of a number of anaemias, e.g. cancer [35], rheumatoid arthritis [36], etc.

At a more fundamental level tracer data have emphasized the fact that most of the iron within the body is intimately concerned in the cycle of blood production and destruction. Only in conditions of iron overload does transfer between other tissues reach significant proportions. Unfortunately ferrokinetic measurements have contributed little so far to our understanding of the turnover and activities of that small, but vital, portion of the body's iron which is contained within enzymes. This aspect of iron metabolism remains a major challenge for future investigation.

REFERENCES

[1] VEALL, N. and VETTER, H., *Brit. J. Radiol.* **25** (1952) 85.

[2] PEACOCK, W. C., EVANS, R. D., IRVINE, J. W. (Jr.), GOOD, W. M., KIPP, A.F., WEISS, S. and GIBSON, J. G., *J. clin. Invest.* **25** (1946) 605.

[3] HAHN, P. F., BALE, W. F., ROSS, J. F., BALFOUR, W. M. and WHIPPLE, G. H., *J. exper. Med.* **78** (1943) 169.

[4] DUBACH, R., MOORE, C. V. and MINNICH, M. S., *J. Lab. clin. Med.* **31** (1946) 1201.

[5] FINCH, C. A., GIBSON, J. G., PEACOCK, W. C. and FLUHARTY, R. G., *Blood* **4** (1949) 905.

[6] BOTHWELL, T. H., CALLENDER, S., MALLETT, B. and WITTS, L. J., *Brit. J. Haematol.* **2** (1956) 1.

[7] DUBACH, R., CALLENDER, S. T. E. and MOORE, C. V., *Blood* **3** (1948) 526.

[8] SAYLOR, L. and FINCH, C. A., *Amer. J. Physiol.* **172** (1953) 372.

[9] BOTHWELL, T. H., PIRZIO-BIROLI, G. and FINCH, C. A., *J. Lab. clin. Med.* **51** (1958) 24.

[10] DUBACH, R., MOORE, C. V. and CALLENDER, S., *J. Lab. clin. Med.* **45** (1955) 599.

[11] FINCH, C. A., *J. clin. Invest.* **38** (1959) 392.

[12] SCHULTZ, J. and SMITH, N. J., *A. M. A. J. Dis. Child.* **95** (1958) 109.

[13] MOORE, C. V., *Amer. J. clin. Nutrition* **3** (1955) 3.

[14] CHODOS, R. B., ROSS, J. F., APT, L., POLLYCOVE, M. and HALKETT, J. A. E., *J. clin. Invest.* **36** (1957) 314.

[15] PIRZIO-BIROLI, G., BOTHWELL, T. H. and FINCH, C. A., *J. Lab. clin. Med.* **51** (1958) 37.

[16] MOORE, C. V., DUBACH, R., MINNICH, V. and ROBERTS, H. K., *J. clin. Invest.* **23** (1944) 755.

[17] BROWN, E. B. (Jr.) and JUSTUS, B., *Amer. J. Physiol.* **194** (1958) 319.

[18] MOORE, C. V. and DUBACH, R., *J. Amer. med. Ass.* **162** (1956) 197.

[19] KINNEY, T. D., HEGSTED, M. and FINCH, C. A., *J. exper. Med.* **90** (1949) 137.

[20] SHARPE, L. M., PEACOCK, W. C., COOKE, R. and HARRISS, R. S., *J. Nutrition* **41** (1950) 433.

[21] BADENOCH, J. and CALLENDER, S. T., *Blood* **9** (1954) 123.

[22] SMITH, M. D. and MALLETT, B., *Clin. Sci.* **16** (1957) 23.

[23] FOY, H. and KONDI, A., *J. trop. Med. Hygiene* **60** (1957) 105.

[24] FINCH, S. C. and FINCH, C. A., *Medicine* **34** (1955) 381.

[25] WALKER, A. R. P. and ARVIDDSON, U. B., *Trans. roy. Soc. trop. Med. and Hyg.* **47** (1953) 536.

[26] BOTHWELL, T. H. and BRADLOW, B. A., *Arch. Path.* **70** (1960) 13.

[27] WYATT, J. P., *Arch. Path.* **61** (1956) 56.

[28] HUFF, R. L., HENNESSY, T. G., AUSTIN, R. E., GARCIA, J. F., ODA, J. M., COCKRELL, M. C. and LAWRENCE, J. H., *J. clin. Invest.* **29** (1950) 1041.

[29] ELMLINGER, P. J., HUFF, R. L., TOBIAS, C. A. and LAWRENCE, J. H., *Acta Haematol.* **9** (1953) 73.

[30] BOTHWELL, T. H., HURTADO, A. V., DONOHUE, D. M. and FINCH, C. A., *Blood* **12** (1957) 409.

[31] FINCH, C. A., COLEMAN, D. H., MOTULSKY, A. G., DONOHUE, D. M. and REIFF, R. H., *Blood* **11** (1956) 807.

[32] STURGEON, P. and FINCH, C. A., *Blood* **12** (1957) 64.

[33] DACIE, J. V., SMITH, M. D., WHITE, J. C. and MOLLIN, D. L., *Brit. J. Haematol.* **5** (1959) 56.

[34] GIBLETT, E. R., COLEMAN, D. H., PIRZIO-BIROLI, G., DONOHUE, D. M., MOTULSKY, A. G. and FINCH, C. A., *Blood* **11** (1956) 291.

[35] MILLER, A., CHODOS, R. B., EMERSON, C. P. and ROSS, J. F., *J. clin. Invest.* **35** (1956) 1248.

[36] FREIREICH, E. J., ROSS, J. F., BAYLES, T. B., EMERSON, C. P. and FINCH, S. C., *J. clin. Invest.* **36** (1957) 1043.

DISCUSSION V

L. Hallberg: In connection with Dr. Bothwell's elegant review on his studies on iron absorption and iron metabolism I would like to mention some data from our laboratory [L. HALLBERG *et al. Acta Med. Scand.* **168** suppl. 358]. I will do this not so much for the discussion of the regulation of iron absorption but rather as an example of the possibilities of using simultaneously more than one radioisotope of the same element in absorption studies. Absorption studies are usually designed as a balance study where the absorption is calculated from the difference between input and output of a certain substance. In such studies no information is obtained on the time element of the absorption. For closer analysis of an absorption process it may be necessary to determine the absorption rate to be able to analyse factors influencing the absorption. In such studies radioisotopes will be of great help. The change of concentration of iron in

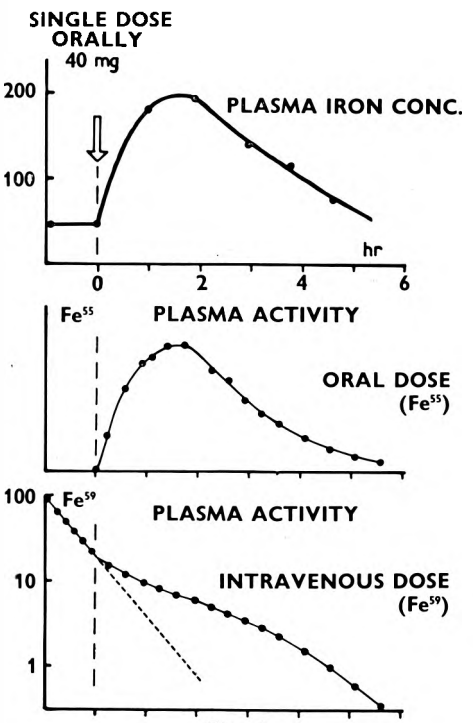


Fig. 1

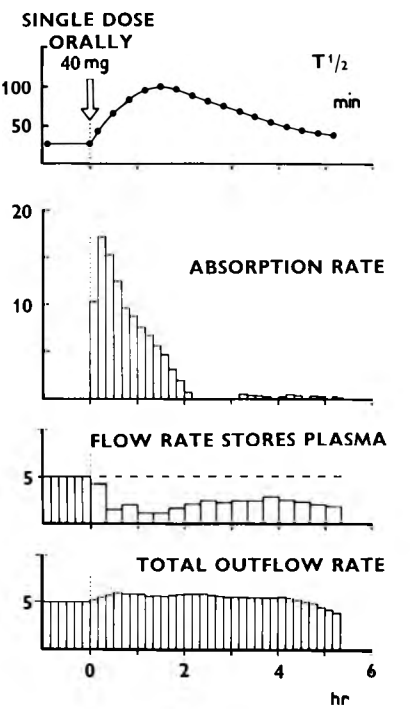


Fig. 2

plasma during the absorption is dependent on the absorption rate, on the outflow of iron from plasma to the bone marrow and storage compartments, on the inflow of iron from catabolized red cells, for instance, and on the plasma volume. If the orally administered iron is labelled with one isotope and if another radioiron isotope is administered intravenously in trace amounts to determine the outflow of iron from plasma it is possible, as illustrated in Figs. 1 and 2, to determine and follow continuously the absorption rate and the in and outflow of iron to and from plasma.

B. W. Subhiya: With regard to iron loss in sweat mentioned by Dr. Bothwell and by Dr. Foy, I should like to report that in Baghdad we have been working

on the problem using the whole-body counting technique described by Dr. Veall. After intravenous administration of Fe^{59}, counting is carried out at 10-day intervals with the patient standing in a "cage" surrounded by eight G.26 GM counters. We have investigated four volunteers and from the results found the effective half-life to range from 38.5 to 41 d, and have calculated the biological half-life as ranging from 260 to 450 d. Now our difficulty is that we have no base line with which to compare the figures we have obtained, and we are planning to repeat the experiments with the same volunteers under colder conditions during the winter, so as to have a basis for comparison. OLIVER and LAJTHA [*Nature* **134** (1959) 1882] concluded, by indirect calculation from daily loss and body stores of iron, that the normal biological half-life of iron is 1400 d.

We have also attempted some short-term experiments taking the form of whole-body counts of patients at the beginning and end of 4-day periods of exercise and rest, but no differences between the two periods have been detected.

G. R. Wadsworth: In Dr. Bothwell's excellent paper precise measurements of the intake of iron into the body from the lumen of the gut were reported, and we have also heard exact data from Dr. Roche on iron losses in hookworm disease. I should like to draw attention to a very serious defect in knowledge caused by our almost total reliance on the analyses given in standard food tables. Perhaps I may illustrate this with one or two examples. Fox, quite a long time ago now, showed that although the average iron content of maize meal in South Africa was 3.8 mg/100 g, the content of individual samples ranged from 1.2 to 7.1 mg [*S. Afr. J. Sci.* **36** (1939) 36]. Some analyses I made of rice bought in shops in London showed iron contents varying from as little as 1.7 to as much as 4.6 mg, about three times as much. In a recent survey in a country in the Western Pacific region it was found that, whilst the iron intake of one group of subjects was 12.7 mg/d as calculated from food tables, strict chemical analysis of food as eaten gave a figure of 82.2 mg. In another group calculated intake was 13.2 mg whereas that based on analysis was 34.3 mg. I think it is clear, therefore, that not much reliance can be placed on measurements of dietary iron intake based on standard food tables.

T. H. Bothwell: I agree wholeheartedly with Dr. Wadsworth about the difficulty in knowing what iron intakes are. In the case of the Bantu of Southern Africa, for example, it is useless to take any statement by a subject as a reliable guide to what he actually has eaten. I feel that the most accurate way of determining intake of food iron is measurement of the faecal content. Absorption is so low that one can be fairly certain that a 24-hour specimen of faeces is very close to what the subject is getting. I have no doubt that for field studies faecal analysis would be the answer to many of our problems in this connection.

C. S. Leithead: I should like to ask Dr. Bothwell if he has considered the possibility that in malaria there may be iron stores which are not available for haemoglobin synthesis, at least for a period immediately following their deposition. I refer to the work of my colleagues Dr. DEEGAN and Professor MAEGRAITH on malarial pigment which they originally identified and have continued to study [*Ann. trop. Med. Parasitol.* **50** (1956) 194 and 212; **52** (1958) 232]. This is an area in which isotopes are likely to be extremely valuable, that is to say in the study of metabolism and distribution of iron in the malarial host. I should like to summarize my colleagues' recent observations on *P. berghei* malaria in rats.

The isotope Fe^{59} was administered subcutaneously as a standard inoculum of 1.0 μc in 1.0 μg total iron. The distribution in the blood, liver, spleen, kidneys and

femur (bone marrow) was followed at intervals for 50 d in groups of normal animals. The isotope was incorporated into erythrocytes within 10 h, indicating a very rapid uptake by the emerging reticulocytes, which could be confirmed autoradiographically. In this way a highly radioactive "batch" of erythrocytes entered the circulation. Thereafter the overall blood content of radioactivity remained constant at about 60% of the total isotope injected.

The original batch of tagged erythrocytes reached the end of their life-span in approximately 60 d and at this point a rise in bone marrow and spleen content of the isotope (but not in that of the liver) was recorded, presumably indicating a turnover of Fe^{59} from the disintegrating cells. The level of activity in the liver, which remained constant subsequent to the tenth day after injection of the isotope and was not affected by the disintegration of the tagged erythrocytes, indicates a remarkably static iron store, which autoradiographs showed was largely in the form of ferritin.

Animals were infected intraperitoneally with *P. berghei* on the twentieth day after inoculation of the isotope, at which point the distribution of Fe^{59} had reached equilibrium. The progress of the infection was subsequently followed, groups of rats being examined at the crisis, i.e. the period of maximal erythrocyte infection (20—25%; about 10 d after inoculation), and subsequently at intervals up to the recovery stage (54—67 d).

The isotope content of the blood of the infected animals fell appreciably during the period of heavy parasitic infection and subsequently returned to a figure lower than that reached in the equilibrium established 20 d after inoculation of the Fe^{59}.

The splenic content rose during the heavy parasitaemia to as much as 12 times the normal. It subsequently fell in the convalescent stage to about three times normal level, where it remained to the end of the experiment.

The liver content was roughly doubled during the parasitaemia and returned slowly to normal in recovery. That of the bone marrow rose at the height of the infection to about 60% above the normal value and fell to the original level in recovery. The content of the kidneys was increased slightly during the parasitaemia in animals exhibiting haemoglobinuria (with visible iron-containing residues in the renal tubular epithelium), and returned to normal in recovery.

The total content of Fe^{59} in the blood and the tissues studied in animals on reaching the recovery stage was about 10% less than in normal animals given the same dose of isotope. As there was no evidence of abnormal excretion of iron in the infected animals, some of the radioactive iron had thus clearly become "unavailable" during the production of reticulocytes in the convalescent period. The proportion of this "lost" iron deposited in other tissues in the form of insoluble haemozoin and that stored as ferritin or haemosiderin was not assessed. Histological studies demonstrated, however, that deposition of the former was considerable.

In the liver there was a considerable rise of Fe^{59} content during active parasitization of the erythrocyte and a fall in the first stage of recovery. Turnover of iron must therefore have been very active and since the synthesis of haemoglobin subsequent to the crisis of the infection involved much less Fe^{59}, the static liver content in recovery must indicate radioactivity due to the accumulated haemozoin (malarial pigment), which was clearly not available for synthesis.

In the case of the spleen the rapidly rising concentration at the crisis and the subsequent persistence of a high level can best be accounted for by deposition of large amounts of unavailable iron.

At the height of infection the spleen contained large deposits of granules of haemozoin and haemosiderin and a blue haze of visible ferritin. Ten days later the only visible iron-containing pigment was haemosiderin (very active autoradiographically). It can be presumed therefore that the high concentration in the crisis resulted from deposition in the tissues of fixed haemozoin plus labile haemosiderin and ferritin. A small proportion of the iron content must have come also from the temporary local accumulation of erythrocytes.

The indications seem to be that at the crisis the turnover of iron in the liver considerably exceeds that in the spleen. During recovery and the associated high production of reticulocytes the amount of Fe^{59} available for resynthesis of haemoglobin is reduced by loss in the form of unavailable haemozoin, and the necessary iron is derived from other sources, including haemosiderin and possibly the homeostatic stores. This possibly explains the lowered Fe^{59} content of the blood in the homeostasis achieved in recovery as compared with the content before infection (See Fig. 1).

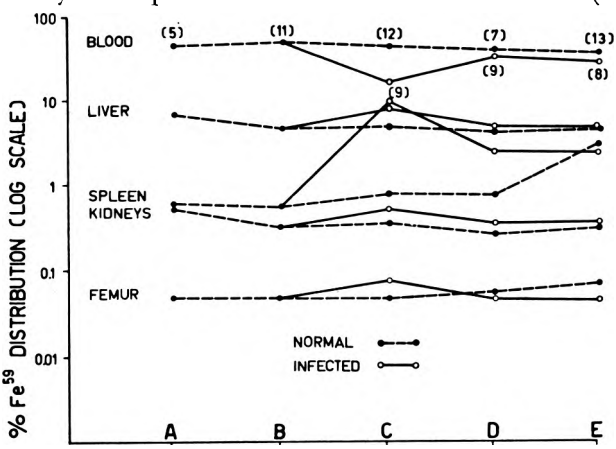


Fig. 1

A. = Administration of Fe^{59}
B. = 20 d after administration of Fe^{59} (Also point of infection)
C. = 30 d after Fe^{59} (10 d after infection; i. e. the crisis)
D. = 45 d after Fe^{59} (Convalescence)
E. = 60 d after Fe^{59} (Length of erythrocyte life-span; recovery stage in malaria).

It is interesting to note that the changes in tissue Fe^{59} concentration associated with the end of the life cycle of the originally tagged erythrocytes (i. e. in the region of 60 d from inoculation) were not evident in the infected animals. This indicates that the initial batch of tagged cells must have been disintegrated during the malarial crisis. Some uninfected cells must have been lysed, since the available Fe^{59} (less that locked up in the haemozoin formed in the infected cells) was incorporated into new cells in a proportion only 10 to 15 % less than in normal homeostasis, during a period in which the erythrocyte count dropped from 7.8 to 3.2 million cells per mm^3.

These experiments demonstrated that the haemozoin produced by the parasites was unavailable immediately for the re-synthesis of haemoglobin. They will be extended to ascertain the role of dietary iron in such re-synthesis.

It would be profitable to examine the iron balance in the human field, and study the synthesis of haemoglobin and the absorption and date of dietary iron not only in malaria, but in other anaemic states including sicklaemia, favism, thalassaemia and especially hookworm infection.

T. H. Bothwell: I am afraid I have no experience at all of this particular problem. We have always assumed that iron in stores is available for haemoglobin production. This is certainly exemplified in the iron storage diseases, such as idiopathic haemochromatosis, where it is often possible to remove a pint of blood every five days over a period of a year or two and yet see haemoglobin levels maintained. Some of these stores must have been present for many years. There is one anomaly, however, and that is in the condition of idiopathic pulmonary haemosiderosis, in which there is repeated bleeding into the lungs. The iron released from these haemorrhages is laid down as haemosiderin. It is, however, not available for blood formation since iron deficiency anaemia often develops in spite of the deposits in the lungs. The reason for this probably relates to the location of iron in idiopathic pulmonary haemosiderosis: it is neither available to transferrin nor are the phagocytes containing iron able to gain access to circulation.

A. S. McFarlane: Dr. Hallberg's interesting demonstration of a slow absorption of mucosal iron suggests that the rate of mucosal regeneration, about which so much more is now known, may be a factor to be considered in any comprehensive explanation of absorption generally. I should be interested to know whether Dr. Hallberg has considered this.

I have also a question to ask Dr. Bothwell: in conditions in which a viable type of abnormal red cell appears in the circulation, is there any possibility that exchange of serum iron with that in the abnormal cells occurs? This would of course greatly affect iron turnover values.

T. H. Bothwell: No evidence has as yet been found that mature red cells can extract iron from the serum in man. It is, however, possible that small amounts may be taken up when large numbers of reticulocytes are in circulation. In this context it is interesting to note that the abnormally high turnovers reported with ineffective erythropoiesis may not always reflect excessive pyrrole pigment metabolism. CROSBY [*Blood* **12** (1957) 165] has produced data to suggest that in conditions associated with blocks in haeme synthesis there may be a significant uptake of iron by red-cell precursors which is not converted into haeme. When such cells entre circulation, the iron granules are rapidly removed by the spleen and re-routed to the marrow. In such conditions there may therefore be very high turnovers of iron as a result of the abnormal shunt.

L. Hallberg: I agree with Dr. McFarlane that the turnover of the mucosal cells may affect the values for mucosal storage of iron. One must suppose, as far as I can see, that cells containing iron and finding their way into the intestines have their iron re-absorbed.

H. Foy: Does Dr. Bothwell, when examining marrows for haemosiderin, find sometimes clumps and sometimes scattered particles, and does he consider that the clumps are probably less utilizable than the scattered particles? Referring to Dr. Leithead's question about the availability of "malarial pigment" for haemoglobin synthesis, it is difficult to distinguish between "malarial pigment" and the clumps of haemosiderin which we find in the marrow. Is this "malarial pigment" utilizable or not?

T. H. Bothwell: I do not know very much about the various types of clumping that are observed, but data have been obtained which suggest that in aplastic states, where there is a slow turnover of iron, the haemosiderin seems to accumulate gradually into large clumps, whereas, in conditions like pernicious anaemia where there is much ineffective erythropoiesis and high turnovers through the marrow, the granules are much smaller. As far as availability is concerned, large clumps are formed in

haemochromatosis and it is possible to pull them out by repeated phlebotomies. I doubt whether the physical form makes much difference; there may be an initial lag, but all the iron is theoretically available.

D. L. Mollin: Professor Dacie has pointed out in this connection that whenever there is an excessive number of siderotic granules in the red cells, this is reflected by the accumulation of a fine dusting of haemosiderin in the reticulum cells in the marrow. Otherwise, it is merely stored in larger clumps.

H. Vetter: In describing methods of calculating plasma turnover, Dr. Bothwell stated that the plasma disappearance curve is an exponential one in most cases. Now, there seems to be evidence that in certain cases, particularly where plasma iron turnover is very rapid, this is not so. I wonder whether he would care to comment on this difficulty which many people have encountered.

T. H. Bothwell: I confess that this was an over-simplification on my part. Although most people using the plasma iron turnover as a measurement of erythropoiesis have ignored minor deviations from the straight line at the lower levels of activity, these deviations have significance, and mean that iron is being fed back from some other pool. Various attempts have been made to fit mathematical models to these data, but I feel that we have not yet sufficient knowledge of where the iron goes and what it is doing for us to be able to obtain biologically valid results from such analyses. On the other hand, this approach has certainly underlined the need for more experimental studies to find out the fate of all the iron leaving the plasma. Meanwhile I think that useful clinical data can still be obtained from simple analysis of ferrokinetic measurements.

H. Vetter: In this matter of over-simplification of the plasma turnover calculation, I think Dr. Bothwell has been extremely modest in not even mentioning one source of error which he himself was the first to point out, namely the diurnal variations in plasma iron concentration and radioiron disappearance rates. I consider this question to be of some importance, and I would welcome a comment from him thereon.

T. H. Bothwell: In normal subjects, the plasma iron level is usually higher in the morning than in the evening. However the plasma iron turnover remains constant. This is accomplished by reciprocal changes in the clearance rate of iron from the plasma. On the other hand there is some evidence that the turnover becomes less during sleep. In pathologic states where there is a relative deficiency of iron (e.g. pernicious anaemia in early remission) any rise in plasma iron is associated with an increase in the turnover rate. These various observations indicate that a plasma iron turnover measured over a short period may not, in fact, reflect the state of affairs over the whole twenty-four hours. At the same time I should like to emphasize again the fact that the plasma iron turnover is only a semi-quantitative measure of erythropoiesis and these discrepancies do not detract from its clinical usefulness.

N. Veall: It occurs to me that one of the factors influencing the plasma iron turnover rate may be the rate of blood flow through the extracting organs and tissues. If the removal is in any way limited by blood flow, it is possible that changes in haemodynamic factors such as cardiac output during sleep account to some extent at least for the diurnal variations.

T. H. Bothwell: This is an intriguing possibility that can certainly not be excluded. It is, I think, generally believed that the variations which have been noted reflect alterations in either reticulo-endothelial or erythropoietic function but the question remains an open one.

Printed in Austria

IRON OVERLOAD

A Familial Study in Idiopathic Hemochromatosis*

T. H. Bothwell, m.d., I. Cohen, m.b., O. L. Abrahams, b.sc. *and* S. M. Perold, b.sc.
Johannesburg, South Africa

It is generally believed that the clinical manifestations of idiopathic hemochromatosis reflect the tissue damage caused by excessive iron in the body [*1*]. Thus the liver, which is the chief reservoir, becomes cirrhotic and the heavy pancreatic deposits lead to diabetes in about 80 per cent of subjects. In addition the skin becomes pigmented. Cardiac involvement is not uncommon.

Since iron overload occupies the central role in the pathogenesis of the disease, rational therapy is directed towards the removal of this iron by repeated venesections [*2,3*]. On this regimen iron is mobilised from stores to satisfy the increased needs of the erythroid marrow. Such therapy has caused amelioration of symptoms and it seems probable that it will improve the prognosis of patients with the disease. However, the degree of improvement must depend on the amount of damage to the organ that has occurred prior to the start of treatment. The ideal subject for therapy would therefore be someone in whom excessive iron stores had not been present in sufficient quantities for a long enough period to cause irreversible tissue damage. Unfortunately these cases are difficult to diagnose as clinical manifestations are evident only when the iron has already led to considerable pathologic changes. Since, however, there is growing evidence that idiopathic hemochromatosis may be a familial inborn error of metabolism [*1*] it is possible to detect a proportion of such cases by investigating relatives of patients with overt disease. Although reported examples of the fully developed clinical disease in relatives have been few, it has been shown that about 20 per cent have elevated serum iron levels [*1,4*].

The present paper records the results of such a survey. These findings have some bearing on the pathogenesis of the condition and also underline the practical importance of detailed familial studies in idiopathic hemochromatosis.

METHODS

Fifty-two immediate relatives (i.e., parents, siblings and children) of six patients with idiopathic hemochromatosis were investigated. The six patients were all subjects in whom the disease had been confirmed histologically. In five it was proved at autopsy and in one by liver biopsy.

Each of the relatives was examined clinically, and fasting serum iron estimations were made by the method of Bothwell and Mallett [*5*] in all except five young children. In this laboratory the average normal figures for serum iron are 114 μg./100 ml. for males and 104 μg./100 ml. for females. Although values above 150 μg./100 ml. have been found in some healthy subjects they have not been constantly raised on repeated testing. In the present study results above 150 μg./100 ml. were therefore regarded as abnormal only when present on two or more successive occasions. In addition the unsaturated iron-binding capacity was estimated in about half the subjects, using the technic of Rath and Finch [*6*]. When possible, liver biopsies were performed in relatives showing persistently raised serum iron levels. Sections of these biopsy specimens were stained with hematoxylin and eosin, and with Prussian blue by Dry's method [*7*].

RESULTS

The salient clinical features of propositi and affected relatives are recorded in the family reports which follow. These reports also include the relevant biochemical abnormalities and, in most instances, the results of liver biopsy. (Figs. 1 and 2.)

Family A. The propositus was a woman who had been followed for several years as an established case of hemochromatosis prior to her death at the age of fifty-eight years. She finally died following a hematemesis.

*From the Department of Medicine and the Department of Pathology and Microbiology, University of the Witwatersrand, Medical School, Johannesburg, South Africa.

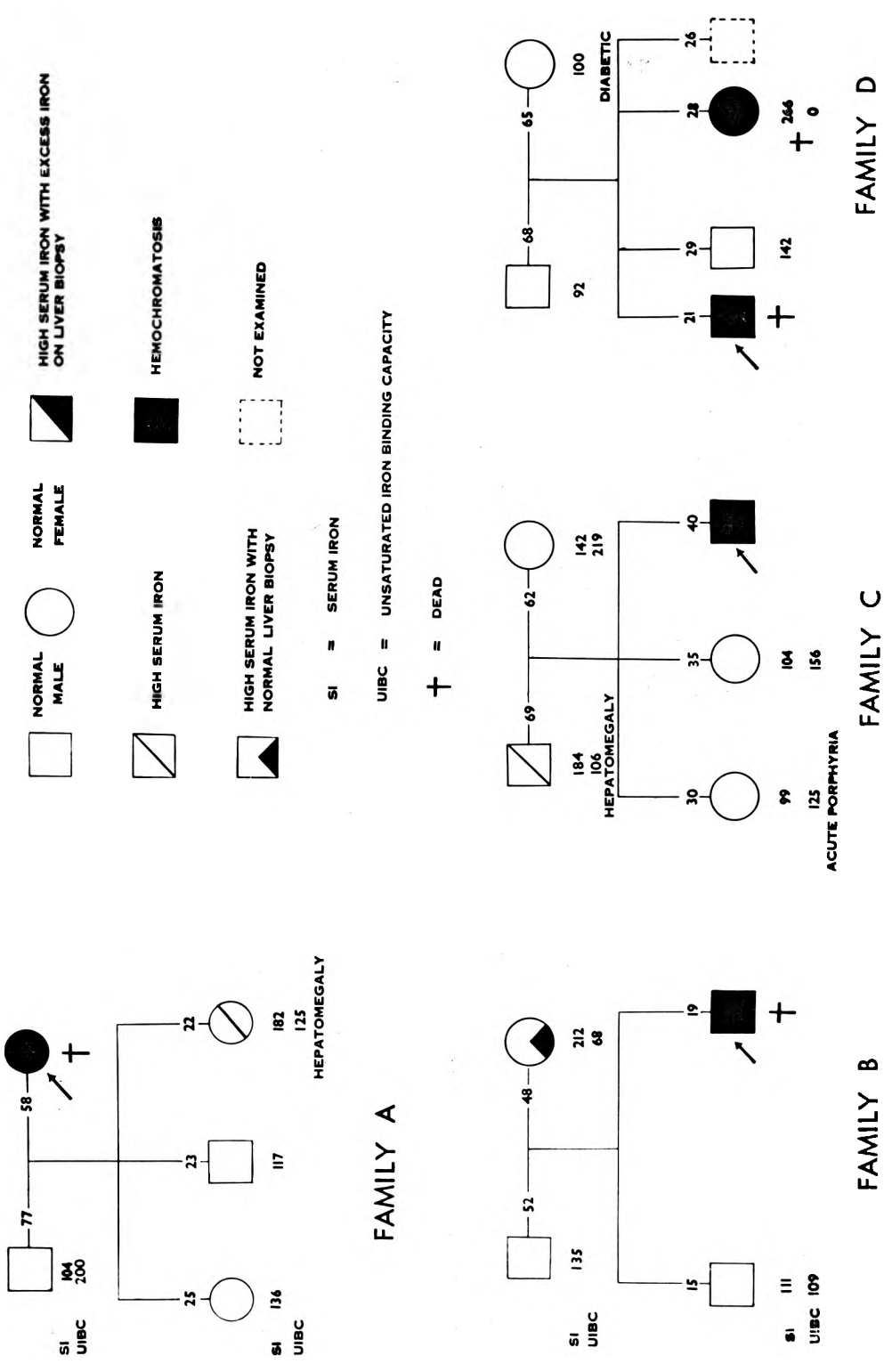


Fig. 1 Pedigrees of four families.

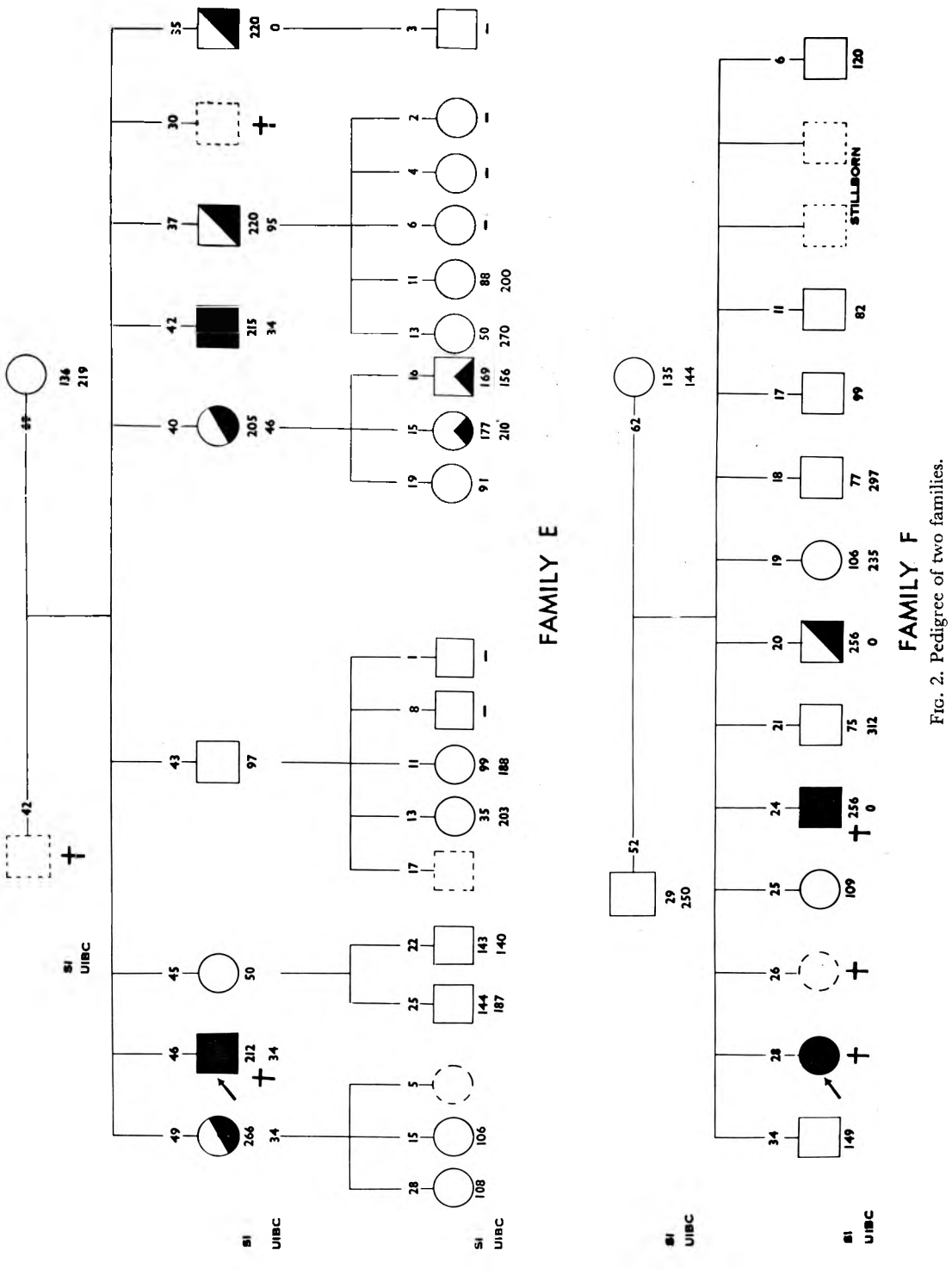


FIG. 2. Pedigree of two families.

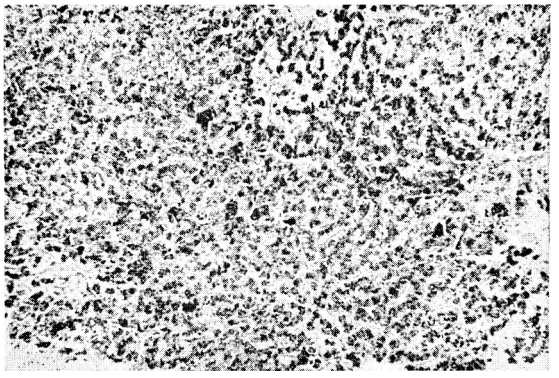


FIG. 3. Family D. Liver biopsy specimen from a twenty-eight year old woman. Hemosiderin granules are present in parenchymal cells. The portal tracts (not shown in figure) show round cell infiltration and slightly increased fibrosis. Perl's stain. Original magnification × 120.

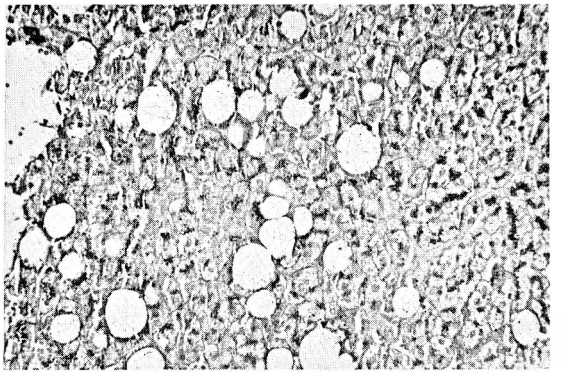


FIG. 4. Family E. Liver biopsy specimen from a forty-nine year old woman. There is marked peripheral fatty change and fine hemosiderin granules are present in parenchymal cells. The portal tracts show no increased fibrosis. Perl's stain. Original magnification × 240.

The husband and three children were examined. The only abnormalities detected were in a daughter, aged twenty-two years, who had a firm two-finger hepatomegaly, a raised serum iron level and a reduced unsaturated iron-binding capacity. No pigmentation of the skin was noted. Menstruation was normal. She refused further investigation.

Family B. The propositus was a young man in whom diabetes developed. He subsequently died in cardiac failure at the age of nineteen years.

The parents and an only brother were examined. All appeared clinically normal but the mother, who was forty-eight years old, had a serum iron level above 200 μg./100 ml. on three separate occasions over a period of eight months. A hysterectomy had been performed three years previously for menorrhagia of several years' duration. Liver function tests were normal as was the blood count (hemoglobin 14.5 gm./100 ml.). Finally a liver biopsy was performed. No abnormalities in the liver were found. Venesections were then started at three weekly intervals. After approximately 1,200 mg. iron had been removed in this way the hemoglobin level was 12.5 gm./100 ml. and the serum iron level was 35 μg./100 ml. At this stage venesections were discontinued.

Family C. The propositus was a forty year old man with diabetes, pigmentation of the skin and testicular atrophy. A diagnosis of hemochromatosis had been established by liver biopsy several years previously. At the time his relatives were studied about 35 gm. iron had already been removed from the patient by phlebotomy.

The parents and two sisters were examined. One sister was known to have acute intermittent porphyria but showed no evidence of iron excess. The only other abnormalities were found in the father who had a firm hepatomegaly and a high serum iron level. In view of his age and apparent good health no further tests were carried out.

Family D. The propositus was a young man, aged twenty-one years, who succumbed to the cardiac complications of the disease. Full details of this patient, including radioiron data, have been recorded elsewhere [*8,9*].

The patient's parents and two siblings were examined. The brother was normal but the sister showed classic clinical features of the disease with pigmentation of the skin, endocrine changes and cardiac signs. The diagnosis was confirmed by liver biopsy. (Fig. 3.) In spite of energetic therapy she died at the age of twenty-eight years in gross congestive failure. (The clinical features of this patient have also been described previously [*10*].) The father appeared normal but the mother was found to be a diabetic of several years standing whose diabetes was adequately controlled on 20 units of protamine zinc insulin daily. In addition she had a two-finger hepatomegaly. In view of these findings a biopsy specimen of the liver was obtained, in spite of the fact that the serum iron level was persistently normal. Histologically the sections appeared normal with no evidence of excessive iron deposits.

Family E. The propositus was a forty-six year old man in whom cardiac failure developed. In addition there was pigmentation of the skin and a three-finger hepatomegaly, but no diabetes. The diagnosis of idiopathic hemochromatosis was supported by skin biopsy findings. He died suddenly several months after the onset of symptoms, and at autopsy showed the typical features of the disease.

The mother was examined and appeared normal. The father was reported to have died at the age of forty-two years from "acute heart failure" but unfortunately no further details were available. In

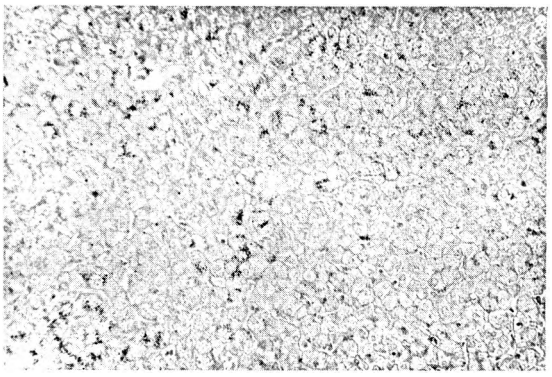


FIG. 5. Family E. Liver biopsy specimen from a forty year old woman. Scattered foci of liver cells contain fine hemosiderin granules. The histology is otherwise normal. Perl's stain. Original magnification × 240.

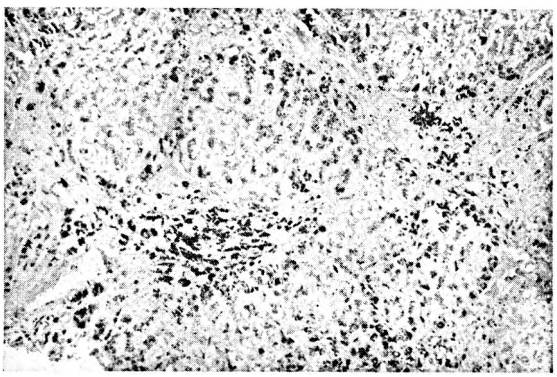


FIG. 6. Family E. Liver biopsy specimen from a forty-two year old man. Coarse hemosiderin granules are present in the parenchymal cells and also in the Kupffer cells and portal tracts. There is well marked fibrosis of the portal tracts with linkage of adjacent tracts. Areas of nodular regeneration are present. Perl's stain. Original magnification × 150.

addition a brother had been killed in an accident at the age of thirty. All the other seven siblings were investigated. Of these only two, a brother and sister, were completely normal. All the remaining siblings showed raised serum iron levels, reduced unsaturated iron-binding capacities and abnormal findings on liver biopsy.

The eldest was a forty-nine year old woman, with three children, the youngest of whom was eight years old. A hysterectomy had subsequently been carried out because of menorrhagia. On examination there was a suggestion of pigmentation of the skin and a one-finger hepatomegaly. The liver biopsy showed scattered deposits of hemosiderin confined to parenchymal cells and well marked fatty infiltration. (Fig. 4.) There was no increased fibrosis of the portal tract. Liver function tests and a glucose tolerance curve were within normal limits.

The other affected sister was a forty year old woman with three adolescent children, who was clinically normal. In view of the serum iron findings a biopsy specimen of the liver was obtained. Fine granules of hemosiderin were present in parenchymal cells but the histologic findings were otherwise normal. (Fig. 5.)

The eldest affected brother was forty-two years old. He gave a history of having suffered from dyspnea on effort for the previous six months. On examination he was found to be in mild congestive failure with cardiomegaly and a gallop rhythm. There was generalised slaty grey pigmentation and a three-finger firm hepatomegaly. The glucose tolerance was normal. A biopsy specimen of the liver showed the typical features of idiopathic hemochromatosis with a well marked monolobular cirrhosis and extensive iron deposits. (Fig. 6.)

Another brother, aged thirty-seven years, gave a history of an excessive alcohol intake but otherwise appeared to be normal. A liver biopsy showed finely dispersed hemosiderin throughout the liver cells but there was no increase in fibrous tissue. (Fig. 7.)

The last affected brother was thirty-five years old, with no subjective complaints. On examination there was some generalised pigmentation with a one-finger hepatomegaly. Liver biopsy showed massive iron deposits with early portal fibrosis. (Fig. 8.)

Seventeen members of the third generation of the family were examined. All were clinically normal but a daughter and son of an affected female, aged fifteen and sixteen years, respectively, showed serum iron levels persistently between 150 and 200 μg./100 ml. Sections from liver biopsy specimens were, however, quite normal. The daughter was subsequently venesected weekly until the hemoglobin level had fallen to 9.0 gm./100 ml. At this stage approximately 1500 mg. iron had been removed.

Family F. The propositus, a yound woman who died at the age of twenty-eight in cardiac failure, has been fully described elsewhere [*11*]. Both parents appeared to be healthy although the mother, who had given birth to fourteen full term infants, complained of menorrhagia. Her serum iron level was only 29 μg./100 ml. but she did not appear to be anemic. A possibly significant point in the family history was the fact that a brother of the mother had died from diabetes at the age of sixty-two years.

Two of the patients' siblings were stillborn and a sister had died at the age of twenty-six years from a "bleeding disorder." The remaining ten siblings were all examined. One brother, aged twenty-four years, was found to be under treatment elsewhere for fully developed hemochromatosis. The salient features were pigmentation of the skin, diabetes and pituitary infantilism. A biopsy specimen of the liver showed the characteristic features of the disease. (Fig. 9.) He subsequently died in heart failure. Another brother, aged twenty years, in apparently good health, showed

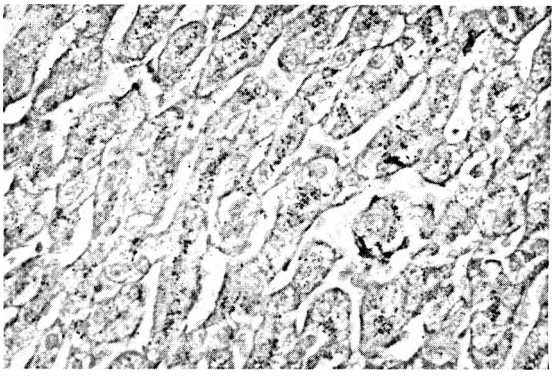


FIG. 7. Family E. Liver biopsy specimen from a thirty-seven year old man. There are fine hemosiderin granules in parenchymal cells. The liver histology is otherwise normal. Perl's stain. Original magnification × 600.

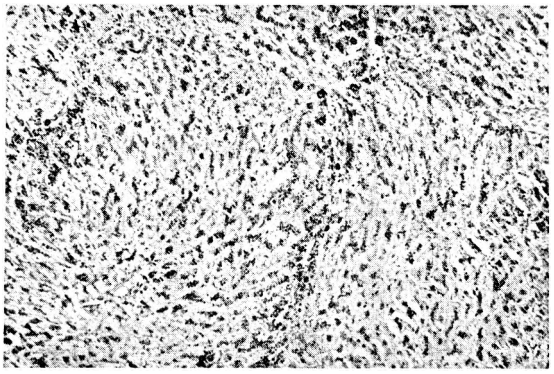


FIG. 8. Family E. Liver biopsy specimen from a thirty-five year old man. Fine and coarse hemosiderin granules are present, chiefly in parenchymal cells but also in Kupffer cells and in the portal tracts. There is a slight increase in portal fibrosis. Perl's stain. Original magnification × 150.

some increased pigmentation, a two-finger hepatomegaly and a raised serum iron level. A liver biopsy specimen showed heavy deposits of iron in hepatic cells with some bands of fibrosis in the portal tracts. (Fig. 10.) Further investigations revealed apparently normal endocrine, hepatic and cardiac function.

COMMENTS

It seems probable that the clinical manifestations of idiopathic hemochromatosis are the end result of tissue damage caused by excessive iron deposits within the body. To understand how such a situation arises it is first necessary to consider some quantitative aspects of iron balance in the normal subject. In an adult male there is about 3 to 4 gm. iron, of which 60 to 70 per cent is in hemoglobin and about 25 per cent in stores. The accumulation of this iron from infancy to adulthood implies a net daily gain of between 0.35 and 0.6 mg. over this period [*12*]. Thereafter the balance in the male remains more or less constant. Only about 0.6 mg. iron is excreted per day and this loss is balanced by the absorption of a similar quantity from the gut [*12*]. In the absence of blood loss the body has little capacity to alter the excretion rate of iron so that when absorption from the gut is increased most of the excess iron is retained within the body. Thus a positive balance of as little as 3 to 4 mg. per day over a period of fifty years will lead to an accumulation of about 50 gm. of iron within the body. From this it is apparent that relatively minor alterations in the absorptive rate of the male can eventually lead to the mas-

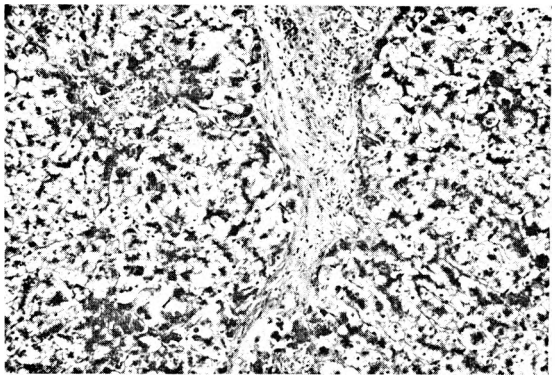


FIG. 9. Family F. Liver biopsy specimen from a twenty-four year old man. Heavy deposits of hemosiderin are present in parenchymal cells and small amounts are also present in Kupffer cells and portal tracts. There is a moderate degree of portal fibrosis. Perl's stain. Original magnification × 150.

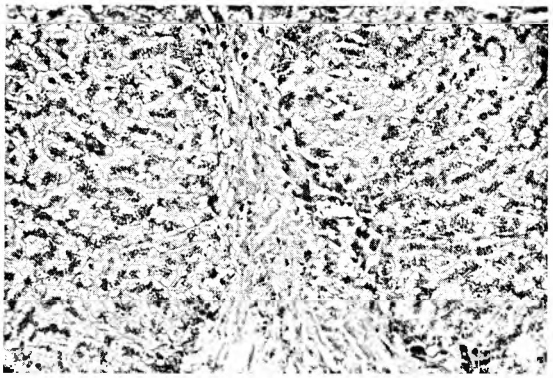


FIG. 10. Family F. Liver biopsy specimen from a twenty year old man. Fine and coarse hemosiderin granules are present in parenchymal cells and to a lesser extent in the portal tracts and Kupffer cells. The portal tracts show a moderate increase in fibrosis with infiltration by lymphocytes, histiocytes and polymorphonuclear leukocytes. Perl's stain. Original magnification × 190.

sive accumulation of iron (30 to 60 gm.) found in idiopathic hemochromatosis at the time when clinical symptoms occur. In the female, however, it is more difficult to establish a positive iron balance since there are the normal losses incurred by menstruation, pregnancies and lactation. Such losses, which probably total between 10 and 15 gm. iron over the average productive life, offer at least a partial explanation for the much lower incidence and the delayed onset of the full blown disease in females [*1*].

Although the clinical features of idiopathic hemochromatosis are relatively characteristic, these only occur at a time when excessive iron has already led to extensive tissue damage, and diagnosis is more difficult in that long latent period when iron is steadily accumulating in the body. Some affected individuals can, however, be detected in the preclinical phase of the disease by other means. It is known that iron overload is usually associated with a rise in the serum iron level and this has proved a useful screening procedure [*1,4*]. In the present study serum iron estimations were carried out on the immediate relatives of patients with idiopathic hemochromatosis and liver biopsy specimens were obtained in most of those with raised levels.

Relatives were chosen as test subjects because there is some evidence that, although most cases of idiopathic hemochromatosis are found sporadically, the disease may be a familial disorder [*1*]. The true incidence is difficult to assess since little attention has been paid to this aspect of the disease. Finch and Finch, who recently reviewed 787 histologically proved cases of hemochromatosis reported in the last twenty years, were able to find only eleven instances in which two or more members of the same family were definitely shown to be suffering from the disease [*1*]. It is, however, doubtful, whether these figures are a true reflection of the familial incidence since there are also a number of instances in which suggestive clinical findings or raised serum iron levels have been described in relatives [*1*]. All except one of these reports [*13*] have recently been extensively reviewed by Boulin and Bamberger [*14*] and by Pirart and Gatez [*15*]. Unfortunately an analysis of the various findings is fraught with difficulty as the criteria for diagnosis have not always been adequate. For example, although the families of thirty-two patients with this disorder appear to have been examined in some detail, only about half of the original cases were proved histologically. In spite of these shortcomings the evidence which is available fits in well with the concept of idiopathic hemochromatosis as a familial disorder.

In the studies on thirty-two families which have been reported in the literature, twenty-six parents were examined. Of these, one was stated to have proved hemochromatosis, five showed clinical evidence of the fully developed disease, two were regarded as subclinical, three were diabetic and one had hepatomegaly. In no instance did more than one parent show suggestive findings.

The results of examination of 107 siblings were even more impressive. There were nine cases proved histologically, twenty-five in whom a firm clinical diagnosis was made, and an additional six who were thought to be probable examples of the disease. In addition, ten subjects had raised serum iron levels, six were diabetic, seven had hepatomegaly and one was suffering from carcinoma of the liver. There was thus some evidence that about half the siblings were affected in varying degrees.

In the present investigation fifty-two immediate relatives of six patients were examined. The most striking findings were in siblings. Of nineteen investigated, three were found to be suffering from the clinical disease and another five showed varying degrees of iron excess on liver biopsy. The incidence of affected siblings may be even higher than these figures suggest since six of the subjects were under the age of twenty years and thus may yet exhibit manifestations at a later date. The significance of the findings in parents and children was more difficult to assess since there was no example of the fully developed disease in a parent and child. There was, however, some evidence to support a hereditary factor. In family B the mother showed a persistently raised serum iron level but the liver biopsy was normal. She gave a history of menorrhagia for many years and it seemed probable that the expression of her metabolic abnormality had been modified by continued blood loss. In family C the father had hepatomegaly and a raised serum iron level but could not be investigated further. It was possible to examine the children of affected persons in only two families. In family A there were three children. One of the daughters, aged twenty-two years, had a high serum iron level and hepatomegaly but refused further investigation. In family F most of the children were very young and appeared normal. However, two adolescent children of an affected female had raised serum

iron levels. In both the liver biopsy findings were normal.

Although these results are not sufficiently complete to permit firm conclusions, they do allow for some speculation as to a possible genetic linkage in idiopathic hemochromatosis. In two of the five families in which parents were examined there was some evidence that one parent in each family was affected. In a third family the mother was diabetic but otherwise normal. In a fourth family the mother, who suffered from menorrhagia and had given birth to fourteen full term infants, appeared healthy but her brother had died previously from diabetes. The significance of these latter findings is difficult to evaluate. (It should, however, be noted that although the diabetes which occurs in idiopathic hemochromatosis is almost certainly due to iron deposits in the pancreas, a higher incidence of diabetes in otherwise unaffected relatives has been noted previously [*14*]. In addition, it has been possible to increase iron absorption experimentally by ligating the pancreatic ducts [*16*] and by damaging the pancreas [*17*]). In the last family the mother was normal but the father had succumbed previously to heart failure at a young age and may possibly have suffered from the disease. All this evidence is at least compatible with the idea that a parent in each of the families examined was suspect in one way or another.

In siblings the findings were much more concrete, since about 50 per cent of those over the age of twenty years showed histologic evidence of varying degrees of iron overload. Finally, there was suggestive evidence that three children of two affected parents were suffering from the metabolic defect. These data therefore fit in well with the concept that idiopathic hemochromatosis is a familial disorder, and they lend some support to Neel and Schull's thesis that it is due to an autosomal gene of incomplete penetrance which is transmitted as a Mendelian dominant [*18*]. Whether, in fact, the penetrance is incomplete is difficult to evaluate from the available data. The present findings would also be compatible with a more complete degree of penetrance but a variable expressivity. The resolution of this problem will be possible only when the nature of the fundamental biochemical defect is discovered. Meanwhile it would be helpful to have data on the rate of absorption of iron from food in seemingly unaffected parents, and to know whether or not the apparently higher incidence of diabetes in relatives, unaccompanied by iron excess, has any significance.

The possibility that common environmental factors, such as unusual eating habits, might explain the present findings seems hardly tenable since affected siblings had been living in different localities for long periods prior to the study. Nevertheless it is probable that the clinical expression of the disease in subjects with a similar degree of absorptive defect can be modified by other factors, such as the amount of iron in the diet and the amount of iron loss from the body, e.g., menstruation and pregnancy. In addition, many workers have noted the higher incidence of alcoholism (± 30 per cent) in patients with the clinical disease [*1*]. Whether such an abnormal diet increases iron absorption or potentiates damage to the liver and thus accelerates the onset of the symptomatic stage of the disease is not known. However, the fact that no example of the fully developed disease in different generations was found in the present series cannot be explained on environmental factors alone and seems rather to represent a variability in expressivity of the abnormal gene. On the other hand the severity of the metabolic defect in affected members of the same generation is remarkably constant. Thus in two of the present families there were five affected siblings, all of whom showed severe involvement in the third decade of life. In a third family four brothers appeared to have an absorptive defect of about the same intensity. In this family two sisters were also probably affected to a similar degree but the iron stores were not as large. This difference could well be explained by the ameloriating effects of blood loss via menstruation.

A surprising finding was the poor correlation between the raised serum iron levels and the degree of iron overload as judged by liver biopsy findings. In subjects with a high serum iron the hepatic histology ranged from normal to the changes of fully developed idiopathic hemochromatosis. In subjects with normal biopsy findings it was thought that there might be still some increase in non-stainable iron in the liver but the response to venesection showed little if any difference from normal. Although the biologic significance of a high serum iron level in subjects with normal iron stores is not clear, it could be a reflection of an abnormal gradient between the iron in cells and plasma. Whatever the explanation it is obvious that a raised serum iron level is not of itself a good indication of the degree of iron overload. In most instances the total iron-binding capacity was also measured.

Although the mean value in affected relatives was somewhat reduced (285 μg./100 ml.), the results were not definite enough to be useful diagnostically.

For a better understanding of the amount of excess iron in relatives with high serum iron levels, liver biopsy was extremely helpful. Using the technic it was possible to demonstrate the various phases in development. In the early stages the iron was found as discrete granules of hemosiderin in hepatic cells, either finely dispersed throughout the liver lobules or in focal scattered areas. With greater quantities, the iron aggregated together into coarser masses and was uniformly distributed throughout the lobules. With marked iron excess there was a slight to moderate increase in fibrosis of the portal tract but a true monolobular cirrhosis was seen only in association with the fully developed clinical disease.

Although idiopathic hemochromatosis is a relatively rare disorder it is probable that familial studies of the present type would uncover a considerable number of affected indivduals, especially in siblings of patients suffering from the disease. The detection of affected subjects in the preclinical phase of idiopathic hemochromatosis is of some practical importance since the excess iron can be removed by repeated venesections [*2,3*]. The rigorous use of such therapy should prevent or arrest the development of tissue damage in such individuals [*19,20*].

SUMMARY

Fifty-two immediate relatives of six patients suffering from idiopathic hemochromatosis were examined. In eleven subjects with raised serum iron levels three were found to have the fully developed disease while five others showed varying degrees of iron excess on liver biopsy.

The present results support the concept that idiopathic hemochromatosis is a familial disorder in which the absorption of excessive amounts of iron from the gut eventually leads to massive accumulation of iron within the body. The disorder is probably the result of an autosomal genetic defect of incomplete penetrance and/or variable expressivity which is transmitted as a Mendelian dominant.

The practical value of familial studies in idiopathic hemochromatosis is stressed since affected persons can in this way be detected and treated in the preclinical phase of the disease.

Acknowledgment: We are indebted to Professor C. A. Finch, Dr. P. V. Tobias and Dr. B. A. Bradlow for helpful criticisms.

REFERENCES

1. Finch, S. C. and Finch, C. A. Idiopathic hemochromatosis, an iron storage disease. *Medicine*, 34: 381, 1955.
2. Finch, C. A. Iron metabolism in hemochromatosis. *J. Clin. Invest.*, 28: 780, 1949.
3. Davis, W. D., Jr. and Arrowsmith, W. R. The effect of repeated bleeding in hemochromatosis. *J. Lab. & Clin. Med.*, 36· 814, 1950.
4. Debre, R., Schapira, G., Dreyfus, J. and Schapira, F., Metabolisme du fer chez les descendants de malades atteints de cirrhose bronzée. *Bull et mém. Soc. méd. d. hôp. de Paris*, 68: 665, 1952.
5. Bothwell, T. H. and Mallett, B. The determination of iron in plasma or serum. *Biochem. J.*, 59: 599, 1955.
6. Rath, C. E. and Finch, C. A. Chemical, clinical and immunological studies on the products of human plasma fractionation. xxxviii. Serum iron transport. Measurement of the iron-binding capacity of serum in man. *J. Clin. Invest.*, 28: 79, 1949.
7. Dry, D. S. Improved methods for the demonstration of mitrochondria, glycogen, fat and iron in animal cells. *South African J. M. Sc.*, 41: 298, 1944.
8. Bothwell, T. H. and Alper, T. The cardiac complications of haemochromatosis. *South African J. Clin. Sc.*, 2: 226, 1951.
9. Alper, T., Savage, D. V. and Bothwell, T. H. Radioiron studies in a case of hemochromatosis. *J. Lab. & Clin. Med.*, 37: 665, 1951.
10. Cohen, I. and Bothwell, T. H. Haemochromatosis in a young female. *South African M. J.*, 32: 629, 1958.
11. Bothwell, T. H., Van Lingen, B., Alper, T. and Du Preez, M. L. The cardiac complications of hemochromatosis. *Am. Heart J.*, 43: 333, 1952.
12. Moore, C. V. Importance of nutritional factors in the pathogenesis of iron deficiency anemia. *Am. J. Clin. Nutrition*, 3: 3, 1955.
13. Houston, J. C. and Zilka, K. J. Haemochromatosis in a family. *Guy's Hosp. Rep.*, 104: 262, 1955.
14. Boulin, R. and Bamberger, J. L'hémochromatose familiale. *Semaine d. hôp. Paris*, 29: 3153, 1953.
15. Pirart, J. and Gatez, P. L'étiologie de l'hémochromatose non transfusionelle. *Semaine d. hôp. Paris*, 34: 1, 1958.
16. Taylor, J., Stiven, D. and Reid, E. W. Experimental and idiopathic siderosis in cats. *J. Path. & Bact.*, 41: 397, 1935.
17. Kinney, T. D., Kaufman, N. and Klavins, J. Effect of ethionine-induced pancreatic damage on iron absorption. *J. Exper. Med.*, 102: 151, 1955.
18. Neel, J. V. and Schull, W. J. Human Heredity. Chicago, 1954. University of Chicago Press.
19. Davis, W. D., Jr. and Arrowsmith, W. R. The treatment of hemochromatosis by massive venesection. *Ann. Int. Med.*, 39: 723, 1953.
20. McAllen, P. M., Coghill, N. F. and Lubran, M. The treatment of haemochromatosis. *Quart. J. Med.*, 26: 251, 1957.

Reprinted from the Nov. issue of *The* American Journal of Medicine
volume XXVII, number 5, pages 730–738, copyright 1959 and printed in the U.S.A.
Published by *The American Journal of Medicine, Inc.*, 11 East 36th Street, New York

S. Afr. J. med. Sci. (1962), **27**, 35-39.

STUDIES ON THE BEHAVIOUR OF TRANSFERRIN IN IDIOPATHIC HAEMOCHROMATOSIS

by

T. H. BOTHWELL*, P. JACOBS† and J. D. TORRANCE
Department of Medicine, University of the Witwatersrand, Johannesburg.
[RECEIVED 20TH FEBRUARY, 1962]

Idiopathic haemochromatosis is the end-result of an inherited metabolic disorder which is associated with an increased absorption of iron; by the time clinical manifestations occur the body iron content is very large. Although it is likely that the primary defect in the condition resides in the mucosal cells of the gut it is still possible that the alteration in mucosal behaviour is secondary to some other intrinsic metabolic derangement. For example, it has been suggested that the transferrin of the plasma (iron-binding protein) may have an abnormal avidity for iron [Crosby, 1958]. This contention is supported to some extent by at least two observations. Firstly it has been noted that the plasma iron level tends to return rapidly to high levels when phlebotomies are stopped in subjects whose stores have been depleted by repeated venesections [Crosby, 1958]. In the second place it has been shown that some affected relatives of patients with idiopathic haemochromatosis have raised plasma iron levels at a time when iron stores are still normal or only moderately increased [Bothwell *et al.*, 1959]. In view of these findings a study was undertaken to compare the *in vitro* and *in vivo* behaviour of transferrin from haemochromatotic individuals with that from normal subjects.

EXPERIMENTAL

The sera used for testing in both the *in vitro* and *in vivo* studies were obtained from two patients with histologically proven idiopathic haemochromatosis whose stores had been depleted by repeated venesections. At the time of the investigations their serum iron levels were within or below the normal range. In all studies the samples were labelled by adding radioiron of high specific activity in quantities which were well within the binding capacities of the sera. In the discussion which follows, haemo chromatotic serum is referred to as H serum and normal serum as N serum.

In vitro studies

(A) *Electrophoretic mobility.* Two samples of H serum and two of N serum were labelled with ^{59}Fe chloride. Aliquots of 0·02 ml. were then subjected to paper electrophoresis for 20 hours at 120 volts in a veronal buffer (pH 8·6). Autoradiographs prepared from these strips revealed a single band of radioactivity in the β_1 globulin fraction in all instances. In a second experiment starch gel electrophoresis was carried out on the same sera using the technique described by Smithies [1955]. Both N and H sera showed the single band Transferrin C. On autoradiography the radioactivity was confined to this band in the test and control sera (Fig. 1).

*This study was supported by a grant from the Council for Scientific and Industrial Research, South Africa, and by a grant (No. A-4912) from the United States Public Health Service.
†In receipt of a bursarship from the Council for Scientific and Industrial Research, South Africa.

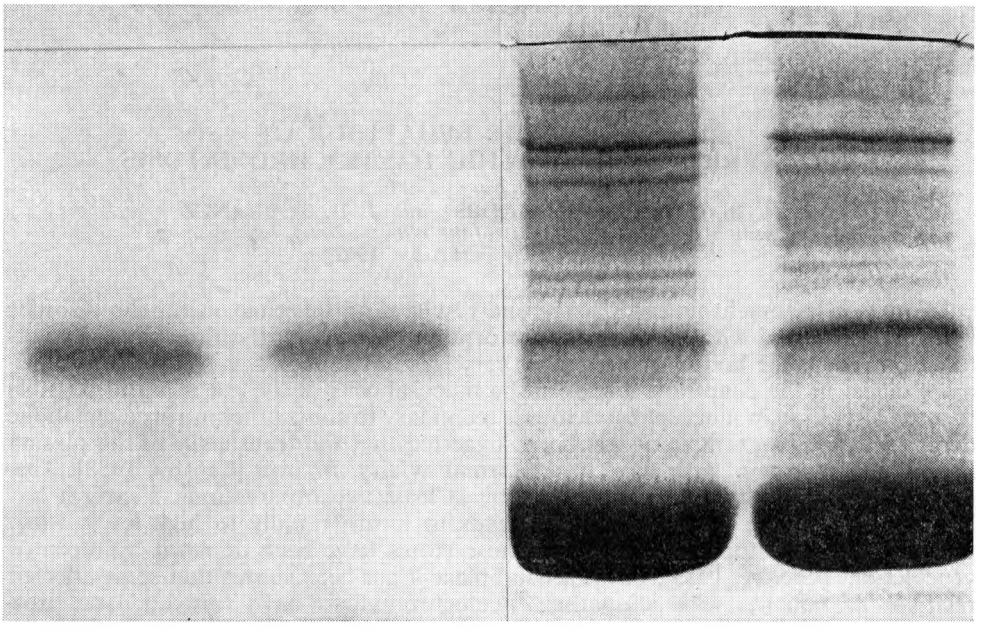


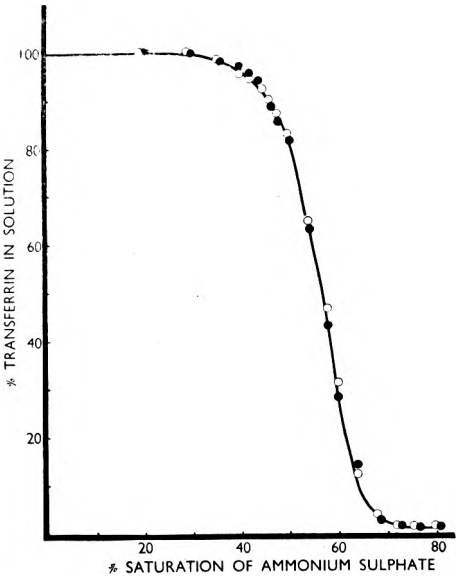
Fig. 1. Fe^{59} autoradiographs of normal and haemochromatotic sera after starch-gel electrophoresis.

N_1, H_1 — Normal and haemochromatotic Fe^{59} autoradiograph.
N_2, H_2 — Normal and haemochromatotic, starch gel stained with Amidoschwarz 10 B to show protein bands.

(B) *Solubility in ammonium sulphate.* Since it has been shown that iron remains attached to transferrin when the protein is precipitated with ammonium sulphate [Feinstein *et al.*, 1953; Itzhaki, 1961] it is possible to use ^{59}Fe as a marker for studying the solubility of transferrin in various concentrations of ammonium sulphate.

Two series of tubes were set up containing quantities of saturated ammonium sulphate (pH 7·5) varying between 2 and 8 ml. In each instance the volume was made up to 9 ml. with 0·08M Tris (tris-(hydroxy-methyl)-amino-methane) buffer (pH 7·5). One ml. aliquots of N and H serum labelled with ^{59}Fe chloride, were then added to each set of tubes. After mixing, the tubes were incubated at 37° C for 4 hours. In addition, duplicate control tubes were set up containing 1 ml. aliquots of serum and 9 ml. Tris buffer. After incubation the solutions were filtered through Whatman No. 542 paper and 4 ml. aliquots were counted in a scintillation counter. Results were expressed as the percentage of transferrin in solution at various saturations of ammonium sulphate. It can be seen (Fig. 2) that there was no difference between the solubilities of the normal and haemochromatotic transferrins.

(C) *Competition between transferrin and EDTA for added iron.* Unsaturated iron-binding capacities were estimated by the method of Bothwell and co-workers [1959] on aliquots of serum collected from 2 normal and 2 haemochromatotic subjects.



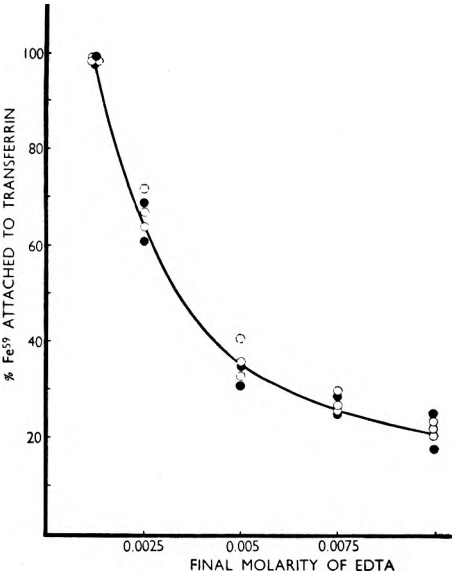


Fig. 2. The percentage of normal transferrin (open circles) and of haemochromatotic transferrin (closed circles) in solution with varying saturations of ammonium sulphate.

Fig. 3. The percentage of Fe^{59} ammonium citrate taken up by the transferrin of 3 normal sera (open circles) and by the transferrin of 2 haemochromatotic sera (closed circles) in the presence of increasing concentrations of EDTA.

Non-active iron in the form of ferric ammonium citrate was then added when necessary to each specimen in order to give a final unsaturated iron-binding capacity of 150 μg/100 ml. The sera were incubated at 37° C for 30 minutes. An 0·1M solution of disodium diamino-ethane-tetra-acetic acid (EDTA) was made up and adjusted to pH 7·5 with NaOH. From this stock solution various dilutions were made up to give molarities of between 0·0025 and 0·020. One ml. aliquots of these solutions were measured into conical flasks and these were followed by 1 ml. samples of serum. The mixtures were allowed to equilibrate at 37° C for 15 minutes. To each was then added 0·2μg. ^{59}Fe ammonium citrate contained in 0·2 ml. (This was prepared as previously described (Bothwell *et al.*, 1959)). After thorough mixing the solutions were incubated for a further 30 minutes at 37° C. At the end of this period the transferrin-bound iron was precipitated by adding 18 ml. saturated ammonium sulphate (pH 7·5) to each flask. After 30 minutes the solutions were filtered through Whatman No. 542 paper and 4 ml. samples of the filtrates were counted in a scintillation counter against appropriate standards. Results were expressed as the percentage of radioactive iron attached to transferrin in the presence of varying molarities of EDTA. The findings in 3 normal and 2 haemochromatotic subjects are shown in Fig. 3. Results in the two groups were similar.

In an extension of this study a fixed concentration of EDTA was used and the reaction was stopped at periods varying from 15 minutes to 4 hours. Again there were no differences between the behaviour of N and H serum.

(D) *Capacity of EDTA to remove transferrin-bound iron.* Two N and two H sera were labelled with tracer quantities of ^{59}Fe ammonium citrate (pH 7·5). After 15 minutes, non-active ferric ammonium citrate was added in amounts calculated to ensure complete or almost complete saturation of the transferrin. After incubation for a further 15 minutes, 1 ml. aliquots were measured into conical flasks and equal volumes of various molarities of EDTA (pH 7·5) were added to each. After incubation at 37° C for 1 hour, 18 ml. of saturated ammonium sulphate were added to each flask. The contents were mixed and allowed to stand for 15 minutes. The solutions were then filtered through Whatman No. 542 paper and the radioactivity in 4 ml. aliquots was counted in a scintillation counter. Duplicate control samples prepared from 1 ml. serum and 19 ml. saline were also counted. Results were expressed as the percentage of radioactivity bound to EDTA (i.e. in the filtrate). It can be seen (Fig. 4) that there was no difference between the results obtained with 2 samples of N serum and 2 samples of H serum.

In vivo studies

The objective of these studies was to compare the rate at which iron bound to N serum was cleared from the plasma in vivo with that of iron bound to H serum.

Heparinised samples of blood were collected from a normal volunteer and from a patient with idiopathic haemochromatosis. Twenty ml. plasma from each subject were then incubated with tracer quantities of radioactive iron. In the one instance 1 μc ^{59}Fe chloride (specific activity 10 $\mu c/\mu g$ iron) was used, and in the other 15

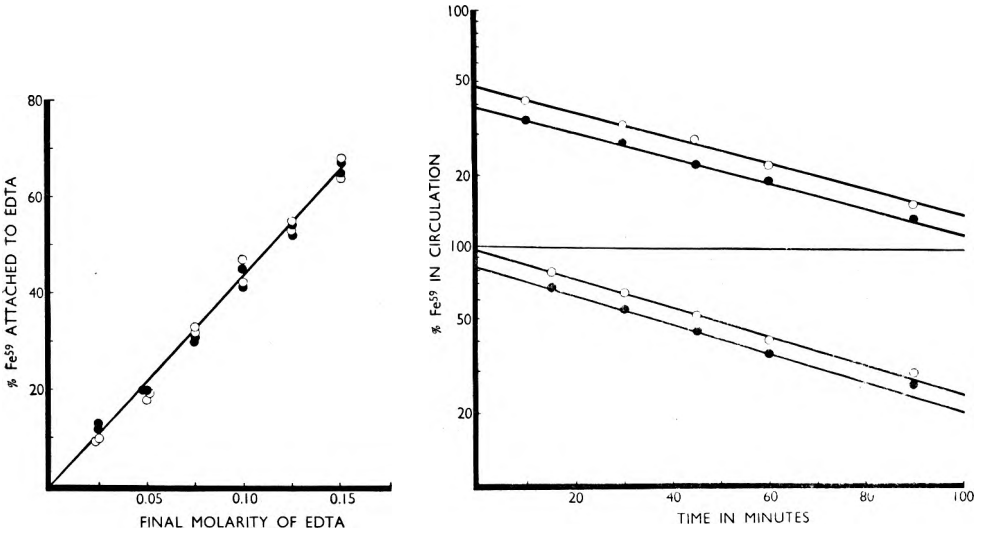


Fig. 4. The percentage of iron detached from the transferrin of normal sera (open circles) and of 2 haemochromatotic sera (closed circles) by incubation with increasing concentrations of EDTA.

Fig. 5. The *in vivo* clearance rates of radioiron bound to normal plasma (open circles) and to haemochromatotic plasma (closed circles) in a normal subject (upper figure) and in a patient with idiopathic haemochromatosis (lower figure).

μc ^{55}Fe chloride (specific activity 8 μc/μg iron). The two samples were then injected intravenously into the normal volunteer and blood samples were collected at regular intervals over the next 90 minutes. Ten ml. aliquots of plasma were digested, electroplated onto copper discs and differentially counted for ^{55}Fe and ^{59}Fe as described by Saylor and Finch, [1953]. In a further experiment, blood was collected from a normal subject and from a second haemochromatotic patient. In this instance the procedure was the same except that the two samples were injected into the subject with haemochromatosis. The results of these two studies are shown in Fig. 5. It can be seen that iron bound to haemochromatotic plasma was cleared from the circulation at the same rate as iron bound to normal plasma.

COMMENT

The present study was undertaken to explore the possibility that the iron-binding protein (transferrin) in idiopathic haemochromatosis is different from normal. However, the results of both *in vitro* and *in vivo* investigations were uniformly similar. The electrophoretic mobility, solubility in ammonium sulphate and capacity to compete for iron with a strong chelate were all normal. In addition the *in vivo* clearance rate of radioiron bound to haemochromatotic plasma was identical with that of radioiron bound to normal plasma in both a normal subject and in a patient with the disease.

Although these results do not totally exclude the possibility that the transferrin in idiopathic haemochromatosis is different from normal they make it unlikely. The question therefore still remains as to why the plasma iron level is sometimes raised in subjects with the disorder at a time when stores are normal or only moderately increased. There is some evidence that the most important regulator of the plasma iron level is normally the reticulo-endothelial system and it is possible that there is some subtle alteration in the gradient for iron between reticulo-endothelial cells and transferrin in idiopathic haemochromatosis. At present there is no evidence in favour of or against such an hypothesis.

SUMMARY

A series of *in vitro* and *in vivo* studies failed to demonstrate any abnormality in the behaviour of transferrin in subjects with idiopathic haemochromatosis.

REFERENCES

BOTHWELL, T H., COHEN, I., ABRAHAMS, O. L. and PEROLD, S. M. (1959). A familial study in idiopathic haemochromatosis. *Amer. J. Med.*, **27**, 730-738.

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**, 93-98.

CROSBY, W. H. (1958). Treatment of haemochromatosis by energetic phlebotomy. One patient's response to the letting of 55 litres of blood in 11 months. *Brit. J. Haemat.*, **4**, 82-88.

FEINSTEIN, A. R. BETHARD, W. F. and MCCARTHY, J. D. (1953). A new method, using radioiron, for determining the iron-binding capacity of human serum. *J. Lab. Clin. Med.*, **42**, 907-914.

ITZHAKI, R. F. (1961). Studies on plasma iron in the rat. I. Investigations *in vitro* on iron binding. *Arch. Biochim. Biophys.*, **92**, 69-73.

SAYLOR, L. and FINCH, C. A. (1953). Determination of iron absorption using two isotopes of iron. *Amer. J. Physiol.*, **172**, 372-376.

SMITHIES, O. (1955). Zone electrophoresis in starch gels: group variations in the serum protein of normal human adults. *Biochem. J.*, **61**, 629-641.

Reprinted from the Archives of Pathology
September 1960, Vol. 70, pp. 279-292
Copyright 1960, by American Medical Association

Siderosis in the Bantu

A Combined Histopathological and Chemical Study

THOMAS H. BOTHWELL, M.D., and BASIL A. BRADLOW, M.B., Ch.B., Johannesburg, South Africa

The widespread incidence of iron overload in the adult Bantu population of South Africa was initially noted by Strachan [1] and has subsequently been confirmed in a number of investigations.[2-7] In addition siderosis has been described in Bantu subjects from Rhodesia,[5,8] Bechuanaland, Nyasaland, Mocambique,[5] and West Africa.[9,10] The material examined has been obtained from subjects dying in the hospital and from subjects dying accidental deaths, and the incidence of siderosis in these different studies has varied between 40 and 88%. All grades of iron overload have been found, varying from a slight excess to amounts of iron comparable to those found in idiopathic hemochromatosis.

Although there is general agreement on the widespread incidence of the condition in Southern Africa, there is some dispute as to both the pathogenesis and the histopathological findings. On the one hand the Gillmans [11] consider that siderosis represents a manifestation of a widespread metabolic defect associated with chronic malnutrition, while Walker and Arvidsson [12] believe that the abnormal deposition of iron in these people is due primarily to their habitually high iron intake. From a pathological point of view the Gillmans [2-11] found that the earliest stage of iron overload was manifest by the appearance of iron granules in parenchymal cells of the liver, while Higginson, Gerritsen and Walker [5] thought that reticuloendothelial involvement represented the earliest stage in evolution.

The most accurate reflection of iron overload in the general Bantu population can be gained by an examination of material from subjects dying traumatic deaths. Of all the previous investigations only one used such material,[2,4] and as this study was completed over 15 years ago it was decided to reinvestigate such a group to find out whether there had been any change in the incidence and histopathological features. Although most attention was focused on the liver findings, the spleen was also examined in a proportion of cases. In addition chemical analyses for iron were carried out on all the tissues examined.

Material and Methods

Pathological Materials.—One hundred and forty-seven liver specimens were obtained from randomly selected autopsies carried out on Bantu subjects dying traumatic deaths. In 50 subjects specimens of spleen were also obtained. The subjects ranged in age between 11 and 70 years, most of them being between the age of 20 and 50 years. There were 131 males and 16 females in the series.

Submitted for publication Dec. 10, 1959.

This work was supported by a grant to one of the authors (T. H. B.) from the Council for Scientific and Industrial Research, South Africa.

From the Department of Medicine and Department of Pathology, Division of Chemical Pathology, University of the Witwatersrand Medical School.

All sections were stained routinely with hematoxylin and eosin. Sections were also stained for hemosiderin using Perl's method without heating and were counterstained with basic fuchsin. Chemical analyses were carried out on formalin-fixed tissues, and no allowance was made for hemoglobin iron since it has been shown that the maximum error introduced in this would be in the order of 0.02 to 0.04% dry weight.[2] The specimens were dried at 110 C for 12 hours and ashed at 500 C. The ash was dissolved in concentrated hydrochloric acid, diluted up to a suitable volume, and iron estimations were then carried out on aliquots of the solution. These were done using a modification of the method of Bothwell and Mallett[13] in which the solution was buffered with sodium acetate, and iron was estimated in the ferrous form using thioglycollic acid as reductant and sulfonated bathophenanthroline[14] as the color reagent. All results were expressed as percentage per 100 gm. dry weight.

Histological Grading of Liver.—The amount of hemosiderin present in parenchymal cells, Kupffer cells, and portal tracts was assessed separately.

(*a*) Parenchymal Cells: Grade 0 = no stainable iron granules; Grade + = a few small fine granules of hemosiderin usually confined to the biliary pole in some, or most, of the liver cells especially in the periportal regions. Grade ++ = numerous fine granules of hemosiderin in most of the cells, and Grade +++ = numerous coarse granules of hemosiderin in most of the liver cells.

(*b*) Kupffer Cells: Grade 0 = no stainable hemosiderin granules. Grade + = occasional fine hemosiderin granules in some of the Kupffer cells. Grade ++ = numerous fine hemosiderin granules in most of the Kupffer cells, and Grade +++ = numerous coarse hemosiderin granules in most of the Kupffer cells. (With Grade +++ the Kupffer cells were usually markedly enlarged.)

(*c*) Portal Tracts: Grade 0 = no stainable hemosiderin granules. Grade + = occasional fine hemosiderin granules in portal tract macrophages. Grade ++ = small clumps of macrophages containing fine and coarse hemosiderin granules, and Grade +++ = coarse irregular intra- and extracellular masses of hemosiderin.

Portal Fibrosis: Grade 0 = essentially normal histology. Grade + = slight thickening of the portal tracts. Grade ++ = moderate thickening of the portal tracts, and Grade +++ = marked thickening of the portal tracts, with early linkage of adjacent tracts in some areas but without distortion of the architectural pattern.

Cirrhosis was only regarded as being present when there was a marked increase in portal fibrosis with linkage of adjacent portal tracts, distortion of the lobular pattern and areas of nodular hyperplasia.

Histological Grading of Spleen.—Grade 0 = no stainable hemosiderin granules. Grade + = occa-

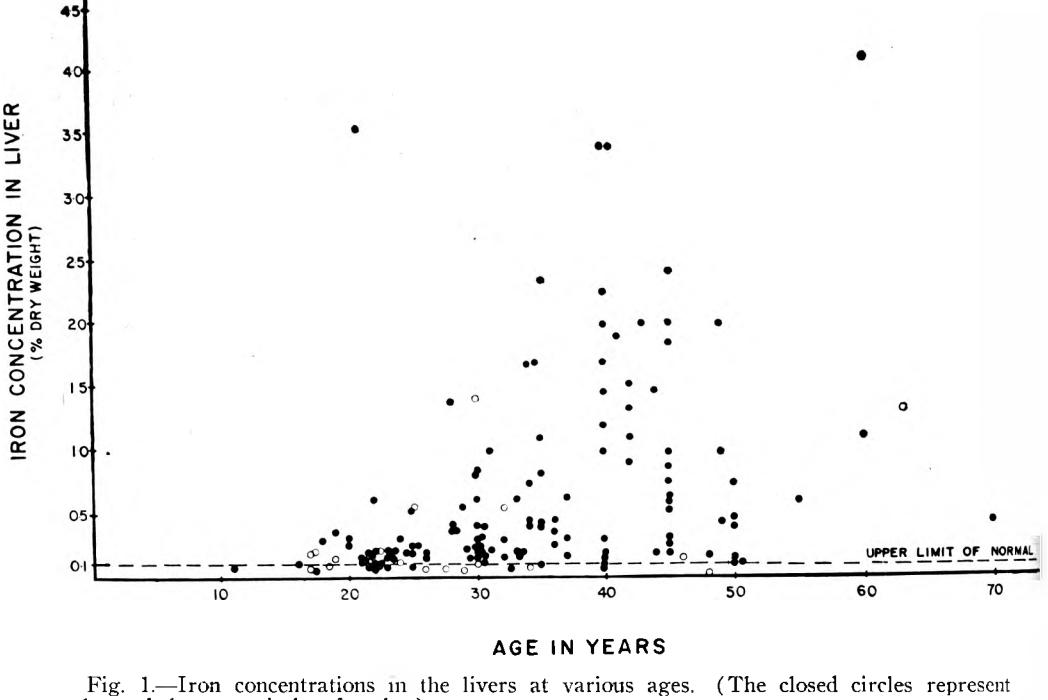


Fig. 1.—Iron concentrations in the livers at various ages. (The closed circles represent males and the open circles, females.)

TABLE 1.—*Relationship Between the Iron Concentrations in the Liver and the Histological Findings*

No. of Cases	Average Age	Iron Content (% Dry Weight)	Liver Histology: Parenchyma				Kupffer Cells				Portal Tracts			
			0	+	++	+++	0	+	++	+++	0	+	++	+++
16	26	<0.1	16	--	--	--	16	--	--	--	16	--	--	--
36	30	0.1-0.19	16	19	1	--	30	6	--	--	36	--	--	--
35	30	0.2-0.39	1	28	6	--	24	11	--	--	32	3	--	--
15	37	0.4-0.59	--	3	12	--	4	9	2	--	9	4	2	--
17	39	0.6-0.99	--	--	17	--	--	4	8	5	3	8	5	1
17	41	1.0-1.99	--	--	8	9	--	2	5	10	--	4	6	7
11	42	>2.0	--	--	--	11	--	--	--	11	--	--	--	11

sional fine granules of hemosiderin in some of the pulp macrophages. Grade ++ = numerous fine and coarse hemosiderin granules in pulp macrophages, and Grade +++ = numerous large masses of coarse intra- and extracellular hemosiderin in the splenic pulp and trabeculae.

Hemosiderin granules were also present in venous endothelial cells and in the subcapsular region in the majority of specimens graded ++ or +++.

Results

Iron Concentration of the Liver at Different Ages.—These results are shown in Figure 1. Only 16 of the subjects had iron concentrations which were less than 0.1%, a figure accepted as the upper limit of normal.[15,16] Although there was a wide scatter, there was a tendency for the iron concentration to rise with age, and maximal concentrations were reached about the age of 40 to 50 years. Between 10 and 19 years the average concentration was 0.16% (S.D. ±0.10), between 20 and 29 it was 0.32% (S.D.±0.17), between 30 and 39 it was 0.50% (S.D.±0.15), between 40 and 49 it was 1.10% (S.D.±0.28), and over 50 years the average concentration was 0.88% (S.D. ±0.11). There were too few female subjects to allow for a comparison between the sexes. Taking the group as a whole 71 subjects showed mild increases in iron concentration (0.10 to 0.39%), 32 showed moderate increase (0.40 to 0.99%), while 28 had marked increases, with iron concentrations above 1.0%.

Comparison of Histological Findings in Liver and Iron Concentrations.—These results are shown in Table 1. It can be seen that with iron concentrations of less than 0.10% iron was not demonstrable histologically. Between 0.10 and 0.19% half the

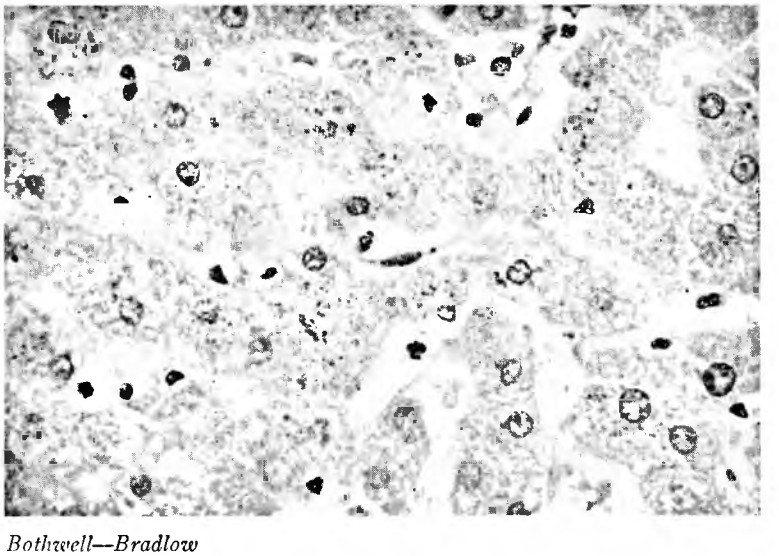


Fig. 2.—Section of liver showing fine hemosiderin granules in parenchymal cells with virtually no hemosiderin in Kupffer cells. Perl's stain; reduced about 30% from mag. ×600. (Iron concentration 0.19% dry weight.)

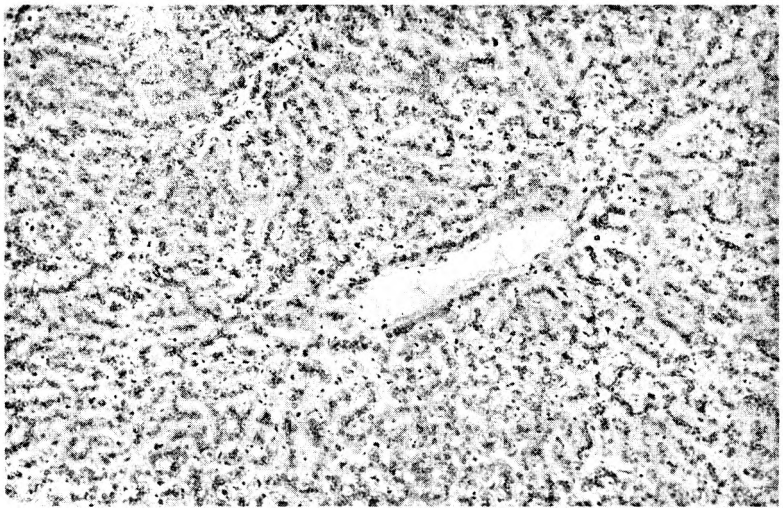


Fig. 3.—Section of liver showing a moderate increase in hemosiderin granules confined to parenchymal cells. Perl's stain; reduced about 30% from mag. × 120. (Iron concentration 0.52% dry weight.)

subjects showed a mild increase in parenchymal cells and 17% had similar changes in Kupffer cells (Fig. 2). (In only 2 subjects was Kupffer cell hemosiderin demonstrable in the absence of parenchymal deposits.) With iron concentrations between 0.20 and 0.39% the majority of subjects had a mild increase in parenchymal cells, and a few showed moderate amounts. About 30% had slight deposits in the Kupffer cells while only 10% showed mild portal tract involvement. Between 0.40 and 0.59% the majority of subjects showed a moderate increase in parenchymal hemosiderin, while most of the cases showed a mild increase in Kupffer cells (Fig. 3). Portal tract deposits were present in less than half the cases and when present were of either mild or moderate degree. With iron concentrations between 0.60 and 0.99% all subjects had moderate parenchymal deposits and the Kupffer cell deposits were of all degrees, most of them being moderate, while the majority of subjects also showed portal tract involvement which was usually mild or moderate (Fig. 4). When the iron concentration varied between 1.0 and 1.9% the parenchymal cell involvement was moderate or severe, and this was true also for Kupffer cells and for portal tracts in most subjects (Fig. 5). However, occasional cases showed only mild Kupffer cell and portal tract involvement in spite of the heavy parenchymal deposits. With iron concentra-

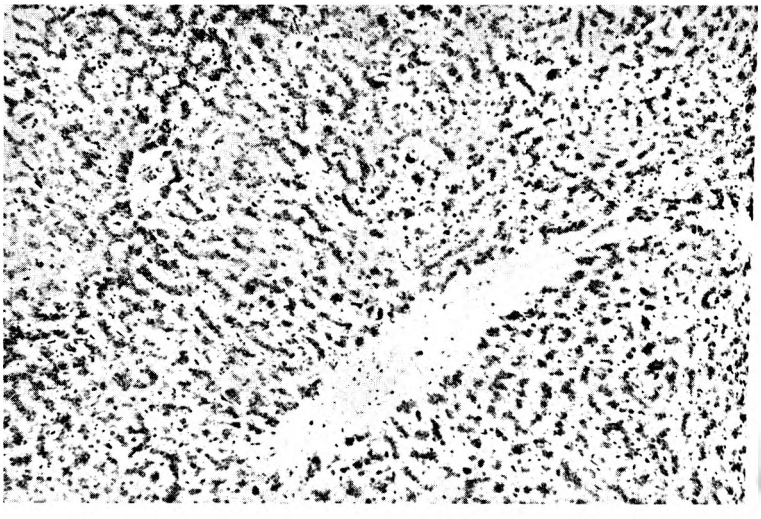


Fig. 4.—Section of liver showing moderate siderosis of parenchymal and Kupffer cells with very little present in the portal tracts. Perl's stain; reduced about 30% from mag. × 120. (Iron concentration 0.96% dry weight.)

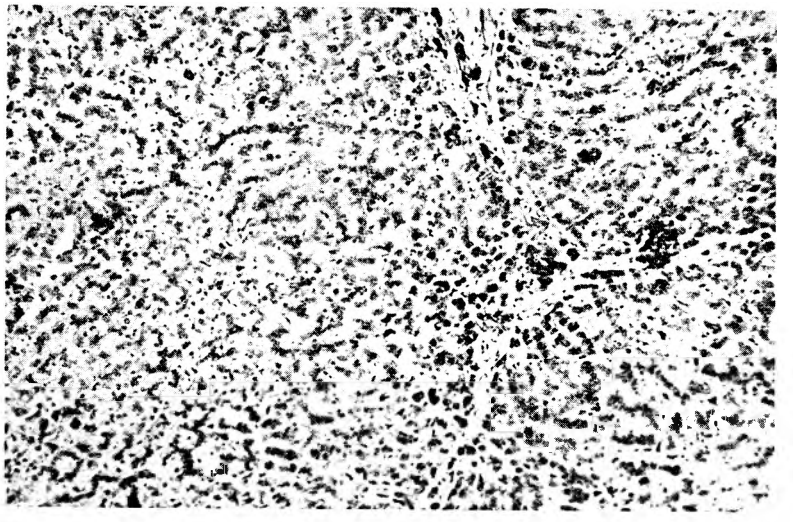


Fig. 5.—Section of liver showing moderately severe siderosis involving parenchymal cells, Kupffer cells and portal tracts. Perl's stain; reduced about 30% from mag. × 120. (Iron concentration 1.47% dry weight.)

tions above 2.0%, all subjects showed heavy deposits in parenchymal cells, Kupffer cells and portal tracts (Fig. 6).

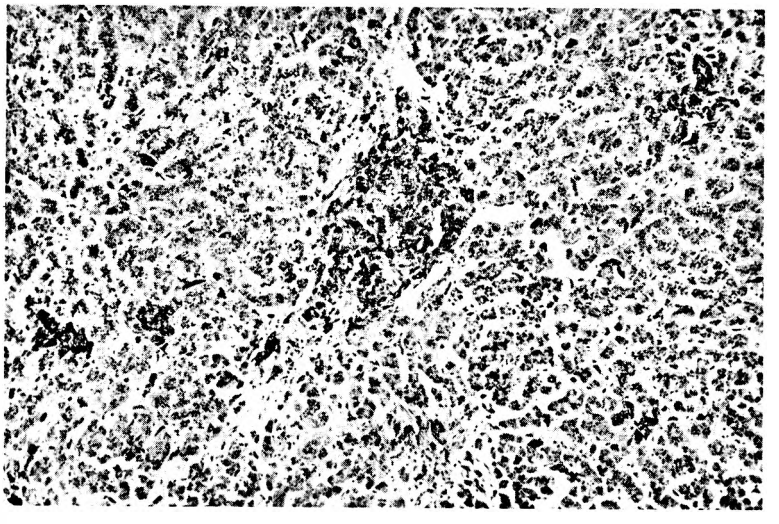


Fig. 6.—Section of liver showing massive hemosiderin deposits in parenchymal cells, Kupffer cells and portal tracts. (Iron concentration 2.0% dry weight.)

Relationship Between Siderosis and Portal Fibrosis.—These results are shown in Table 2. It can be seen that only 5 of 96 subjects with iron concentrations of less than 0.50% showed portal fibrosis, and this was mild in 4 of them. One subject in this group with an iron concentration of 0.20% had findings suggestive of subacute hepatitis with moderate portal fibrosis and granulomatous lesions compatible with chronic bilharzial infections. Of 40 subjects with iron

TABLE 2.—*Relationship Between the Iron Concentration in the Liver and Portal Fibrosis*

No. of Cases	Average Age	Iron Content of Liver (% Dry Weight)	Portal Fibrosis				
			0	+	++	+++	Cirrhosis
96	30	<0.5	91	4	1	-	-
40	39	0.5-1.99	31	8	1	-	-
11	41	>2.0	-	2	5	2	2

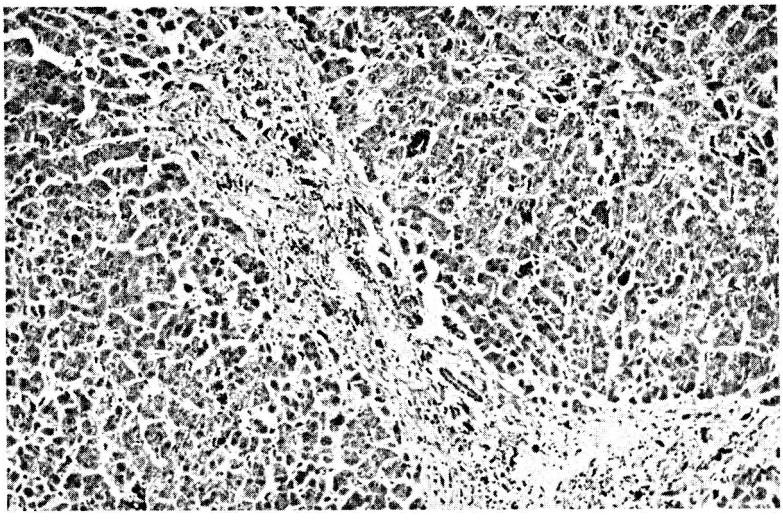


Fig. 7.—Section of liver showing moderate portal fibrosis. Hematoxylin and eosin; reduced about 30% from mag. × 120. (Iron concentration 2.0% dry weight.)

concentrations between 0.50 and 1.99%, 8 showed mild portal fibrosis and one had portal fibrosis of a moderate degree. In striking contrast were the findings in 11 subjects with iron concentrations above 2.0%. Portal fibrosis of varying degrees was present in all these subjects. In two it was mild, in five moderate (Fig. 7), in two severe, while the last two showed a septal cirrhosis (Fig. 8).

Other Histological Features in the Liver. Mild to moderate fatty infiltration was found in seven subjects; miliary tuberculosis was noted in one subject, and findings compatible with acute hepatitis and subacute hepatitis respectively were present in two others. Cellular infiltration in the portal tracts of varying degrees was present in 60 specimens. In 95 subjects (average age 31 years) with iron concentrations of less than 0.50% there were 26 who showed mild or moderate cellular infiltration, while in 52 subjects (average age 39 years) with iron concentrations above 0.50% there were 34 cases showing all grades of cellular infiltration.

Comparison Between the Concentration of Iron in the Liver and Spleen.—These results are shown in Figure 9. It can be seen that there was a close correlation between the rise in concentrations of iron in the liver and in the spleen. In even the mildest cases

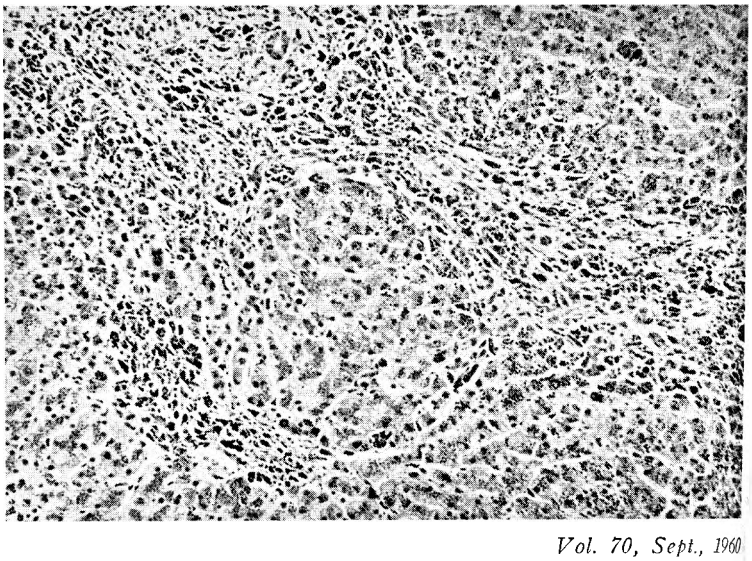


Fig. 8.—Section of liver showing an early septal cirrhosis with portal fibrosis, cellular infiltration of the portal tracts and a hyperplastic nodule. Hematoxylin and eosin; reduced about 30% from mag. × 120. (Iron concentration 3.4% dry weight.)

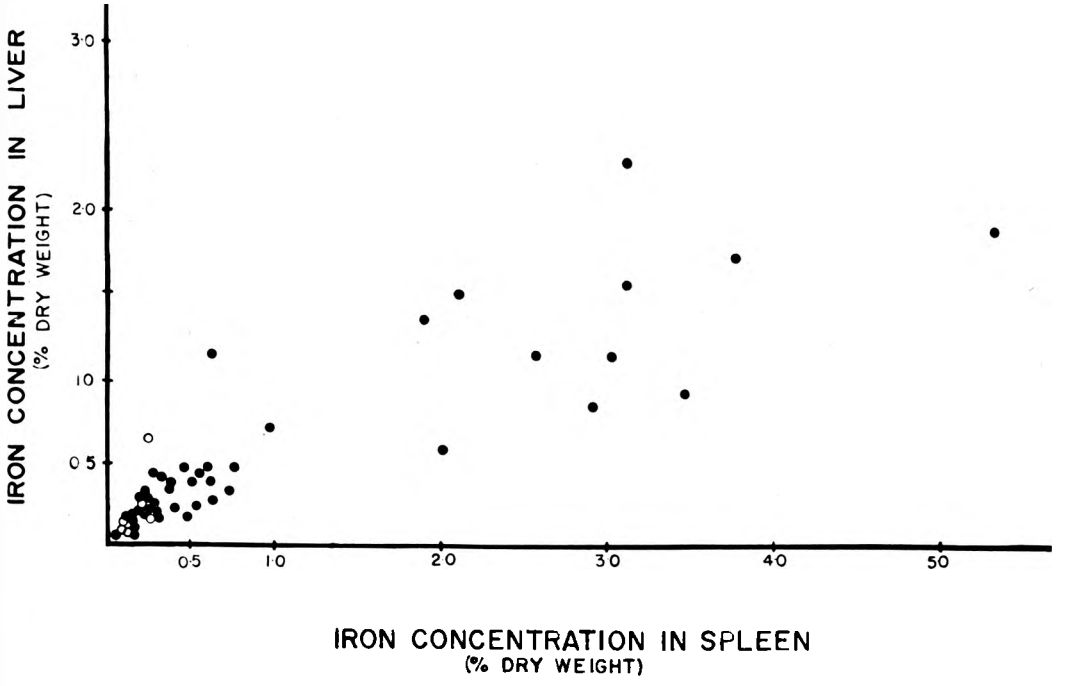


Fig. 9.—A comparison of the iron concentrations in liver and spleen. (The closed circles represent males and the open circles, females.)

of hepatic iron overload the splenic concentration of iron was usually above the average normal value of 0.14%.[16] In mild cases, the degree of rise in iron concentrations was approximately of the same order in liver and spleen while in subjects with moderate and marked increases in liver iron content the splenic concentration always tended to be higher. There was a good correlation between the concentration of iron in the spleen and the histological findings (Table 3). With iron concentration of less than 0.15% hemosiderin was not demonstrable histologically in the spleen except in one case. With concentrations between 0.15 and 0.49% the majority showed slight deposits (Fig. 10), with concentrations between 0.50 and 2.0% most showed moderate deposits (Fig. 11), while with concentrations above 2.0% all showed massive iron deposits (Fig. 12).

Comment

The results of this study confirm the high incidence of siderosis in the adult male Bantu of Johannesburg. Judging by the findings in the liver and spleen the siderosis first becomes manifest in late adolescence and reaches its greatest degree in the forties. In the present series the subjects varied in age from 11 to 70 years, although most were between 20 and 50. Of the cases 89%

TABLE 3.—*Relationship Between the Iron Concentration of the Spleen and the Histological Findings*

No. of Cases	Average Age	Iron Content (% Dry Weight)	Spleen Histology			
			0	+	++	+++
8	30	<0·15	7	1	--	--
20	31	0.15-0.49	2	18	--	--
11	37	0.50-2.0	--	3	8	--
11	42	2.0 -5.32	--	--	--	11

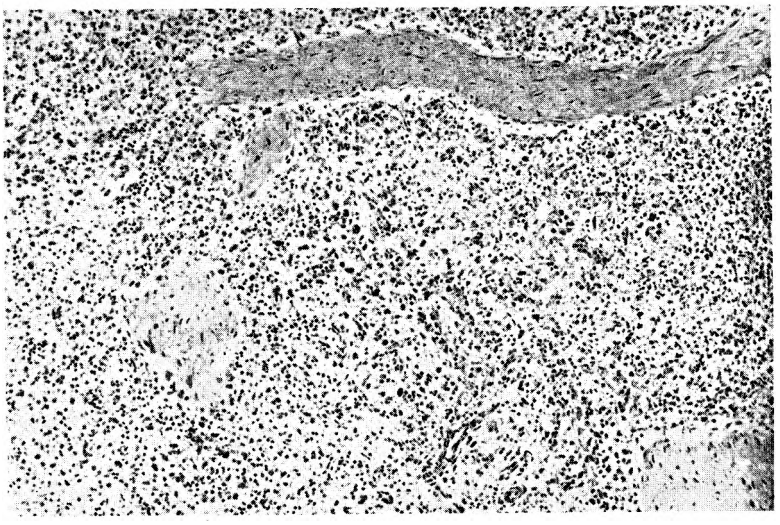


Fig. 10. — Section of spleen showing hemosiderin granules in occasional pulp macrophages. Perl's stain; reduced about 30% from mag. × 120. (Iron concentration 0.21% dry weight; corresponding liver concentration 0.26% dry weight.)

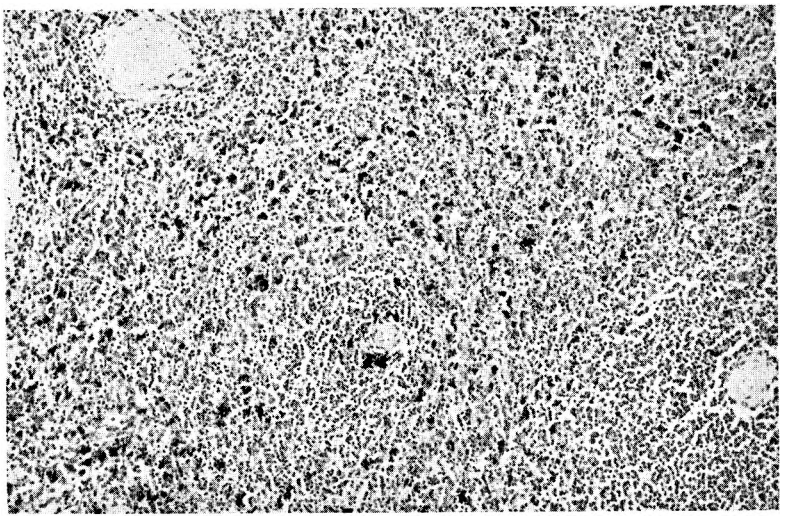


Fig. 11. — Section of spleen showing large hemosiderin granules in macrophages throughout the pulp. Perl's stain; reduced about 30% from mag. × 120. (Iron concentration 0.98% dry weight; corresponding liver concentration 0.68% dry weight.)

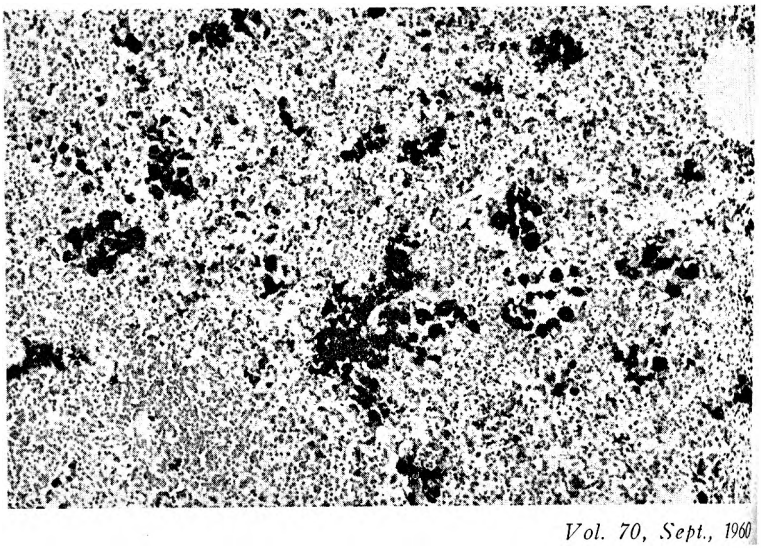


Fig. 12. — Section of spleen showing large masses of intra- and extracellular hemosiderin. Perl's stain; reduced about 30% from mag. × 120. (Iron concentration 3.02% dry weight; corresponding liver concentration 1.19% dry weight.)

showed varying degrees of hepatic siderosis. However in 59% the siderosis was of mild degree (between 0.10 and 0.39%). At the other end of the scale there were 19% with iron concentrations above 1.0% which is in the range found in idiopathic hemochromatosis.[15]

A comparison of the histological findings in liver and spleen with the chemical concentrations of iron in these organs made it possible to trace the sequence of events in the pathogenesis of the condition. In the majority of subjects with iron concentrations in the liver between 0.1 and 0.4% fine hemosiderin granules were visible in parenchymal cells, especially in the periportal areas. In a small proportion of these cases hemosiderin was also present in Kupffer cells. With concentrations of iron between 0.4 and 0.6% moderate amounts of iron were present in parenchymal cells, and in most cases there were also minimal deposits in Kupffer cells while a few showed early portal tract involvement. In subjects with levels above 0.6% increasing amounts of hemosiderin were present in parenchymal cells, in Kupffer cells and in portal tracts, and when the concentrations of iron was 2.0% or more, deposits were maximal in all three sites.

These results are in moderately close agreement with those previously reported by Gillman and Gillman [2,3,11] and by Wainwright [6] and do not support the contention of Higginson, Gerritsen and Walker [5] that reticuloendothelial involvement is present in the liver from the beginning. It is possible that this difference may be due to the fact that these last workers regarded a diffuse blue flush in Kupffer cells as indicative of increased iron while in the current study and in those of the Gillmans iron was only regarded as being present when hemosiderin granules were visible. One discrepancy between the present results and those of the Gillmans [2] was their observation that iron granules were sometimes present in parenchymal cells even with normal iron concentrations. On this basis they suggested that the earliest stage in the genesis of the condition was a disruption of mitochondria with the release of iron already present.[11] However, it should be noted that they also found stainable hemosiderin in 25% of medicolegal autopsies carried out on white subjects,[4] and it seems more likely that the discrepancy is due to different staining techniques since the Gillmans used a method in which heat is applied while the present results were obtained using cold potassium ferrocyanide.

Although it is evident that Bantu siderosis initially manifests in the liver as parenchymal deposits, it was of interest to find that the splenic concentration of iron rose at the same time as did the liver concentration and that with increasing levels of iron the splenic concentration rose to higher figures than did the liver. These findings, as previously pointed out by Higginson, Gerritsen and Walker,[5] are different from those in idiopathic hemochromatosis where reticuloendothelial deposits are not prominent even at advanced stages of the condition.[15] On the other hand, this distribution is very similar to that reported in animals placed on deficient diets with heavy iron supplements.[17-19] In all such studies iron deposits have been found in the spleen and in the parenchymal and Kupffer cells of the liver relatively early in the development of iron overload. Although the reason for these different patterns of iron distribution is not immediately apparent they can possibly be explained if attention is paid to the normal transport mechanism of iron within the body.

The plasma iron occupies a central role in the transfer of iron from one part of the body to another, and approximately 35 mg. iron entering this small pool of about 3 mg. is derived from the breakdown of red cells in the reticuloendothelial system, while the major portion leaving the plasma is directed towards the bone marrow for the formation of new red cells. Other tissues appear to have little ability to take up iron from the plasma, and the only other receptor which has any capacity for removing iron from the plasma is the liver.[22-24] This capacity is, however, an extremely limited one

when compared with that of the erythroid bone marrow.[24] When iron is absorbed from the bowel, it enters the plasma pool, but the amounts absorbed from a normal diet are so small that they do not significantly raise the level of iron in the plasma. On the other hand, the level of plasma iron can be raised by feeding large amounts of iron orally [25] or by injecting nonviable erythrocytes.[26] When this is done the amount of iron passing through the plasma is not significantly increased in normal subjects,[27] although the subsequent distribution of the iron is altered with an increased uptake by the liver.[17] While the reason for this increased hepatic uptake is not known, in vitro studies have shown that the amount of iron taken up from the iron-binding protein of the plasma by liver slices is increased when the iron level is high.[28] From these various observations it might therefore be anticipated that subjects continually absorbing excessive amounts of iron from the gut have high plasma iron levels and increased deposits of iron in the liver. In the Bantu there is good evidence that such a situation exists since the iron content of the diet is known to be high [12] and the average plasma iron levels in apparently normal Bantu males is above normal (229μg/100 ml. as compared to 113μg/100 ml. in white males).[29] Most of the absorbed iron probably passes directly into the plasma, but lymphatic absorption may also contribute to some extent as it has been shown that heavy deposits of iron are often present in the lamina propria of the duodenum and in the related lymph glands.[5,11]

Although the hepatic deposits found in early iron overload can be reasonably explained, it is more difficult to account for the splenic deposits since kinetic measurements suggest that the spleen takes up little if any iron directly from the plasma.[22-24] It is therefore possible that the deposition of iron in the spleen and other reticuloendothelial tissues may, at least in part, be due to impaired release of iron from these sites. Little is known of the factors which affect the release of iron from the reticuloendothelial cells, but it is conceivable that a decreased release might occur in situations where the plasma iron is continually raised as a result of increased absorption from the intestine. If this were so, a gradual build-up of iron derived from hemoglobin breakdown would occur in the spleen and other reticuloendothelial tissue. However, such an explanation probably does not explain all the iron present, and it appears likely that some of the portal tract deposits in the advanced stages of siderosis are derived from the breakdown of iron-laden hepatic parenchymal cells.

There are several views on the cause of the excessive iron absorption which must be present in Bantu subjects who develop iron overload. It has been demonstrated that the average Bantu diet contains large amounts of iron with quantities often exceeding 100 mg. per day and occasionally even double this figure.[12] Although the basic foodstuffs, such as maize, consumed by the Bantu contain moderate amounts of iron, most of the ingested iron is derived from the iron utensils used in cooking and in the preparation of fermented beverages. On the basis of these findings it has been suggested that iron overload in Bantu subjects is primarily due to their habitually high iron intake.[12] This view is supported by the observation that the amount of iron retained in the body increases with the size of the dose.[30,31] However, little is known of the long-term results of oral iron overload, although there have been isolated reports of the development of hemochromatosis in subjects taking excessive iron over long periods.[32] From a quantitative point of view the amounts absorbed daily in the Bantu would not have to be large to account for the deposits usually found by the ages of 40 to 60 years. Thus a daily retention in the male of 2.5 mg. per day between the ages of 20 and 40 years would lead to an accumulation of about 10 gm. by the age of 40, which is probably somewhat more than the average amount present in Bantu subjects. However, it is still not clear whether the amount of iron in the diet is the only factor in the patho-

genesis of siderosis in the Bantu, and it remains possible that other constituents and their relative proportions may be of importance. For example, it is difficult to overload animals with iron unless the phosphorus content of the diet is low.[19,33] Although the over-all phosphorus content in the average Bantu diet is if anything greater than normal,[12] the amounts present in kaffir beer, which is a major source of iron, are relatively low. Whether this influences the quantities absorbed is not known. Finally, it is possible that the behavior of the bowel mucosa may be altered in Bantu subjects who develop siderosis, and the Gillmans [11] have postulated that the increased absorption is the result of a widespread metabolic defect induced by chronic malnutrition. However, it should be remembered that the present material was obtained from subjects in apparently good health and although malnutrition, as evidenced by such manifestations as kwashiorkor in infants and pellagra in alcoholics certainly does occur in the general Bantu population, it is doubtful whether it is as widespread as the incidence of siderosis would suggest. In fact, there is some evidence that the average diet of urban Bantu in Johannesburg, although by no means optimal, may not be as inadequate as is commonly supposed. For example, one study done recently in the Johannesburg area showed that the average Bantu diet contained 25 gm. of animal protein, 39 gm. of vegetable protein, 37 gm. of fat, and 442 gm. carbohydrate without taking into account the amounts of carbohydrate consumed as beer.[34] It is therefore somewhat difficult to relate the siderosis produced in animals by feeding diets of corngrit and lard [17] or of uncooked corn meal,[19] together with iron supplements, to the situation in the Bantu. However, it should be noted that even in animals on grossly deficient diets iron over-load can largely be prevented by the addition of phosphates.[33] This finding suggests that the excessive absorption in these animals is related more to the iron/phosphorus ratio of the diet than to an alteration in the behavior of the intestinal mucosa.

Although cirrhosis of the liver is a constant feature in idiopathic hemochromatosis attempts to reproduce such findings in animals subjected to iron overload have been uniformly unsuccessful.[35] It is, however, noteworthy that in most of the experiments in which massive overload has been produced iron has been administered in forms which are predominantly taken up by the reticuloendothelial system, and little redistribution has occurred even after long periods.[35] Studies of this type therefore only illustrate how well reticuloendothelial tissues tolerate iron overload and do not necessarily apply to the effects of excessive iron in epithelial tissues. In the Bantu it has been difficult to elucidate the relationship between siderosis of the liver and secondary pathological changes. In their initial studies, Gillman, Mandelstam and Gillman [2] came to the conclusion that although cirrhosis was common in subjects with advanced siderosis the two were not causally related since cirrhosis was found in subjects with all grades of siderosis. In addition, Higginson, Gerritsen and Walker [5] found no correlation between the degree of fibrosis in liver and the amount of iron present. However, in a further study in which the material was drawn from 876 consecutive necropsies on Bantu patients dying in hospital, Higginson, Grobbelaar and Walker [36] found a marked association between siderosis and the more severe degrees of diffuse portal fibrosis. In another recent study in which liver biopsy specimens were examined from 200 adult male Bantu patients with suspected liver disease siderosis of varying degrees was found in 88% of cases.[7] Portal fibrosis was present in 43% of all patients, portal cirrhosis in 18.5% and postnecrotic scarring in 10.5%. Portal fibrosis and cirrhosis were closely correlated with advanced portal siderosis, and it was suggested that the presence of iron in the portal tracts might provoke fibrosis or cirrhosis, especially in the pres-

ence of chronic malnutrition. In the present study the over-all incidence of liver disease was not high. In the series of 147 liver specimens which were examined, one showed miliary tuberculosis, one showed changes compatible with acute hepatitis, 7 showed mild fatty infiltration, 13 showed evidence of very mild portal fibrosis, 7 had moderate portal fibrosis, 2 had severe portal fibrosis and 2 showed a septal cirrhosis. The correlation between the iron content of the liver and the presence of portal fibrosis was a striking one. Thus in the 96 subjects with less than 0.50% iron there were 4 cases with mild portal fibrosis and 1 with subacute hepatitis showed moderate portal fibrosis. With iron concentrations between 0.50% and 1.99% there were 7 cases with mild fibrosis and 1 with moderate portal fibrosis. In sharp contrast were the findings in 11 subjects with iron concentrations over 2.0%. There was increased portal fibrosis in all cases. In 7 it was either moderate or severe, while in 2 a frank cirrhosis was present. Although the differences in the incidence of fibrosis between those with mild and marked iron overload might be explained by differences in the ages of the two groups, this certainly did not apply to the comparison between those with moderate and marked degrees of iron overload, since the average ages in these two groups were very similar. In the present findings there appears, therefore, to be a close correlation between gross iron excess and the presence of portal fibrosis or cirrhosis. Although it is possible that iron under these circumstances only potentiates the effects of some other unknown fibrogenic agent or agents, the relationship may well be a direct one.

Since it appears that siderosis in the Bantu may lead to pathological changes in the liver, the question arises as to the frequency and significance of disturbance in other organs. By the time clinical manifestations appear in idiopathic hemochromatosis deposits of iron are present in many organs including the pancreas, stomach, heart, thyroid and adrenals, and patients with the disease commonly have diabetes and cardiac failure in addition to the signs of cirrhosis of the liver.[32] However, in Bantu subjects with siderosis epithelial deposits are relatively rare, and it has been suggested that there is no evidence that iron overload per se is harmful to the body.[12] Whether this is in fact true is open to question. From the present study and those of others it is obvious that although siderosis is common it is only rarely of severe degree. In the pathogenesis of any form of oral iron overload the liver takes up most of the excessive iron, and it is only when heavy hepatic deposits are present that siderosis develops in other epithelial tissues.[18,32] It might therefore be expected that only a very small proportion of Bantu subjects would accumulate amounts of iron as great as those eventually present in idiopathic hemochromatosis, especially as it is known that the rate of intestinal absorption decreases when the stores are enlarged.[30] In the small group who do accumulate enough iron over a long enough period significant deposits may be found in many epithelial tissues, and except for the heavy splenic deposits the histological findings are very similar to those in idiopathic hemochromatosis.[11,37] As yet no diligent clinical research for subjects of this type has been made, but there is already evidence that diabetes may supervene in Bantu subjects with massive iron overload.[37,38]

Summary

A series of 147 liver specimens obtained from 129 male and 18 female Bantu subjects dying acute traumatic deaths were examined histologically and chemically for the presence of iron. In addition 50 specimens of spleen were also examined. The subjects ranged in age between 11 and 70 years, most of them being between the ages of 20 and 50 years.

Hepatic siderosis of varying degrees was present in 89% of the cases and in 18% it was severe, with iron concentrations above 1.0% dry weight. The degree of siderosis tended to increase with age up to 40 to 50

years. There was a close correlation between the iron concentration and the histological findings. With mild degrees of iron overload iron was usually present only in the parenchymal cells of the liver. With increasing concentrations iron appeared in the Kupffer cells and in the portal tracts. With gross degrees of iron overload heavy deposits were present in all three sites. The concentration of iron in the spleen was usually increased with all degrees of iron overload and in advanced cases tended to be somewhat higher than that present in the liver.

There was a close correlation between the iron concentration in the liver and the presence of hepatic fibrosis or cirrhosis. Although the incidence of hepatic fibrosis and cirrhosis in the group as a whole was not high (15.6% and 1.4% respectively), all the 11 subjects with iron concentrations above 2.0% dry weight showed either portal fibrosis or cirrhosis.

We are indebted to Dr. J. Friedman for access to the material used in this study and to Professor B. J. P. Becker for making facilities available in his department. Thanks are also due to Mr. D. S. F. Treurnich who prepared the histological sections and to Mrs. R. Benson and Mrs. R. Melrose who carried out the chemical estimations.

Dept. of Medicine, Medical School, University of Witwatersrand.

REFERENCES

1. Strachan, A. S.: Haemosiderosis and Haemochromatosis in South African Natives, with a Comment on the Aetiology of Haemochromatosis, M.D. Thesis, Glasgow, 1929.

2. Gillman, J.; Mandelstam, J., and Gillman, T.: 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), South African J.M. Sc. 10:109 (Dec.) 1945.

3. Gillman, J., and Gillman, T.: Structure of the Liver in Pellagra, Arch. Path. 40:239 (Oct.) 1945.

4. Gillman, J., and Gillman, T.: Liver Disease in Johannesburg: Relation to Pellagra, Lancet 1:169 (Jan. 31) 1948.

5. Higginson, J.; Gerritsen, T., and Walker, A. R. P.: Siderosis in the Bantu of Southern Africa, Am. J. Path. 29:779 (July-Aug.) 1953.

6. Wainwright, J.: Siderosis in the African, South African J. Lab. & Clin. Med. 3:1 (March) 1957.

7. Gillman, T.; Hathorn, M., and Lamont, N. M.: Liver Disease in Durban Africans: Histopathological Findings in Biopsies, South African J.M. Sc. 23:187 (Dec.) 1958.

8. Gelfand, M.: Bantu Siderosis, Tr. Roy. Soc. Trop. Med. & Hyg. 49:370 (July) 1955.

9. Edington, G. M.: Haemosiderosis and Anemia in the Gold Coast African, West African M.J. 3:66 (June) 1954.

10. Edington, G. M.: Nutritional Siderosis in Ghana, Central African J. Med. 5:186 (April) 1959.

11. Gillman, J., and Gillman, T.: Perspectives in Human Malnutrition, New York, Grune & Stratton, Inc., 1951.

12. Walker, A. R. P., and Arvidsson, U. B.: Iron "Overload" in the South African Bantu, Tr. Roy. Soc. Trop Med. & Hyg. 47:536 (Nov.) 1953.

13. Bothwell, T. H., and Mallett, B.: The Determination of Iron in Plasma or Serum, Biochem. J. 59:599 (April) 1955.

14. Trinder, P.: The Improved Determination of Serum Iron, J. Clin. Path. 9:170 (May) 1956.

15. Sheldon, J. H.: Haemochromatosis, New York and London, Oxford University Press, 1935.

16. Gross, H.; Sandberg, M., and Holly, O. M.: Changes in Copper and Iron Retention in Chronic Diseases Accompanied by Secondary Anemia: II. Changes in Liver, Spleen and Stomach, Am. J.M. Sc. 204:201 (Aug.) 1942.

17. Kinney, T. D.; Hegsted, D. M., and Finch, C. A.: The Influence of Diet on Iron Absorption: I. The Pathology of Iron Excess, J. Exper. Med. 90:137 (Aug.) 1949.

18. Finch, C. A.; Hegsted, M.; Kinney, T. D.; Thomas, E. D.; Rath, C. E.; Haskins, D.; Finch, S., and Fluharty, R. G.: Iron Metabolism: The Pathophysiology of Iron Storage, Blood 5:983 (Nov.) 1950.

19. Gillman, T.; Hathorn, M., and Canham, P. D.: Experimental Dietary Siderosis, Am. J. Path. 35:349 (March-April) 1959.

20. Huff, R. L.; Hennessey, T. G.; Austin, R. E.; Garcia, J. F.; Roberts, B. M., and Lawrence, J. H.: Plasma and Red Cell Iron Turnover in Normal Subjects and in Patients Having Various Hematopoietic Disorders, J. Clin. Invest. 29: 1041 (Aug.) 1950.

21. Bothwell, T. H.; Callender, S.; Mallett, B., and Witts, L. J.: The Study of Erythropoiesis Using Tracer Quantities of Radioactive Iron, Brit. J. Haematol. 2:1 (Jan.) 1956.

22. Elmlinger, P. J.; Huff, R. L., and Oda, J. M.: Depression of Red Cell Iron Turnover by Transfusion, Proc. Soc. Exper. Biol. & Med. 79:16 (Jan.) 1952.

23. Huff, R. L.; Tobias, C. A., and Lawrence, J. H.: A Test for Red Cell Production, Acta. haemat. 7:129 (March) 1952.

24. Elmlinger, P. J.; Huff, R. L.; Tobias, C. A., and Lawrence, J. H.: Iron Turnover Abnormalities in Patients Having Anemia: Serial Blood and in Vivo Tissue Studies with Fe^{59}, Acta haemat. 9:73 (Feb.) 1953.

25. Josephs, H. W.: Absorption of Iron as a Problem in Human Physiology, Blood 13:1 (Jan.) 1958.

26. Noyes, W. D.; Bothwell, T. H., and Finch, C. A.: The Role of the Reticulo-Endothelial Cell in Iron Metabolism, to be published.

27. Bothwell, T. H.; Hurtado, A. V.; Donohue, D. M., and Finch, C. A.: Erythrokinetics: IV. The Plasma Iron Turnover as a Measure of Erythropoiesis, Blood 12:409 (May) 1957.

28. Jandl, J. H.; Inman, J. K.; Simmons, R. L., and Allen, D. W.: Transfer of Iron from Serum Iron-Binding Protein to Human Reticulocytes, J. Clin. Invest. 38:161 (Jan.) 1959.

29. Gerritsen, T., and Walker, A. R. P.: Serum Iron and Iron-Binding Capacity in the Bantu, South African M.J. 27:577 (July 11) 1953.

30. Bothwell, T. H.; Pirzio-Biroli, G., and Finch, C. A.: Iron Absorption: I. Factors Influencing Absorption, J. Lab. & Clin. Med. 51:24 (Jan.) 1958.

31. Smith, M. D., and Pannacciulli, I. M.: Absorption of Inorganic Iron from Graded Doses: Its Significance in Relation to Iron Absorption Tests and the "Mucosal Block" Theory, Brit. J. Haemat. 4:428 (Oct.) 1958.

32. Finch, S. C., and Finch, C. A.: Idiopathic Hemochromatosis, an Iron Storage Disease: A. Iron Metabolism in Hemochromatosis, Medicine 34:381 (Dec.) 1955.

33. Hegsted, D. M.; Finch, C. A., and Kinney, T. D.: The Influence of Diet on Iron Absorption: II. The Inter-Relation of Iron and Phosphorus, J. Exper. Med. 90:147 (Aug.) 1949.

34. Oettle, A. G.: Personal communication.

35. Brown, E. B., Jr.; Dubach, R.; Smith, D.; Reynafarje, C., and Moore, C. V.: Studies on Iron Transportation and Metabolism, J. Lab. & Clin. Med. 50:862 (Dec.) 1957.

36. Higginson, J.; Grobbelaar, B. G., and Walker, A. R. P.: Hepatic Fibrosis and Cirrhosis in Man in Relation to Malnutrition, Am. J. Path. 33:29 (Jan.-Feb.) 1957.

37. Bradlow, B. A.; Dunn, J. A., and Higginson, J.: Tissue Iron Distribution in Bantu Siderosis, to be published.

38. Isaacson, C.; Seftel, H., and Bothwell, T. H.: Siderosis and Diabetes in the Bantu, to be published.

S. Afr. J. med. Sci. (1960), **25**, 89-98.

THE RELATIONSHIP BETWEEN SIDEROSIS AND DIABETES IN THE BANTU

by

H. C. SEFTEL, C. ISAACSON and T. H. BOTHWELL*

South African Institute for Medical Research, Baragwanath Hospital and Department of Medicine, University of the Witwatersrand, Johannesburg.

[RECEIVED 1ST JUNE, 1960]

Although there is general agreement on the widespread incidence of iron overload in the adult Bantu population of South Africa, there is still some controversy as to the aetiology, pathogenesis and significance of the condition [Gillman and co-workers, 1945a, 1945b; 1948; 1951; 1958; Higginson *et al.*, 1953; Wainwright, 1953; Bothwell and Bradlow, 1960]. Originally it was postulated that the increased absorption of iron from the gut was secondary to changes in the behaviour of the bowel mucosa induced by chronic malnutrition [Gillman and Gillman, 1951], while more recently it has been suggested that it may be primarily due to the high iron content of the average Bantu diet [Walker and Arvidsson, 1953]. Although both factors may play a part the average Bantu diet definitely contains large amounts of iron and it seems probable that oral iron overload is the single most important factor in the aetiology of siderosis in the Bantu. Whatever the aetiology of the condition there is no doubt that excessive deposition of iron occurs in the majority of Bantu adults [Gillman and Gillman, 1951; Higginson *et al.*, 1953; Wainwright, 1957; Gillman *et al.*, 1958]. In males the degree of siderosis tends to increase with age up to between 40 and 50 and, although females are also affected, the incidence and severity in younger age groups tends to be lower than in males [Wainwright, 1957]. This sex difference in young subjects may well be due to the increased loss of iron via menstruation, pregnancy and lactation in the female.

Most of the absorbed iron is initially deposited in the parenchymal cells of the liver but significant quantities are also found in reticulo-endothelial tissues such as the spleen [Higginson *et al.*, 1953; Bothwell and Bradlow, 1960]. With increasing degrees of iron overload the involvement of liver and spleen becomes progressively greater and between the ages of 40 and 50 the average iron concentration in these organs have been found to be 1·1 and 1·7% dry weight respectively [Bothwell and Bradlow, 1960]. At this stage the liver usually shows a characteristic appearance with heavy haemosiderin deposits in parenchymal cells, Kupffer cells and portal tracts while the spleen contains large amounts of intra- and extra-cellular iron in the pulp. However, in spite of the marked involvement of both the liver and reticulo-endothelial system it is uncommon to find significant deposits in epithelial tissues [Higginson *et al.*, 1953; Wainwright, 1957]. In the majority of cases the histological features are therefore unlike those in idiopathic haemochromatosis in which widespread epithelial involvement is a characteristic feature [Finch and Finch, 1955]. On the other hand there is no doubt that a small percentage of Bantu subjects with siderosis do show deposits throughout the body [Gillman and Gillman, 1951], but no data are available as to the clinical significance of such findings.

*In receipt of a grant from the Council for Scientific and Industrial Research, Pretoria, South Africa.

The present study was undertaken to find out whether iron overload in the Bantu ever gives rise to any of the clinical features associated with idiopathic haemochromatosis. Since diabetes occurs in approximately 85% of subjects with this disease [Finch and Finch, 1955] it was decided to review the autopsy records of all diabetic Bantu subjects who had died in a General Hospital over a four-year period.

MATERIAL AND METHODS

Approximately 400 autopsies are performed annually at the Baragwanath Hospital and from the records available 20 were listed as having been diagnosed as diabetic prior to death. It was possible to obtain a number of tissues which had been fixed in 10% formol saline from 18 of these subjects. Sections were cut from these specimens and were routinely stained with haematoxylin and eosin and for iron using Perl's method. In addition chemical analyses were carried out on a number of specimens of tissue. Such specimens were dried at 110° C for 12 hours and ashed at 500° C. The ash was dissolved in concentrated hydrochloric acid, diluted to a suitable volume and iron estimations carried out on aliquots of the solution. These were done using a modification of the method of Bothwell and Mallett [1955] in which the solution was buffered with sodium acetate and iron was estimated in the ferrous form using thioglycollic acid as reductant and sulfonated bathophenanthroline [Trinder, 1956] as colour reagent. All results were expressed as a percentage per 100 g. dry weight.

RESULTS

On reviewing the histological specimens from 18 cases it was found that 7 had no demonstrable iron in any organ, while in 6 there was a mild siderosis present in the liver but no deposits were found in epithelial tissues. One subject showed marked siderosis of the liver involving parenchymal cells, Kupffer cells and portal tracts but there was no cirrhosis, and deposits in epithelial tissues were not present. In the remaining 4 subjects a widespread siderosis was present with histological findings very similar to those in idiopathic haemochromatosis. The histological appearances in various representative organs are shown in Figs. 1-8. Chemical analyses on a number of tissues from these 4 subjects confirmed the histological findings (Table 1).

In 2 futher cases tissues were not available for histological or chemical analyses. However, from the full autopsy records available on these 2 subjects it was evident that siderosis was not a feature in either.

TABLE I

Iron concentrations in different organs of 4 *Bantu subjects with diabetes and marked iron overload. Results are expressed as percentages per* 100 *g. dry weight.*

Case No.	Age	Sex	Liver	Spleen	Heart	Pancreas	Thyroid
1	46	F	3·5	2·1	—	0·9	0·2
2	66	M	4·6	10·6	0·4	1·8	0·5
3	42	F	3·8	3·7	0·8	4·8	—
4	51	F	1·3	0·9	0·8	2·2	0·6
Average Concentration and Range in Idiopathic Haemochromatosis (Sheldon, 1935)			3·6 (1·0-7·6)	0·6 (0·2-0·8)	0·5 (0·2-1·0)	2·0 (0·06-5·0)	0·5 (0·2-1·2)

Clinical details of the 4 subjects with advanced generalised siderosis were obtained from the hospital files and these together with the detailed post-mortem findings are given below.

CASE 1: A female patient was aged 41 years when first seen at Baragwanath Hospital in November, 1950. At that time she was admitted for stabilisation of diabetes which had been present since 1945. She was obese, with a blood pressure of 140/95 mm. Hg. The rest of the physical examination was negative.

Laboratory Data. Urine analysis: 4 plus sugar; fasting blood sugar: 252 mg. %; serum proteins: 8·3 g. % (albumin 3·0 and globulin 4·3). X-ray of the chest: normal.

Subsequent Course. The diabetes was stabilised and the patient was discharged after 4 weeks on 40 units of Protamine Zinc insulin daily.

In May, 1951, she was re-admitted suffering from a left lower lobe pneumonia which responded well to treatment. At the time she was on 20 units of Protamine Zinc insulin daily. (A note in the records of this admission mentions that she was still menstruating normally at this time.)

In August, 1955, the patient was again admitted with pneumonia and on this occasion the liver was noted to be enlarged and firm. The spleen was also palpable. Response to therapy was again good and she was discharged from hospital on this occasion on a maintenance dose of 40 units of Isophane Insulin daily. However, she was re-admitted 2 weeks later in coma. On physical examination it was found that the blood pressure was 100/64 mm. Hg., the pulse rate was 80 per minute and the temperature was 98° F. Crepitations were present over the left lung base and hepato-splenomegaly was again noted. The urine was found to contain 2 plus sugar but no acetone or diacetic acid. Three blood sugars within 36 hours of admission gave values of 210, 317 and 200 mg. %. The blood urea was 58 mg. % and the cerebrospinal fluid was normal. The cause of the coma was not elucidated and treatment was mainly directed towards the correction of the mild diabetes. In spite of therapy the patient's condition deteriorated and she died 3 days after admission.

Autopsy Findings. The body was that of a well-nourished elderly Bantu female. The heart, which weighed 260 g., appeared normal. The liver (weight 1,750 g.) was nodular and of firm consistency with markedly increased fibrosis. The nodules varied in size from 1 to 4 mm. in diameter. The pancreas was brownish in colour and the spleen (weight 190 g.) was slightly enlarged and of firm consistency.

Microscopic Findings. The lungs showed evidence of an acute bronchiolitis. The cardiac muscle fibres contained only very occasional iron granules. The stomach contained heavy deposits of haemosiderin in epithelial cells while moderate amounts of haemosiderin were present in the lamina propria of the jejunum. The liver showed moderately severe fibrosis of the portal tracts with infiltration by lymphocytes and occasional polymorphs. Bile duct proliferation was a feature. Regenerating nodules were prominent but these were not large and did not generally exceed 3 to 4 mm. in diameter. The collagenous bands were not very thick and the overall histological appearances conformed with the septal type of cirrhosis as described by Baggenstoss and Stauffer [1952]. Heavy iron deposits were noted in the liver cells, Kupffer cells and portal tracts. Haemosiderin was also present in the epithelium of the bile ducts. The pancreas showed increased fibrosis and deposits of haemosiderin were present in the acinar cells, in the islets of Langerhans and in the interstitial connective tissue. Extensive haemosiderosis was present in the spleen. The kidney showed small amounts of haemosiderin in the tubular cells. Small amounts of haemosiderin were also noted in the thyroid and adrenal. In the thyroid the deposits were almost exclusively in the follicular-lining cells and in the adrenal and zona glomerulosa of the cortex was most affected.

CASE 2: The patient, who was a male aged 66 years, was admitted to hospital in September, 1955, complaining of a backache which had followed the lifting of a heavy weight. On questioning he admitted to both polyuria and polydipsia. On examination he was emaciated and appeared slightly jaundiced. The blood pressure was 100/70 mm. Hg. The liver was enlarged 4 fingers below the costal margin and was firm in consistency. In addition a moderate amount of free fluid was present in the abdominal cavity. The lower limbs were oedematous. Apart from mild tenderness over the lumbar spine the remainder of the examination was negative.

Laboratory Data. Urine analysis: 2 plus sugar but no acetone; fasting blood sugar: 328 mg. %; blood urea 24 mg. %. A liver biopsy specimen was reported as showing "siderosis and cirrhosis".

Subsequent Course. A diagnosis of haemochromatosis was made and therapy was started with insulin. Response was adequate and the patient was discharged 4 weeks later on a schedule of 50 units of Lente insulin daily.

Over the next 4 months the patient was re-admitted several times. On three occasions it was for the relief of ascites and twice it was for coma or stupor due to hypoglycaemia. These hypoglycaemic

attacks occurred while the patient was receiving a maintenance dose of 30 units of Lente insulin daily. On his final admission in February, 1956, the patient was extremely emaciated but only moderate ascites was present. The urine contained 2 plus sugar but no acetone or diacetic acid was present and the blood sugar was 300 mg. %. Eighteen hours after coming into hospital the patient died suddenly. The reason for this was not apparent although it may have been due to hypoglycaemia since he was given 20 units of soluble insulin 12 and 6 hours before death.

Autopsy Findings. The subject was an extremely emaciated middle-aged Bantu male. Fifty ml. of straw-coloured fluid was present in the peritoneal cavity. The heart weighed 200 g. and showed marked brownish discolouration of the myocardium. There was, however, no evidence of dilatation or hypertrophy. The stomach mucosa was intensely brown in colour and a similar though less marked appearance was noted in the duodenum and jejunum. The liver was enlarged and weighed 3,100 g. It was firm in consistency and brown in colour. The surface was irregular due to the presence of small nodules 1 to 4 mm. in diameter. The parenchyma showed increased fibrosis and nodular hyperplasia, the appearances being those of a septal cirrhosis. The pancreas was normal in size but was brown in colour as were the parotid glands. The spleen was enlarged (weight 300 g.), and of soft consistency. The capsule was thickened and the parenchyma was brownish in colour.

Microscopic Findings. The skin was free of iron pigment. A few haemosiderin laden macrophages were present in the alveoli and alveolar septa of the lungs. The heart showed heavy deposits of haemosiderin in the muscle fibres, most of them being present in the perinuclear region. The stomach contained heavy deposits of haemosiderin in the epithelial cells, particularly in the deeper parts of the mucosa. Small focal deposits were found in the jejunum lying mainly in the connective tissue of the lamina propria. The liver showed a well-marked cirrhosis. There was increased fibrosis of the portal tracts with chronic inflammatory cell infiltration and bile duct proliferation. Many of the fibrous tracts were linked together but the majority of fibrous bands were not coarse. Regenerating nodules of parenchymal tissue were seen, most of which were comparatively small. Due to the difficulties in determining the morphogenetic pathway of the cirrhotic process in the terminal stages it was not possible to be certain whether the cirrhosis was of the post-necrotic or septal variety. However, the macroscopic appearances and the comparatively small regenerating nodule favoured a diagnosis of a septal type of cirrhosis. Heavy deposits of haemosiderin were present in liver cells, Kupffer cells, bile ducts and portal tracts. Large deposits of haemosiderin were present in the pancreas. These were noted in acinar-lining cells, in the islets of Langerhans and in the histiocytes of the interstitial tissue. The spleen contained heavy deposits of haemosiderin and the fibrous trabeculae showed foci of calcification. The kidney contained small quantities of haemosiderin in a few tubular cells and in the cells of the glomerular tufts. Many of the macrophages in the bone marrow were heavily laden with haemosiderin. The pituitary did not contain any iron pigment. Both the thyroid and adrenal glands showed heavy deposits of haemosiderin. In the thyroid they were particularly marked in the follicular-lining cells. In the adrenal haemosiderin was prominent in both the interstitial cells and in the parenchymal cells, the zona glomerulosa containing the heaviest deposits. In the testes there were a few scattered foci of haemosiderin in the interstitial tissue and similar findings were noted in the parotid gland.

CASE 3: A female, aged 42 years, was admitted to hospital in semi-coma in February, 1956. No history was available. On examination she was found to be obese and dehydrated. The breathing was acidotic, the conjunctivae were jaundiced and the lower limbs were moderately oedematous. The temperature was 99·4° F and the blood pressure was 120/60 mm. Hg. The liver was 5 fingers enlarged and on rectal examination a melaena stool was observed. Shortly after admission the patient had three episodes of Jacksonian epilepsy involving the right upper limb.

Laboratory Data. Haemoglobin: 9·7 g. %; urine analysis; 4 plus sugar, 4 plus acetone; blood sugar 600 mg. %; blood urea: 92 mg. %; electrolytes: Na 130 mEq./l., Cl. 100 mEq./l., K 5·1 mEq./l. and HCO_3 13 mEq./l. The cerebrospinal fluid was normal apart from a high sugar content (312 mg. %).

Subsequent Course. The patient was diagnosed as suffering from diabetic coma plus cirrhosis of the liver with ruptured oesophageal varices. On therapy with soluble insulin, intravenous fluids, blood transfusion and antibiotics the ketosis, dehydration and anaemia were largely corrected within 36 hours but the patient remained semi-comatose. She continued in this state for 3 days and then died after a massive haematemesis.

Autopsy Findings. The body was that of a rather obese Bantu female. There was no obvious gross jaundice of the conjunctivae. The peritoneal cavity contained 600 ml. of straw-coloured fluid. The heart and lungs appeared normal. The oesophagus showed the presence of dilated tortuous varicosities at its lower end and the stomach contained some old clotted blood. The liver was markedly enlarged (weight 2,300 g.). The serosal surface was irregular and nodular, the nodules varying in diameter between 1 and 3 mm. The cut surface showed similar nodules and a considerable increase in fibrous

tissue was also noted. The appearances were those of a septal cirrhosis. The biliary passages were normal. The hilar, tracheo-bronchial and mesenteric lymph nodes were brownish in colour. The pancreas was also dark brown in colour. Apart from an acute cystitis the remaining morbid anatomical findings were essentially normal.

Microscopic Findings. The heart showed heavy haemosiderin deposits in all the muscle fibres. Heavy deposits were also present in the epithelial cells of the stomach mucosa but only a few small granules of haemosiderin were noted in the lamina propria of the small bowel. The liver showed a septal type of cirrhosis. There was mild fatty change of the hepatic cells. A considerable amount of haemosiderin was present in the parenchymal cells and significant deposits were also present in Kupffer cells, in the fibrous tissue septa and in the epithelial lining of the bile ducts. The pancreas contained very heavy haemosiderin deposits in the acinar cells and in the islets of Langerhans but only scanty amounts were found in the interstitial connective tissue. Moderate amounts of haemosiderin were present in the splenic pulp and a few haemosiderin granules were also seen in the tubular epithelium of the kidneys.

CASE 4: A female, aged 51 years, was admitted to hospital in March, 1957, complaining of swelling of the abdomen of 3 months' duration. On further questioning a history of alcoholism (skokiaan) going back 14 years was obtained. The menopause had occurred at the age of 45 years. On examination the patient was wasted and appeared slightly jaundiced. There was hyperpigmentation of the skin of the face and dorsal aspects of the hands and feet and the skin was dry and atrophic. Axillary hair was completely absent and pubic hair was diminished. The blood pressure was 120/70 mm. Hg. The liver, which was 3 fingers enlarged, was hard and nodular. The spleen was also palpable and the abdomen contained a considerable amount of free fluid. There was marked sacral and pedal oedema.

Laboratory Data. Urine analysis was completely normal. Haemoglobin: 14·6 g. %; serum proteins: 6·6 g. % (albumin 1·6, globulin 5·0); prothrombin index: 76 %; serum bilirubin 1·5 mg. %; "Liver function" tests: thymol turbidity 7·5 units, thymol flocculation 4 plus, colloidal red 4 plus, Takata-Ara 3 plus. The ascitic fluid was straw-coloured with a specific gravity of 1010 and a protein content of 4·0 g. %.

Subsequent Course. A diagnosis of cirrhosis of the liver was made. Ascitic fluid (4 litres) was removed by paracentesis and treatment was continued using a low salt diet and mercurial diuretics. The patient was fit enough to be discharged after 2 weeks in hospital.

In November, 1958, she was re-admitted because of the onset of "fits". On examination the physical signs were essentially the same as on her previous admission but in addition she was found to be markedly dehydrated and urine analysis revealed 4 plus glycosuria and acetonuria. The blood sugar was 290 mg. %. A few hours after admission she developed clonic movements involving the left upper limb. Examination of the central nervous system was otherwise normal and a specimen of cerebrospinal fluid was also normal. The patient was treated with intravenous fluids and soluble insulin. On this therapy she made a slow recovery over a period of 2 weeks. She was eventually discharged on a maintenance dose of 50 units of Lente insulin daily.

In March, 1959, the patient was re-admitted in semi-coma. At the time she was severely dehydrated and wasted. The urine was found to contain 4 plus sugar and acetone was also present. The blood sugar at this time was 580 mg. %. Treatment with soluble insulin, intravenous fluids and antibiotics corrected the dehydration and ketosis but failed to bring about any improvement in the mental state and 2 days after admission the patient died.

Autopsy Findings. The subject was a wasted middle-aged female. The peritoneal cavity contained 1,500 ml. of straw-coloured fluid. The stomach showed mucosal congestion with brownish discolouration. The small bowel appeared normal and showed no macroscopic evidence of siderosis. The liver, which weighed 2,100 g., was of firm consistency and had a markedly thickened capsule. The cut surface showed the features of a septal cirrhosis with increased fibrous tissue and numerous regenerating nodules, most of which were between 2 and 3 mm. in diameter. The pancreas was of normal size but was brown in colour. The spleen was enlarged (weight 200 g.) and on section the capsule was thickened and the parenchyma had a faint brownish discolouration. The respiratory, cardiovascular, genito-urinary and nervous systems presented no abnormal morbid anatomical features.

Microscopic Findings. The lungs showed severe congestion but were otherwise normal. Heavy iron deposits were present in the majority of the muscle fibres of the heart. The stomach contained excessive haemosiderin deposits in mucosal cells while sections of the small bowel showed only a few granules of haemosiderin in the lamina propria of the jejunum. In the liver thick collagenous septa separated the liver lobules although the lobular architecture was not completely destroyed. There was marked bile duct proliferation and lymphocytic infiltration in the fibrous tracts. Regenerating nodules were prominent. The appearances were those of a cirrhosis but it was not possible to determine the morphogenetic pathway. Large deposits of haemosiderin were present in the parenchymal and Kupffer

cells, and in the fibrous tissue. The epithelium of the bile ducts also contained iron granules. The pancreas showed increased interstitial fibrosis with atrophy of the secreting cells. Many of the islets of Langerhans showed atrophy with foci of degeneration and necrosis. Deposits of haemosiderin were present throughout the organ but were particularly heavy in acinar cells and in the islets. Small deposits of haemosiderin were present in the pulp of the spleen. Occasional granules of haemosiderin were present in the lining epithelial cells of the convoluted tubules in the kidney. In the thyroid large amounts of iron were found in the cells lining the follicles and the suprarenals showed heavy deposits particularly in the cells of the zona glomerulosa.

DISCUSSION

Excessive deposits of iron in the body may arise in three ways. In idiopathic haemochromatosis there is an alteration in the behaviour of the bowel mucosa so that increased quantities are absorbed from the normal diet [Finch and Finch, 1955]. Since the body's capacity to excrete iron is an extremely limited one it slowly accumulates and by the time clinical manifestations appear between 40 and 50 g. are usually present [Finch and Finch, 1955]. In certain refractory anaemias excessive quantities of iron may also be found in the body, but in these circumstances most of the iron is derived from repeated blood transfusions [Finch and Finch, 1955]. Lastly, in the Bantu of Southern Africa increased iron deposits are present in the majority of adult subjects [Gillman and Gillman, 1951; Higginson *et al.*, 1953; Wainwright, 1957; Bothwell and Bradlow, 1960], and it seems probable that this is primarily due to the large quantities of iron present in their diets [Walker and Arvidsson, 1953].

Although the histopathological findings in these three types of iron excess have been well defined there is still controversy as to the role of the iron deposits in the production of tissue damage. In idiopathic haemochromatosis the correlation between the presence of large iron deposits and pathological changes appears to be a close once since the common clinical manifestations such as cirrhosis, diabetes and cardiac failure can be explained by the heavy accumulations of iron which are found in the liver, pancreas and heart [Finch and Finch, 1955]. In spite of this some workers are doubtful whether the relationship between iron overload and tissue damage is as direct as these findings suggest [Dubin, 1955; Golberg, 1957; Brown *et al.*, 1957]. It has been pointed out that the fundamental defect in idiopathic haemochromatosis is as yet unknown and although there is certainly an alteration in the behaviour of the bowel mucosa this may not be the primary defect in the condition [Brown *et al.*, 1957]. It therefore remains possible that the increased absorption and deposition of iron are only by-products of some other metabolic disorder and that pathological changes in various organs are not the result of iron at all. This thesis has gained some support from the fact that a number of workers have been uniformly unsuccessful in their attempts to reproduce the pathological features of idiopathic haemochromatosis in several different animal species subjected to oral iron overload [Brown *et al.*, 1957]. There are, however, several possible explanations for these negative results. In studies of this type the iron has either been added in large quantities in the diet or it has been administered parenterally and there are certain drawbacks to both methods. When the oral route is used it is difficult to achieve concentrations of iron in the body as high as those found in idiopathic haemochromatosis. On the other hand when large amounts are given parenterally most of the administered iron is taken up by the reticulo-endothelial system and the distribution of iron is thus very different from that found in idiopathic haemochromatosis [Brown *et al.*, 1957]. In addition, the observation period in most animal studies has been very short when compared with the many years necessary for the development of organ damage in

idiopathic haemochromatosis. In only one study in dogs was the follow-up period a relatively long one (4-7 years) but it is difficult to interpret the significance of the negative findings obtained since parenteral iron was given and even at the end of the experiment most of the iron was still located in the reticuloendothelial system [Brown *et al.*, 1957]. It is therefore difficult to apply the results of animal experiments to the situation in idiopathic haemochromatosis and, even if it were possible to do so, the results obtained in this way might not apply to man since there may be differences in the tolerance of various species to iron overload.

In the siderosis that ensues after giving repeated blood transfusions to patients with refractory anaemias the initial distribution is very similar to that produced in animals given parenteral iron since the iron released from broken down red cells is found primarily in the reticulo-endothelial system [Kleckner *et al.*, 1954; Cappell *et al.*, 1957; Oliver, 1959]. In this site iron appears to exert little if any deleterious effects and a number of subjects have tolerated many blood transfusions without apparent harm [Kleckner *et al.*, 1954; Cappell *et al.*, 1957; Oliver, 1959]. However, with the passage of time re-distribution of body stores tends to occur and eventually histopathological findings not unlike those in idiopathic haemochromatosis may be found [Oliver, 1959]. Little is known of the factors which promote this re-distribution but the element of time appears to be the most important one [Finch, 1958]. In such cases clinical features suggestive of haemochromatosis may develop. For example a comprehensive review of the literature revealed that 20 out of 69 subjects of this type showed glycosuria [Oliver, 1959]. Increased portal fibrosis of the liver was present in the majority of cases but was rarely severe, while cardiac failure was uncommon. Although such findings have been regarded by some authors as strong corroborative evidence in favour of the pathogenic role of iron overload [Finch and Finch, 1955], others believe that the pathological changes may be the result of other factors such as chronic anaemia, frequent transfusion reactions, viral hepatitis and severe nutritional disturbance [Brown *et al.*, 1957].

Against this background of controversy the findings in Bantu subjects with iron overload are of particular interest. Although siderosis is found in the majority of adult Bantu it is usually not of severe degree and in most cases significant deposits are only present in the liver and reticulo-endothelial system [Gillman and Gillman, 1945; Higginson *et al.*, 1953; Wainwright, 1957; Bothwell and Bradlow, 1960]. While deposits in the parenchymal cells of the liver are a characteristic feature even in the earliest stages of Bantu siderosis [Gillman and Gillman, 1945; Bothwell and Bradlow, 1960] the well-marked reticulo-endothelial involvement serves to distinguish the condition from idiopathic haemochromatosis [Higginson *et al.*, 1953; Bothwell and Bradlow, 1960]. On the other hand the findings are very similar to those present in experimental animals given large amounts of oral iron [Kinney *et al.*, 1949; Gillman *et al.*, 1959]. Although the reason for these differences in iron distribution is not known, it may well be due to variations in the rate of absorption from the gut [Bothwell and Bradlow, 1960]. In idiopathic haemochromatosis the deviation from normal is not a great one and at any one time the amounts of iron absorbed from the gut are only slightly greater than normal [Finch and Finch, 1955], while in Bantu siderosis and in animal studies iron overload occurs at a much greater rate. Whatever the explanation for the distribution of iron present in most Bantu subjects with iron overload there is no doubt that the major impact is on the liver and reticulo-endothelial system. Even with severe degrees of siderosis in these sites it is uncommon to find significant deposits elsewhere in the body [Higginson *et al.*, 1953; Wainwright, 1957]. In fact, this is not altogether surprising since it has been calculated that the

amount of excess iron in Bantu subjects over 40 years of age rarely exceeds 10 g. [Bothwell and Bradlow, 1960]. This is well below the quantities found in idiopathic haemochromatosis and at these levels it might be anticipated that there would be little uptake of iron by other organs. As most of the excess iron is contained in the liver it has been of interest to correlate the degree of siderosis in this organ with other histological features.

In three recent studies in which material was obtained from patients dying in hospital [Higginson *et al.*, 1957], from liver biposy specimens [Gillman *et al.*, 1958] and from subjects dying acute traumatic deaths [Bothwell and Bradlow, 1960], a marked association was found between siderosis and the severer grades of portal fibrosis. In one of these investigations chemical estimations were also carried out on the tissues examined and it was found that portal fibrosis or cirrhosis was present in all specimens in which the iron concentration was above 2·0% dry weight [Bothwell and Bradlow, 1960]. On the other hand there appears to be no association between the presence of siderosis and post-necrotic scarring [Gillman *et al.*, 1958]. The present findings support these general conclusions since well marked cirrhosis was present in each of the 4 subjects examined. Although it was sometimes difficult to determine the morphogenetic pathway from the final picture [Baggenstoss and Stauffer, 1952] the microscopic and macroscopic features in these cases were almost certainly those of septal cirrhosis. These various observations suggest that large concentrations of iron in the liver are definitely fibrogenic. However, it is not yet certain whether this is a direct relationship since it is possible that the iron may only potentiate the effects of other unknown fibrogenic agents [Gillman *et al.*,1958].

Epithelial deposits of significant degree are extremely rare in Bantu siderosis. For example, in two studies in which several hundred cases were reviewed none showed the histological picture of idiopathic haemochromatosis [Higginson *et al.*, 1953; Wainwright, 1957]. Although such cases definitely do occur [Gillman and Gillman, 1951] they must be extremely rare and there is no good evidence as to whether they ever exhibit the clinical features present in idiopathic haemochromatosis. If they did it might be expected that a proportion would develop diabetes since this is present in over 80% of patients with idiopathic haemochromatosis [Finch and Finch, 1955]. It was for this reason that the histopathological findings at autopsy in all known diabetic Bantu subjects dying in hospital over a four year period were reviewed in the present study. In 4 out of 20 patients the histological findings were indistinuishable from those in idiopathic haemochromatosis and chemical analyses of liver, pancreas, thyroid and heart confirmed that the concentrations of iron were well within the ranges described in this disease. Although it was theoretically possible that these subjects were suffering from the genetic disorder there were several reasons which made such a possibility untenable. In the first place the number found in this series would suggest an incidence of the disease far in excess of that described elsewhere, secondly, the sex incidence was unusual since 3 out of 4 were females while idiopathic haemochromatosis is predominantly found in males [Finch and Finch, 1955], and lastly, the concentrations of iron in the spleen were a good deal greater than those described in the classical disease [Sheldon, 1935]. On the evidence, therefore, there seems to be no doubt that these subjects represent the most advanced degree of siderosis in the Bantu. At this stage organ damage similar to that found in idiopathic haemochromatosis occurs. Since the only apparent common denominator of the two conditions is an excess of iron in various body organs it seems reasonable to assume that manifestations of tissue damage such as cirrhosis of the liver and diabetes are secondary to excess iron. From a practical point of view the

present findings are of some importance since diabetes is not uncommon in the Bantu and it may well be that a significant proportion of such cases are due to iron overload. If this is so then methods must be developed for recognising them clinically so that venesection therapy can be instituted. This may not prove easy since siderosis of lesser degrees is so common that the finding of raised plasma iron levels and of siderosis on liver biopsy specimens would not necessarily imply that significant deposits were present in the pancreas. However, there is some evidence that pancreatic involvement is much more common when the liver shows cirrhotic changes (Bradlow, 1960; Isaacson *et al.*, 1960) and this may prove a useful pointer in future studies.

SUMMARY

Although siderosis is common in adult Bantu subjects it is usually confined to the liver and reticulo-endothelial system. However, in a small proportion of subjects widespread deposits do occur and the histopathological findings in this group are very similar to those in idiopathic haemochromatosis. In order to assess whether such cases ever develop clinical manifestations similar to those in idiopathic haemochromatosis the autopsy findings in a group of 20 diabetic Bantu subjects were reviewed. In 4 of these the histopathological picture was indistinguishable from idiopathic haemochromatosis; these findings were confirmed by chemical analyses for iron on various tissues. The results of this limited study suggest that massive iron overload is one of the causes of diabetes in the Bantu.

The authors wish to thank Dr. I. Frack, Superintendent of Baragwanath Hospital, for permission to publish the case reports. They are also indebted to the Director, South African Institute for Medical Research for granting facilities, to Professor B. J. P. Becker for reviewing the pathological findings and to Mr. M. Ulrich who prepared the photomicrographs.

REFERENCES

BAGGENSTOSS, A. H. and STAUFFER, M. H. (1952). Posthepatic and alcoholic cirrhosis; clinicopathologic study of 43 cases of each. *Gastroenterol.*, **22**, 157-180.

BOTHWELL, T. H. and MALLETT, B. (1955). The determination of iron in plasma or serum. *Biochem. J.*, **59**, 599-602.

BOTHWELL, T. H. and BRADLOW, B. A. (1960). Siderosis in the Bantu. *Arch. Path.* (In press).

BRADLOW, B. A. Personal communication.

BROWN, E. B., Jr., DUBACH, R., SMITH, D. E., REYNAFARJE, C. and MOORE, C. V. (1957). Studies in iron transportation and metabolism. *J. Lab. Clin. Med.*, **50**, 862-893.

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**, 245-264.

DUBIN, I. N. (1955). Idiopathic hemochromatosis and transfusional siderosis; a review. *Amer. J. Clin. Path.*, **25**, 514-542.

FINCH, S. C. and FINCH, C. A. (1955). Idiopathic haemochromatosis, an iron storage disease. A. Iron metabolism. *Medicine*, **34**, 381-430.

FINCH, C. A. (1958). *Discussion of hemosiderosis and hemochromatosis. Iron in Clinical Medicine.* (University of California Press.) p. 131.

GILLMAN, J. and GILLMAN, T. (1945). Structure of the liver in pellagra. *Arch. Path.*, **40**, 239-263.

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**, 109-136.

GILLMAN, J. and GILLMAN, T. (1948). Liver disease in Johannesburg. Relation to pellagra. *Lancet*, **i**, 169-173.

GILLMAN J. and GILLMAN, T. (1951). *Perspectives in Human Malnutrition.* (Grune and Stratton, New York).

GILLMAN, T., HATHORN, M. and LAMONT, N. M. (1958). Liver disease in Durban Africans. Histopathological findings in biopsies. *S. Afr. J. Med. Sci.*, **23**, 187-200.

GILLMAN, T., HATHORN, M. and CANHAM, P. A. (1959). Experimental dietary siderosis. *Amer. J. Path.*, **35**, 349-367.

GOLBERG, L. (1957). Hemosiderosis and hemochromatosis; The question of pathogenesis. *Post-grad. Med.*, **22**, 382-388.
HIGGINSON, J., GROBBELAAR, B. G. and WALKER, A. R. P. (1957). Hepatic fibrosis and cirrhosis in man in relation to malnutrition. *Amer. J. Path.*, **33**, 29-53.
HIGGINSON, J., GERRITSEN, T. and WALKER, A. R. P. (1953). Siderosis in the Bantu of Southern Africa. *Amer. J. Path.*, **29**, 779-815.
ISAACSON, C., SEFTEL, H. C. and BOTHWELL, T. H. (1960). Unpublished data.
KINNEY, T. D., HEGSTED, D. M. and FINCH, C. A. (1949). The influence of diet on iron absorption. I. The pathology of iron excess. *J. Exper. Med.*, **90**, 137-146.
KLECKNER, M. S., Jr., BAGGENSTOSS, A. H. and WEIR, J. F. (1954). Hemochromatosis and trans-fusional hemosiderosis. A clinical and pathologic study. *Amer. J. Med.*, **16**, 382-394.
OLIVER, R. A. M. (1959). Siderosis following transfusions of blood. *J. Path. Bact.*, **77**, 171-194.
SHELDON, J. H. (1935). *Haemochromatosis.* (Oxford Univ. Press.)
TRINDER, P. (1956). The improved determination of iron in serum. *J. Clin. Path.*, **9**, 170-172.
WAINWRIGHT, J. (1957). Siderosis in the African *S. Afr. J. Lab. Clin. Med.*, **3**, 1-28.
WALKER, A. R. P. and ARVIDSSON, U. B. (1953). Iron "overload" in the South African Bantu. *Trans. R. Soc. Trop. Med. Hyg.*, **47**, 536-548.

LEGENDS TO PLATES 1 and 2

FIG. 1. Section of liver showing septal cirrhosis. The regenerating nodules are small and are separated by fairly dense bands of collagen. Heavy deposits of haemosiderin are present in the hepatic cells, Kupffer cells and collagenous septa. Perl's Stain (x 30).

FIG. 2. Heavy deposits of intra and extracellular iron in the splenic pulp. Perl's Stain (x 120).

FIG. 3. High-power view of the pancreas showing large haemosiderin deposits in the acinar-lining cells. (Other fields showed iron in islets of Langerhans). Perl's Stain (x 480).

FIG. 4. Section of stomach mucosa showing large deposits of haemosiderin in the epithelial cells, particularly in the deeper layers. Perl's Stain (x 120).

FIG. 5. Section of thyroid showing haemosidern in the epithelial cells lining the follicles. Perl's Stain (x 120).

FIG. 6. Section of an adrenal gland containing enormous quantities of haemosiderin. The deposits are most marked in the secreting cells of the Zona glomerulosa. Perl's Stain (x 120).

FIG. 7. Section of the pituitary showing haemosiderin deposits in the secreting cells. Perl's Stain (x 120).

FIG. 8. Section of heart muscle showing heavy accumulations of haemosiderin in the myofibrils. Perl's Stain (x 480).

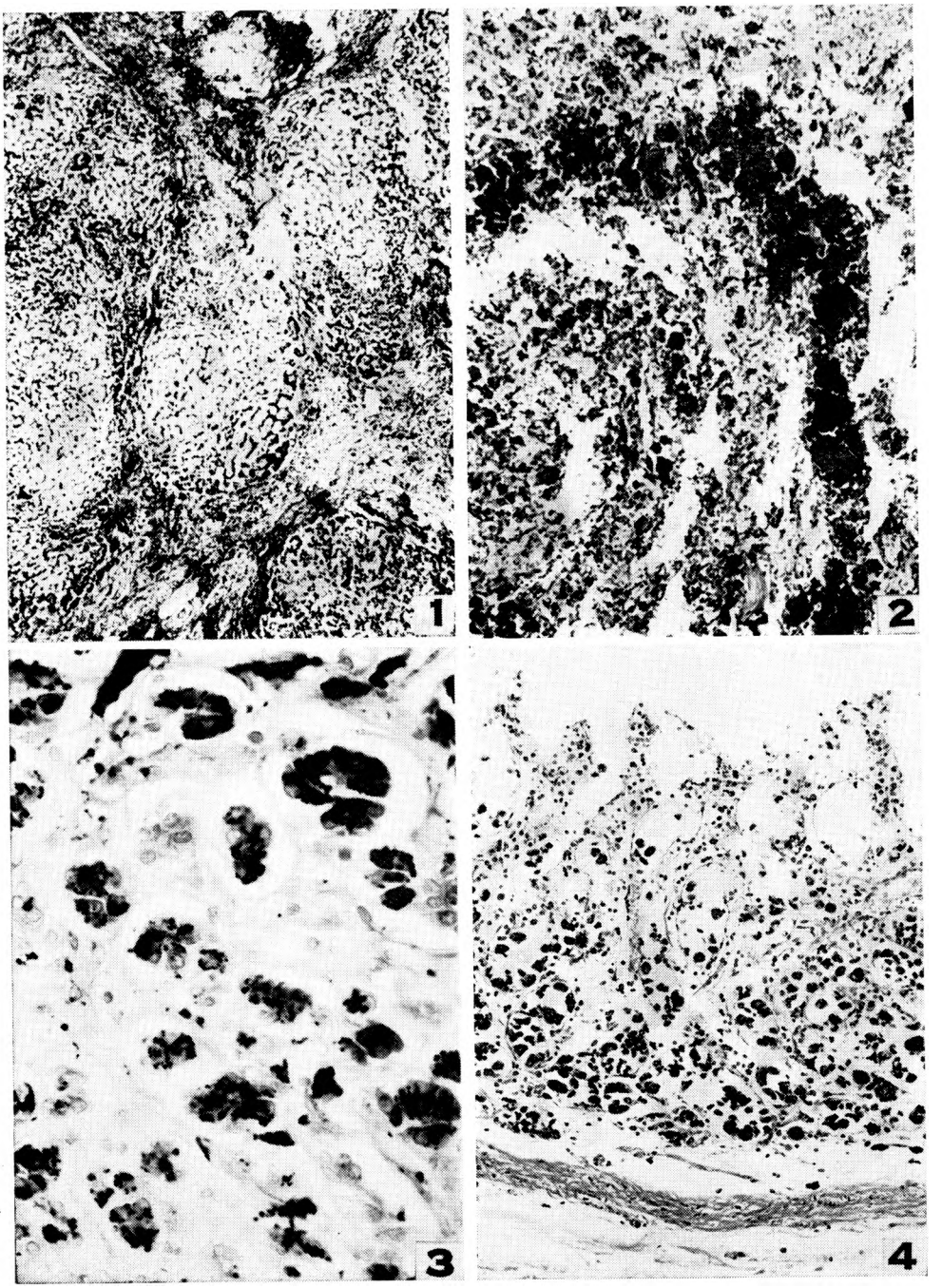
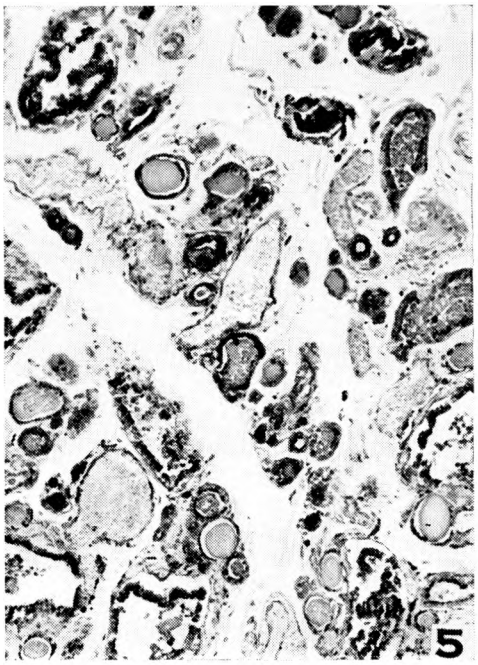
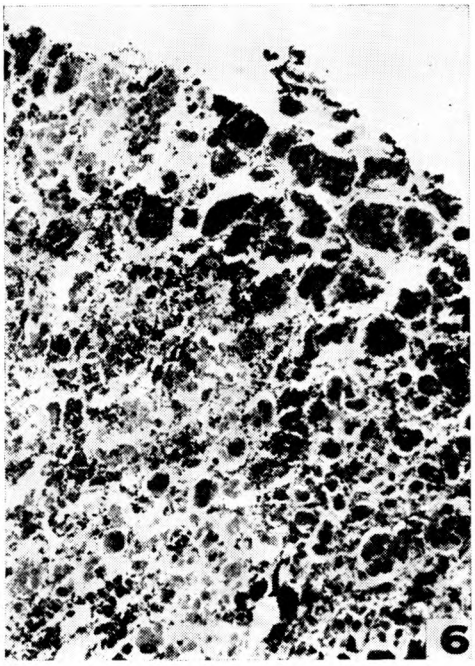


PLATE I





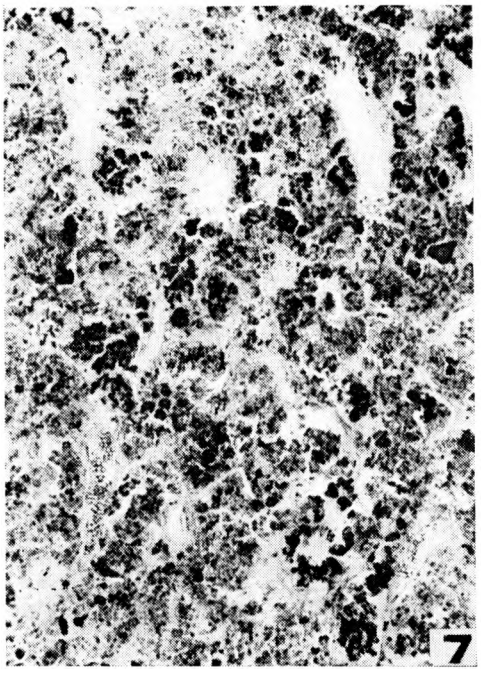




PLATE 2

SIDEROSIS IN THE BANTU: THE RELATIONSHIP BETWEEN IRON OVERLOAD AND CIRRHOSIS

C. ISAACSON, M.B. (Rand), D.C.P. (London), Dip. Path.
H. C. SEFTEL, Dip. Med. (Rand)
K. J. KEELEY, M.B. (Rand), M.R.C.P. (Edinburgh)
and
T. H. BOTHWELL, M.D. (Rand), M.R.C.P. (London)
Johannesburg, South Africa

From the South African Institute for Medical Research, Baragwanath Hospital, and the Department of Medicine, University of the Witwatersrand

Reprinted from

THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE

St. Louis

Vol. 58, No. 6, Pages 845-853, December, 1961

(Copyright © 1961 by The C. V. Mosby Company)
(Printed in the U. S. A.)

Siderosis in the Bantu: The relationship between iron overload and cirrhosis

C. ISAACSON, M.B. (RAND), D.C.P. (LONDON), DIP. PATH.,
H. C. SEFTEL, DIP. MED. (RAND),
K. J. KEELEY, M.B. (RAND), M.R.C.P. (EDINBURGH), and
T. H. BOTHWELL, M.D. (RAND), M.R.C.P. (LONDON)*
Johannesburg, South Africa

The Bantu of Southern Africa consume a diet with a high iron content and the majority of adults at autopsy show varying degrees of siderosis. Since previous studies have demonstrated a marked association between severe hepatic siderosis and portal fibrosis, an investigation was carried out to ascertain the pattern and degree of iron overload in consecutive cases of portal cirrhosis coming to autopsy. Severe hepatic siderosis was present in 22 out of 24 cases. In addition, iron deposits were found in a number of epithelial tissues including the pancreas, thyroid, and adrenals and the iron concentrations were within the range found in idiopathic hemochromatosis. These findings were different from those in a control group with severe hepatic siderosis but no cirrhosis. Under such circumstances the iron was confined to the liver and the reticuloendothelial system.

In idiopathic hemochromatosis there is an alteration in the behavior of the gut mucosa and excessive quantities of iron are absorbed from the diet.[1] By the time clinical manifestations appear, widespread deposits are present in most of the body tissues and the concentrations are particularly high in the liver and pancreas. The clinical manifestations of the disease are related closely to the iron deposits, since portal cirrhosis invariably is present and diabetes is found in approximately 80 per cent of cases.[1] There is, however, some dispute as to the role which iron plays in the production of tissue damage and it has been argued

From the South African Institute for Medical Research, Baragwanath Hospital, and the Department of Medicine, University of the Witwatersrand, Johannesburg, South Africa.

Received for publication Dec. 14, 1960.

*Recipient of a grant from the Council for Scientific and Industrial Research, Pretoria, South Africa.

that the pathologic changes eventually found in the condition could equally well be a direct result of the metabolic defect that leads to excessive iron absorption.[2] If iron is itself the primary factor it might be anticipated that changes similar to those found in idiopathic hemochromatosis would occur in other forms of oral iron overload. In this context the findings in adult Bantu have special significance, since the majority show varying degrees of siderosis at autopsy.[3-5] In this condition there is good evidence that the most important etiological factor is the high iron content of the Bantu diet.[6]

Although iron overload is very common in Bantu adults it is not usually of a severe degree and it has been calculated that the total content of the body rarely exceeds 10 Gm., much less than is present in idiopathic hemochromatosis.[7] In such cases the iron is usually confined to the liver and the reticuloendothelial system.[4] Since the liver bears the major brunt of the iron load most investigators have concentrated on the findings in this organ. In early studies no correlation was found between the degree of hepatic siderosis and portal fibrosis,[4] but more recently a marked association has been noted.[7-9] In one of these investigations the iron concentrations in liver specimens were also estimated and it was found that hepatic fibrosis or frank portal cirrhosis was present in all those with iron concentrations above 2 per cent dry weight.[7] Because of these various findings a prospective study was undertaken to find out what proportion of subjects showing portal cirrhosis at autopsy had heavy iron deposits in the liver. In addition a number of other organs were examined so that a comparison could be made with the findings in idiopathic hemochromatosis. At the same time 2 other groups were examined, those with advanced hepatic siderosis but no cirrhosis, and those with postnecrotic cirrhosis.

Materials and methods

Consecutive cases of cirrhosis coming to autopsy over a period of approximately 20 months were examined both histologically and biochemically for their iron content. During this time approximately 700 autopsies were carried out and, of these, 38 showed cirrhosis. The distinction between portal and postnecrotic cirrhosis was based on certain morbid anatomic and histologic features previously described.[8, 10] In addition, 20 subjects with obvious macroscopic evidence of marked hepatic siderosis but with no cirrhosis were selected at random.

Postmortem tissues were fixed in 10 per cent formol mercury, embedded in paraffin wax, and stained with hematoxylin and eosin. In addition, they were stained for iron, using Perls' method. The iron concentration in most of the tissues was estimated chemically as previously described.[7] Results were expressed as a percentage per 100 Gm. dry weight.

Results

Severe siderosis without cirrhosis. Twenty subjects were examined. The average age was 51 years (range: 35 to 70 years). The striking biochemical findings were the high concentrations of iron in the livers and spleen. The mean concentration was 2.21 per cent (range: 0.86 to 7.55 per cent) in the liver and 3.38 per cent (range: 0.29 to 11.1 per cent) in the spleen. The average concentrations in other organs were as follows: pancreas, 0.09 per cent (range: 0.3 to 0.24 per cent); adrenal, 0.11 per cent (range: 0.03 to 0.26 per cent); thyroid,

0.08 per cent (range: 0.03 to 0.33 per cent); heart, 0.05 per cent (range: 0.03 to 0.13 per cent); testis, 0.07 per cent (range: 0.04 to 0.15 per cent); and kidney, 0.11 per cent (range: 0.04 to 0.62 per cent). These average concentrations are within or just above the normal range.[11]

Histologically, the iron in the liver was present in large amounts in hepatic cells, Kupffer cells, and portal tracts. There was a moderately close correlation between the concentration of iron and increased portal fibrosis. In the 10 cases where the concentration was 2 per cent or higher, portal fibrosis was severe in 7, moderate in 2, and mild in 1. On the other hand, in those cases with levels lower than 2 per cent, one had moderate portal fibrosis, 4 had mild portal fibrosis, and the remaining 5 were normal. These differences did not appear to be on account of disparities in age since the average ages in the 2 groups were 50 and 51 years, respectively. Apart from the liver and spleen, iron deposits were not striking in any other organs except the upper small bowel, where the iron was present mostly in the lamina propria. In other tissues it was confined totally to the reticuloendothelial cells and was never found in parenchymal cells.

The subjects in this group succumbed to a number of diseases. These included idiopathic heart failure (6 cases), miliary tuberculosis (3 cases), ruptured syphilitic aneurysms (2 cases), cerebral hemorrhage (2 cases), carcinoma of the stomach (1 case), peritonitis (1 case), rheumatic heart failure (1 case), pneumonia (1 case), pyelonephritis (1 case), acute pancreatitis (1 case), and unknown causes (1 case).

Postnecrotic cirrhosis. There were 14 cases in this group, an incidence of 2 per cent. The average age was 40 years (range: 18 to 60 years). There was a wide variation in the iron concentrations in liver (0.03 to 1.87 per cent) and in spleen (0.06 to 4.10 per cent). The average levels of 0.38 and 0.67 per cent, respectively, however, were much lower than are found in apparently normal Bantu in the same age groups.[7] Iron concentrations in other organs, with one exception, were within the normal range. This subject had the highest concentration in the liver (1.87 per cent) and the concentrations in other organs were as follows: spleen, 1.31 per cent; pancreas, 1.22 per cent; adrenal, 0.14 per cent; thyroid, 0.10 per cent; pituitary, 0.11 per cent; heart, 0.22 per cent; testis, 0.02 per cent; and kidney, 0.08 per cent.

In 13 of the 14 cases, iron, when present, was confined to the liver and reticuloendothelial system. In the last case, which was the one with the highest hepatic concentration, deposits were also present in parenchymal cells elsewhere in the body; those in the pancreas were particularly striking.

Seven subjects died from malignant hepatomas, 4 from ruptured varices, 2 from hepatic failure, and 1 from rheumatic heart failure.

Portal cirrhosis. There were 24 cases of portal cirrhosis, an incidence of 3.4 per cent. Three of the cases were of the fatty or nutritional variety. Excessive hepatic deposits of iron were a feature in all except 2 cases, 1 of which was fatty (Table I). The livers of the remaining cases were macroscopically brown in color due to the iron deposits. The average iron concentration in the whole group was 2.31 per cent (range: 0.07 to 8.16 per cent). In the spleen the iron concentration was also very high, with a mean value of 2.63 per cent (range:

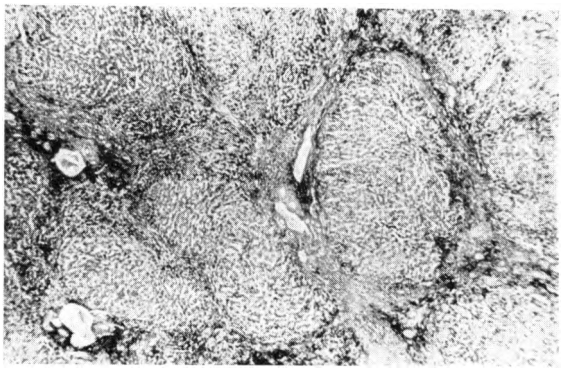


Fig. 1. Section of the liver showing portal cirrhosis and heavy hemosiderin deposits in hepatic cells, Kupffer cells, and portal tracts. (Perls' stain. Original magnification × 30.)

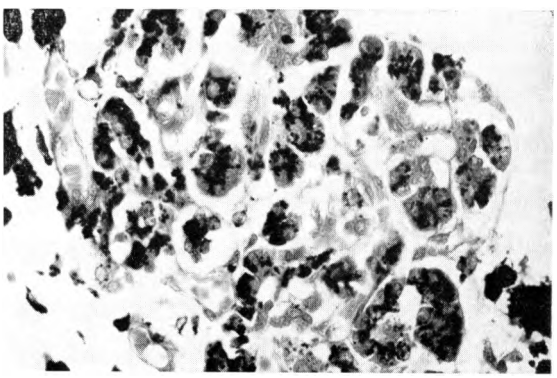


Fig. 2. Section of the pancreas showing hemosiderin deposits in the acinar-lining cells. (Perls' stain. Original magnification × 600.)

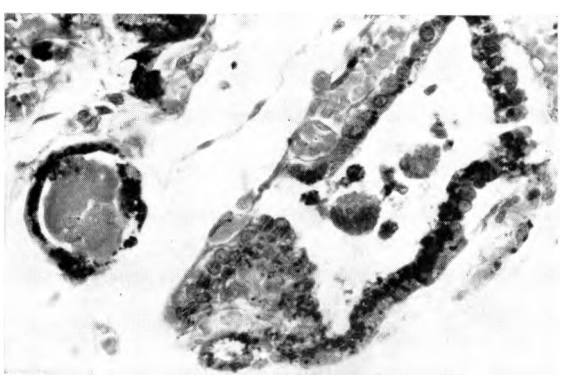


Fig. 3. Section of the thyroid showing hemosiderin in the epithelial cells lining the follicles. (Perls' stain. Original magnification × 600).

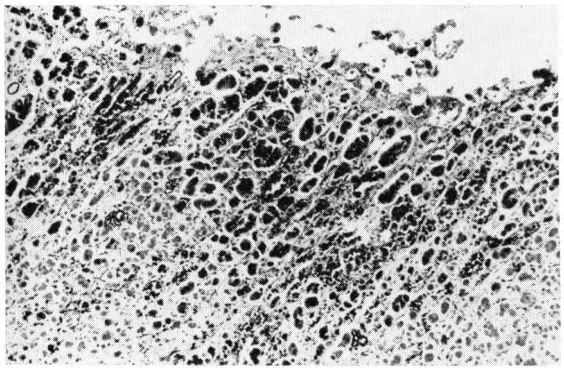


Fig. 4. Section of the adrenal gland showing heavy hemosiderin deposits which are mostly in the secreting cells of the zona glomerulosa. (Perls' stain. Original magnification × 120.)

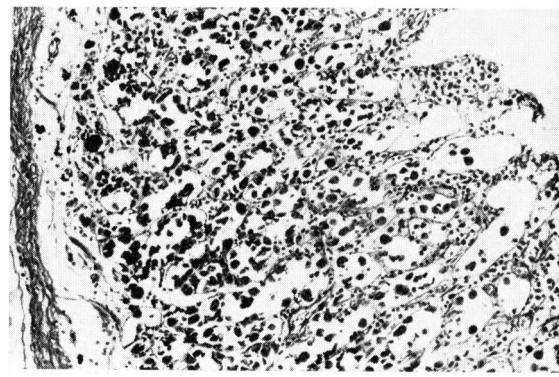


Fig. 5. Section of the stomach mucosa showing large deposits of hemosiderin in the epithelial cells particularly in the deeper layers. (Perls' stain. Original magnification. × 200.)



Fig. 6. Section of heart muscle showing heavy accumulations of hemosiderin in the myofibrils. (Perls' stain. Original magnification × 600.)

Table I. Iron concentrations (per cent dry weight) in organs of subjects with portal cirrhosis

Case No.	*Sex*	*Age*	*Liver*	*Spleen*	*Pancreas*	*Adrenal*	*Thyroid*	*Pituitary*	*Heart*	*Testis*	*Kidney*	*Cause of death*
1	M	75	8.16	3.65	2.01	1.20	0.55	—	0.10	0.26	0.18	Hepatic failure
2	M	37	6.84	7.36	0.69	0.14	—	—	0.08	0.21	0.14	Hepatic failure, tuberculosis
3	F	61	5.25	3.02	0.86	—	—	—	0.14	—	0.25	Hepatic failure
4	F	80	4.63	5.64	0.45	0.62	0.12	0.11	0.07	—	0.12	Cerebral hemorrhage
5	M	29	3.15	4.14	0.51	0.20	0.11	—	0.06	0.83	0.08	Malignant hepatoma
6	M	31	3.01	5.77	1.17	0.30	0.44	—	0.27	0.48	0.12	Hemothorax (? cause)
7	M	68	2.71	2.70	0.27	0.12	—	0.09	0.23	0.05	0.07	Hepatic failure
8	M	35	2.24	2.94	1.10	0.61	0.82	0.22	0.17	0.11	0.14	Ruptured varices (diabetes)
9	M	39	2.13	0.31	2.98	0.24	0.70	0.34	0.31	—	0.07	Ruptured varices
10	M	74	2.12	1.41	0.16	0.29	0.10	0.14	0.05	0.05	0.06	Pyelonephritis, renal failure
11	M	42	2.12	1.30	0.73	0.38	0.17	0.25	0.13	0.13	0.15	Ruptured varices (diabetes)
12	F	60	2.00	1.54	0.70	0.23	0.94	0.18	0.20	—	0.13	Hepatic failure
13	M	51	1.90	4.20	—	0.36	0.26	0.20	0.07	—	0.41	Ruptured varices
14	F	57	1.50	1.52	1.06	0.40	0.84	0.33	0.08	—	0.10	Ruptured varices
15	F	29	1.37	4.12	—	—	0.03	—	0.03	—	0.05	Hepatic failure
16	F	44	1.30	0.90	2.20	—	0.60	—	0.80	—	—	Diabetic coma and hepatic failure
17	M	48	0.93	1.55	0.57	—	0.20	—	0.09	—	0.16	Meningitis, pyelonephritis and hepatic failure
18	M	41	0.84	1.15	1.71	0.40	0.08	—	0.48	0.01	0.11	Hepatic failure and bronchopneumonia
19	F	80	0.84	0.83	0.41	0.09	0.45	0.16	0.10	—	0.09	Hepatic failure (diabetes)
20*	M	32	0.84	6.13	0.08	0.24	0.05	0.10	0.08	0.06	0.07	Hepatic failure
21	M	55	0.77	0.87	1.22	0.10	0.16	—	0.18	0.07	0.09	Hepatic failure (diabetes)
22	M	49	0.48	1.05	0.81	0.09	0.17	—	0.07	0.05	0.07	Hepatic failure
23*	F	40	0.12	0.59	0.04	0.04	0.04	—	0.03	—	0.04	Hepatic failure and ruptured varices
24*	F	50	0.07	0.49	—	—	0.05	0.05	0.02	—	0.04	Hepatic failure and widespread tuberculosis
Average		51	2.31	2.63	0.94	0.32	0.33	0.18	0.16	0.20	0.12	

*Fatty liver.

0.31 to 7.36 per cent). In addition, significant deposits were present in a number of other organs. The average concentrations were as follows: pancreas, 0.94 per cent; thyroid, 0.33 per cent; adrenal, 0.32 per cent; testis, 0.20 per cent; pituitary, 0.18 per cent; heart, 0.16 per cent; and kidney, 0.12 per cent.

On histologic section the liver showed heavy deposits in hepatic cells, Kupffer cells, and portal tracts. A striking feature in this group was the presence of excessive iron in organs other than the liver and spleen. Deposits were particularly prominent in the pancreas, where they were found in both the acinae and islets of Langerhans. In the thyroid the deposits were almost exclusively in the follicular-lining cells, while in the adrenal the zona glomerulosa of the cortex was most affected. Hemosiderin granules were present in myocardial fibers in 15 cases and in 5 of these the deposits were moderate or heavy. Involvement of the epithelial cells of the stomach was noted in 16 cases but in 6 they were extremely scanty and irregular in distribution. Representative sections of various tissues from Case 6 (Table I) are shown in Figs. 1 through 6.

Discussion

The present study was undertaken to find out the pattern and degree of iron overload in the South African Bantu showing cirrhosis at autopsy. Since iron overload is so common in the Bantu, a control group of cases showing marked hepatic siderosis without cirrhosis was also examined. In this group the features were similar to those found by previous workers since the iron deposits were virtually confined to the liver and reticuloendothelial system.[4] Although varying degrees of portal fibrosis were present in all cases in which the hepatic concentration of iron was above 2 per cent dry weight, death did not seem to be attributable to the iron deposits since the subjects succumbed to a variety of unrelated causes.

In postnecrotic cirrhosis, variable deposits were found but the average concentration in the liver was a good deal lower than is found in the general Bantu population at comparable ages. It therefore appears that excessive deposits of iron play no part in the genesis of postnecrotic cirrhosis and when the two do occur together it is only because varying degrees of iron overload occur so commonly in the Bantu.

In cases with portal cirrhosis the pattern was completely different since iron overload was a feature in all except 2 persons. The mean concentration in the liver was approximately twice as great as occurs at comparable ages in the general population[7] and, in fact, was very similar to that obtained in the group selected solely because of marked hepatic siderosis. The second feature of note was the distribution of the iron. Not only was it present in liver and reticuloendothelial system but in addition was found in a number of epithelial tissues. The histologic findings were therefore virtually indistinguishable from those in idiopathic hemochromatosis and the only feature that served to distinguish the two conditions was the relatively high concentration of iron in the spleens of Bantu cases.

These results raise the question as to the role which iron deposits play in the production of portal cirrhosis. In both this study and a previous one[7] it was

demonstrated that the incidence of portal fibrosis increases as the concentration of iron in the liver rises. This suggests that there is a direct association between iron deposits and fibrosis. However, it is noteworthy that several of the cases of portal cirrhosis in the present series had concentrations of iron in the liver which were lower than those with marked siderosis but no cirrhosis. This difference was not explicable on age or sex differences. Although it is feasible that the iron concentration in a single aliquot of cirrhotic liver may not necessarily be a valid reflection of the over-all concentration, these findings raise the possibility that some other factor such as malnutrition or an extrinsic "toxin" may accelerate the development of cirrhosis in some subjects. It has been shown experimentally that cirrhosis develops rapidly in the liver overloaded with iron when the animal is exposed to toxic agents or to a deficient diet[12] and it is possible that a similar situation may exist in the Bantu. It should be noted that a large proportion of the iron ingested by the Bantu is derived from the containers used in the preparation of alcoholic drinks so that those with marked siderosis are usually heavy drinkers. It is therefore possible that the insult of alcoholism may contribute to the subsequent cirrhosis.

The second problem relates to the effect of portal cirrhosis on the distribution of iron within the body since it is apparent from the data that parenchymal deposits occur only in other organs when cirrhosis is present. This pattern of distribution does not seem to be related to the type of cirrhosis, as it was also found in 1 case of postnecrotic cirrhosis with severe hepatic siderosis. Although it is possible that the raised plasma iron levels and the increased saturation of circulating transferrin occurring under such circumstances may facilitate epithelial uptake, there are at present insufficient data to support this.

Whatever the explanation for the findings in the present study, there is no doubt that a proportion of Bantu adults develop pathologic findings which are virtually indistinguishable from those in idiopathic hemochromatosis. The similarity between the two conditions is apparent in the clinical manifestations also. Apart from symptoms referable to portal cirrhosis, 5 out of 22 patients were diabetic, an incidence of 22.7 per cent. This is 10 times greater than the 2. 2 per cent incidence which has been found previously in 700 nonhemochromatotic subjects of comparable age distribution coming to autopsy at the same hospital[13] and it is also several times greater than the reported incidence of diabetes in cirrhotic subjects without siderosis.[14] Although the incidence of diabetes is still much lower than that found in idiopathic hemochromatosis,[1] this is possibly because of the fact that the cirrhosis in the Bantu is more active and severe. Death, therefore, may supervene in the majority of cases before epithelial deposits have reached sufficient concentrations to give rise to pathologic effects.

We wish to thank Dr. I. Frack, Superintendent of Baragwanath Hospital, for access to case records, and the Director, South African Institute for Medical Research, for granting facilities.

REFERENCES

1. Finch S. C., and Finch, C. A.: Idiopathic Hemochromatosis in Iron Storage Disease. A. Iron Metabolism in Hemochromatosis, Medicine 34:381, 1955.

2. Brown, E. B., Jr., Dubach, R., Smith, D., Reynafarje, C., and Moore, C. V.: Studies in Iron Transportation and Metabolism. X. Long-Term Iron Overload in Dogs, J. LAB. & CLIN. MED. 50: 862, 1957.
3. Gillman, J., and Gillman, T.: Liver Disease in Johannesburg. Relation to Pellagra, Lancet 1: 169, 1948.
4. Higginson, J., Gerritsen, T. and Walker, A. R. P.: Siderosis in the Bantu of Southern Africa, Am. J. Path. 29: 779, 1953.
5. Wainwright, J.: Siderosis in the African, South African J. Lab. & Clin. Med. 3: 1, 1957.
6. Walker, A. R. P., and Arvidsson, U. B.: Iron "Overload" in the South African Bantu, Tr. Roy. Soc. Trop. Med. & Hyg. 47: 536, 1953.
7. Bothwell, T. H., and Bradlow, B. A.: Siderosis in the Bantu. A Combined Histopathological and Chemical Study, A.M.A. Arch. Path. 70: 279, 1960.
8. Higginson, J., Grobbelaar, B. G., and Walker, A. R. P.: Hepatic Fibrosis and Cirrhosis in Man in Relation to Malnutrition, Am. J. Path. 33: 29, 1957.
9. Gillman, T., Hathorn, M., and Lamont, N.: Liver Disease in Durban Africans, South African J. M. Sc. 23: 187, 1958.
10. Higginson, J., and Keeley, K. J.: Liver Disease in the South African Bantu. A Review of Liver Biopsies From 262 Bantu Patients, Gastroenterolgy 38: 332, 1960.
11. Sheldon, J. H.: Haemochromatosis, London, 1935, Oxford University Press.
12. Golberg, L., and Smith, J. P.: Iron Overloading and Hepatic Vulnerability, Am. J. Path. 36: 125, 1960.
13. Seftel, H. C.: Unpublished data.
14. Reinberg, M. H., and Lipson, M.: The Association of Laennec's Cirrhosis With Diabetes Mellitus, Ann. Int. Med. 33: 1195, 1950.

Reprinted from THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, St. Louis.
Vol. 58, No. 6, Pages 837-844, December, 1961. (Printed in the U. S. A.)
(Copyright © 1961 by The C. V. Mosby Company)

Siderosis in the Bantu: The clinical incidence of hemochromatosis in diabetic subjects

H. C. SEFTEL, DIP. MED. (RAND), K. J. KEELEY, M.B. (RAND), M.R.C.P. (EDINBURGH), C. ISAACSON, M.B. (RAND), D.C.P. (LONDON), DIP. PATH., and T. H. BOTHWELL, M.D. (RAND), M.R.C.P. (LONDON)*
Johannesburg, South Africa

In a previous necropsy study on adult Bantu it was shown that when moderate or marked hepatic siderosis was present together with portal cirrhosis the findings elsewhere in the body were virtually indistinguishable from those in idiopathic hemochromatosis. Pancreatic deposits were particularly striking and diabetes was present in about 20 per cent of cases. In view of these findings, a random group of 100 adult Bantu diabetic subjects were investigated to discover what proportion were suffering from hemochromatosis. Plasma iron levels were estimated in all subjects and liver biopsies were then carried out on the majority of those in whom the plasma iron was high. Seven showed the changes of hemochromatosis. The clinical presentation in these subjects was different from that usually found in adult Bantu diabetic persons.

Although siderosis is common in Bantu adults, the iron deposits are usually confined to the liver and reticuloendothelial system.[1] The pathologic picture, therefore, is different from that found in idiopathic hemochromatosis, since widespread epithelial deposits are a striking feature in this disease.[2] On the other hand, there is some evidence that a small proportion of Bantu

From Baragwanath Hospital, the South African Institute for Medical Research, and the Department of Medicine, University of the Witwatersrand, Hospital Street, Johannesburg, South Africa.
Received for publication Dec. 14, 1960.
*Recipient of a grant from the Council for Scientific and Industrial Research, Pretoria, South Africa.

Table I. Liver and gastric biopsy findings in Bantu diabetics with high plasma iron lev

Diagnosis	*Case No.*	*Sex*	*Age*	*Plasma iron (μg/100 ml.)*	*Total iron-bindi capacity (μg/100 ml.)*
Hepatic siderosis	1	F	60	171	250
with portal	2	F	50	237	316
cirrhosis	3	F	67	198	237
	4	M	50	312	330
	5	M	55	180	216
	6	M	35	174	183
	7	M	56	246	300
Hepatic siderosis	8	M	40	217	301
without cirrhosis	9	M	42	196	226
	10	M	40	240	240
	11	F	40	240	240
Incomplete cases	12	M	47	165	235
	13	M	50	222	248
	14	M	45	232	243

+ = Mild, − = Absent, ++ = Moderate, and +++ = Severe.

subjects do eventually develop pathologic changes similar to those found in idiopathic hemochromatosis. In one study the autopsy records of 20 known diabetic subjects were reviewed and in 4 the histologic and chemical findings were virtually indistinguishable from those in the idiopathic disease.[3] Since portal cirrhosis was a feature in each of these cases a further prospective investigation was carried out in which detailed chemical and histologic examinations were performed on every case of portal cirrhosis at autopsy in a Bantu hospital over an 18 month period.[4] The majority showed heavy hepatic siderosis and significant deposits were found in a number of epithelial tissues, with the highest concentrations in the pancreas. In addition, it was noteworthy that 22 per cent of the subjects were diabetic and that in each of them the chemical concentrations of iron in the pancreas were within the range of those found in idiopathic hemochromatosis.

These various observations strongly suggest that oral iron overload in the Bantu can eventually be associated with diabetes which is secondary to deposits in the pancreas. Since no diligent attempt has yet been made to diagnose such cases clinically a study was undertaken in which various tests were carried out on an unselected group of diabetic Bantu hospital patients. The plasma iron was estimated on all subjects and liver biopsies were performed on those in which it was raised. The presence of moderate or marked siderosis together with portal cirrhosis was taken as indicative of hemochromatosis, since in the previous pathologic study[4] significant pancreatic deposits invariably were present under such circumstances. In addition, gastric biopsies were carried out when possible.

Liver biopsy					*Gastric biopsy: Iron distribution*	
Iron distribution			*Portal cirrhosis*	*Portal fibrosis*		
ver cells	*Kupffer cells*	*Portal tracts*			*Epithelium*	*Interstitium*
+++	+++	+	Present		–	–
+++	+++	+++	Present		+	+
+++	+++	+++	Present		–	+
++	++	++	Present		–	–
+++	+++	+++	Present		+	–
++	++	++	Present		+	–
+++	+++	+++	Present		+	?
+++	+++	+	–	++	–	+
+++	+++	+++	–	++	–	–
+++	+++	++	–	–		
+++	++	+	–	–		
					–	–
					–	–

Materials and methods

Clinical material. One hundred unselected Bantu diabetic subjects living in the urban area of Johannesburg were studied. Most of these were over 40 years of age. The majority were outpatients attending the Baragwanath Hospital Diabetic Clinic; the remainder were hospital patients. The diagnosis of diabetes mellitus was confirmed in all cases by blood sugar estimations.

Methods. The plasma iron level was estimated on all subjects by a modification of the method of Bothwell and Mallett,[5] in which sulfonated bathophenanthroline was used as the color reagent and the unsaturated iron-binding capacity was measured by a method of Bothwell and co-workers.[6] Liver biopsies were carried out in the majority of those with plasma iron levels above 150 μg per 100 ml. and gastric biopsies also were done in some. The specimens obtained were fixed in 10 per cent formol saline and sections were stained routinely with hematoxylin and eosin and, with Perl's method, for iron.

Results

A. Plasma iron levels. In 86 (29 men and 57 women) of the 100 diabetics the plasma iron levels were not raised. The mean value in men was 82 μg per 100 ml. (range: 34 to 126) and in women was 83 μg per 100 ml. (range: 41 to 139). The total iron-binding capacity varied from 192 to 475 μg per 100 ml. (mean: 291) in men; 168 to 458 μg per 100 ml. (mean: 306) in women.

Ten men and 4 women had plasma iron levels above 150 μg per 100 ml. (Table I). In each the finding was confirmed on a repeat estimation. The mean plasma iron level in this group was 216 μg per 100 ml. (range: 165 to 312) and the mean total iron-binding capacity was 255 μg per 100 ml. (range: 183 to 330).

B. Liver and gastric biopsy findings (Table I). Liver biopsies were carried out on 11 subjects with raised plasma iron levels. In 4 men and 3 women the liver biopsy showed moderate to marked siderosis, with iron present in parenchymal cells, Kupffer cells, and portal tracts. In addition, portal cirrhosis was present, the diagnosis being based on the presence of small, regular regenerating nodules surrounded by thin fibrous tracts and on the absence of collapse and gross architectural distortion. In 4 of the 7 patients gastric biopsy revealed epithelial deposits of iron. The absence of such deposits in the remaining 3 cases was not altogether surprising, since they were scanty or absent in about 50 per cent of a previous pathologic series of Bantu hemochromatotics.[4] All 7 cases were regarded as having fully developed hemochromatosis, and this was confirmed both histologically and chemically in 2 who subsequently died. The concentrations of pancreatic iron in these 2 subjects were found at autopsy to be 1.2 and 1.1 per cent dry weight, respectively.

In 3 men and 1 woman marked hepatic siderosis was present without cirrhosis. In 2 patients gastric biopsies were carried out but no epithelial deposits were found. It was felt that the pattern of iron distribution in these cases was that found in the majority of Bantu adults[1] and that it was extremely unlikely that epithelial deposits were present in the pancreas.

In the remaining 3 cases liver biopsy material was not obtained, either because the attempt at biopsy failed or because it was not possible to trace the patients. In 2 of these cases gastric biopsies were done and in both the findings were normal.

In order to test the validity of the screening procedure liver biopsies were carried out on 10 randomly selected cases from the control group with normal plasma iron levels. In 6 the findings were entirely normal, while the remaining 4 showed minimal or mild deposits of iron. In the 5 cases in which gastric biopsy was done the mucosa was found to be normal.

C. Clinical features of Bantu subjects with hemochromatosis. The clinical features in the 7 cases diagnosed on laboratory criteria during the study were analyzed (Table II, Cases 1 through 7). In addition, another 4 hospital patients who were not part of the random series were included also (Table II, Cases 8 through 11). In each of these 4 cases the plasma iron level was found to be high. Liver and gastric biopsies were then done in 3 of these cases. Marked hepatic siderosis together with cirrhosis was present in all of them and the epithelium of the stomach contained iron deposits in 2. The diagnosis of hemochromatosis was confirmed in the last case at autopsy, the concentration of iron in the pancreas being 0.7 per cent dry weight.

The findings in the 11 subjects were compared with those of 100 diabetics with normal plasma iron levels who were drawn at random from the Baragwanath Hospital Diabetic Clinic. The group was confined to patients over the age of 40 years since all but one of the subjects with hemochromatosis were over this age, as are three-quarters of all Bantu diabetics seen in hospital practice.

SEX. Of the 11 subjects with hemochromatosis 7 were men; there was a female-to-male ratio of 1.8 to 1.0 in the control group.

WEIGHT. Nine of the subjects with hemochromatosis were thin or wasted, 1 was of normal weight, and only 1 could be described as obese. In contrast about two-thirds of the control group were overweight.

CONSUMPTION OF ILLICITLY BREWED ALCOHOL. All subjects with hemochromatosis gave a history of consuming illicitly brewed alcoholic drinks. In the control group only 22 per cent admitted to taking such concoctions.

LIVER DISEASE. In every subject with hemochromatosis the liver was enlarged to palpation, often considerably so. The enlarged liver was usually firm in consistency. In the control group hepatomegaly was detected in only 10 per cent of cases. Evidence of liver failure or portal hypertension was present in 7 of the 11 subjects with hemochromatosis and was not seen in the control group.

PORPHYRIA. The urine was examined spectroscopically for porphyrins in 9 of the hemochromatotic subjects and excessive amounts were found in 3. Two of these cases showed the facial hyperpigmentation which is often present in Bantu cases of porphyria cutanea tarda.[7] Apart from these, the other subjects with hemochromatosis were not excessively pigmented. In the control group an excess of porphyrins in the urine was found in only 2 per cent of cases.

TUBERCULOSIS. Four of the subjects with hemochromatosis were found to have pulmonary tuberculosis as compared with an incidence of 9 per cent in the control group.

PROGNOSIS. The prognosis of patients with hemochromatosis was much more serious than that of subjects in the control group and 4 of them died within a year of the onset of symptoms. There were two major reasons for this. Liver failure and/or portal hypertension were present in 7 of the subjects with hemochromatosis and these complications were largely responsible for 3 of the 4 deaths in this series. Second, in about half the cases the diabetes could be described as severe. Other factors which probably contributed to the poor prognosis were alcoholism and the significant incidence of pulmonary tuberculosis. In contrast, the subjects in the control group were usually stable, ketosis was infrequent, and the diabetes could often be controlled by diet or by oral hypoglycemic agents.

Discussion

The results of a previous pathologic study indicated that when moderate or marked hepatic siderosis is found together with portal cirrhosis at autopsy significant epithelial deposits are always present in other organs, the highest concentrations being found in the pancreas.[4] On this basis 7 per cent of a random series of 100 diabetic Bantus attending hospital were diagnosed as having fully developed hemochromatosis. The incidence is an extremely high one when compared with the 0.16 per cent incidence of the idiopathic disease in an American series of 30,000 diabetic subjects.[8] It should, however, be emphasized that the present figures refer to a hospital population only.

Although the findings in Bantu diabetics with hemochromatosis resemble those found in idiopathic hemochromatosis there are certain notable differences. The cirrhosis in Bantu subjects tended to be rapidly progressive and was the principle cause of death, while in idiopathic hemochromatosis

Table II. Clinical features of 11 Bantu diabetics with hemochromatosis

Case No.	*Sex*	*Age*	*Illicit alcohol intake*	*Mode of presentation*	*Habitus*	*Signs of liver disease (finger)*	*Porphyria*	*Tubercle*	*Daily insulin dose*	*Remarks*
1	F	60	Moderate	Diabetic coma	Obese	4f. hepatomegaly	+	–	40	Enlarged heart on x-ray; no cardiac symptoms
2	F	50	Heavy	Diarrhea; glycosuria on routine urinalysis	Thin	4f. hepatomegaly	–	–	50	Stopped taking insulin thrice, followed by ketosis twice and death the third time; no autopsy obtained; course 9 months
3	F	67	Heavy	Polydipsia and polyuria	Thin	3f. hepatomegaly and 1f. splenomegaly			50	
4	M	50	Moderate	Polydipsia and polyuria	Thin	2f. hepatomegaly	+	+	80	Peri-orbital pigmentation
5	M	55	Moderate	Polydipsia, polyuria, and swelling of feet	Thin	3f. hepatomegaly, 2f. splenomegaly, ascites, leg edema, and gynecomastia	–	–	40	Death largely due to liver failure with progressive ascites, edema, wasting, and jaundice; course 8 months, hemochromatosis confirmed at autopsy
6	M	35	Heavy	Swelling of abdomen; glycosuria on routine urinalysis	Thin	2f. hepatomegaly and ascites	+	+	96	Patchy facial pigmentation; death mainly due to liver failure with progressive ascites, wasting, and hematemesis; course 11 months, hemochromatosis with ruptured varices at autopsy

7	M	56	Moderate	Clonic movements left leg and arm; glycosuria on routine urinalysis	Normal	3f. hepatomegaly and 1f. splenomegaly	–	–	See under *Remarks*	No cause found for clonic movements; initially stabilized on 120 units insulin daily but requirements fell until control achieved with tolbutamide alone
8*	F	45	Heavy	Cough and weight loss with pulmonary tubercle; glycosuria on routine urinalysis	Thin	4f. hepatomegaly and 1f. splenomegaly	–	+	40	
9*	M	49	Heavy	Weakness, headache, and polyuria	Thin	3f. hepatomegaly		+	80	
10*	M	70	Heavy	Abdominal pain	Thin	5f. hepatomegaly	–	–	20	First presented in 1955; jaundiced in 1957; diabetes discovered in 1960; one hypoglycemic episode while on 20 units insulin daily
11*	M	42	Moderate	Swelling of abdomen; glycosuria on routine urinalysis	Thin	4f. hepatomegaly, ascites, and edema	–	–	See under *Remarks*	Diabetes responded to soluble insulin but patient became comatose and died 4 days after admission; hemochromatosis with ruptured varices at autopsy

*These 4 cases were not part of the randomly selected series.

it is relatively benign and normally runs a protracted course.[2] There are several possible reasons for the difference. In addition to their high iron content[9] the alcoholic concoctions consumed by the Bantu are adulterated with a variety of toxic substances. It is feasible that these unidentified substances may, together with alcohol and malnutrition, be responsible for potentiating the fibrogenic action of the hepatic iron deposits and thus hastening the downhill course. Unknown toxic substances also may be responsible for the porphyria found in a significant proportion of Bantu cases but not seen in idiopathic hemochromatosis. With the cirrhosis dominating the clinical picture it is not surprising that cardiac complications, which are so common in idiopathic hemochromatosis,[2] are not a feature in Bantu cases, since the heart is one of the last organs to accumulate iron deposits[4] and the cirrhosis tends to cause death before they have reached significant proportions. One final difference between the present findings and those in idiopathic hemochromatosis was the high incidence of tuberculosis. Although there are several factors which probably contribute to its development, the high exposure rate among the Bantu population may well be the most important.

We wish to thank Dr. I. Frack, Superintendent of Baragwanath Hospital, and the Director, South African Institute for Medical Research, for granting facilities for this work.

REFERENCES

1. Higginson, J., Gerritsen, T., and Walker, A. R. P.: Siderosis in the Bantu of Southern Africa, Am. J. Path. 29: 779, 1953.
2. Finch, S. C., and Finch, C. A.: Idiopathic Hemochromatosis an Iron Storage Disease. A. Iron Metabolism in Hemochromatosis, Medicine 34: 381, 1955.
3. Seftel, H. C., Isaacson, C., and Bothwell, T. H.: The Relationship Between Siderosis and Diabetes in the Bantu, South African J. Clin. Sc. 25: 89, 1960.
4. Isaacson, C., Seftel, H. C., Keeley, K. J., and Bothwell, T. H.: Siderosis in the Bantu. The Relationship Between Iron Overload and Cirrhosis, J. Lab. & Clin. Med. 58: 845, 1961.
5. Bothwell, T. H., and Mallett, B.: The Determination of Iron in Plasma or Serum, Biochem. J. 59: 599, 1955.
6. Bothwell, T. H., Jacobs, P., and Kamener, R.: The Determination of the Unsaturated Iron-Binding Capacity of Serum Using Radioactive Iron, S. African J. Med. Sc. 24: 93, 1959.
7. Eales, L.: Cutaneous Porphyria. Observations on 111 Cases in Three Racial Groups, S. African J. Lab. & Clin. Med. 6: 63, 1960.
8. Marble, A., and Bailey, C. C.: Hemochromatosis, Am. J. Med. 11: 590, 1951.

Reprinted from the BRITISH MEDICAL JOURNAL
February 24, 1962, *vol. i, pp.* 522–524

SIDEROSIS IN THE BANTU

A COMPARISON OF INCIDENCE IN MALES AND FEMALES

BY

T. H. BOTHWELL,* M.D., M.R.C.P.
Tutorial Physician, Department of Medicine, University of Witwatersrand, Medical School, Hospital Street, Johannesburg

AND

C. ISAACSON, M.B., D.C.P., D.Path.
Pathologist, South African Institute for Medical Research and Baragwanath Hospital, Johannesburg

The diet of the Bantu in South Africa contains large amounts of iron, most of which is derived from the containers used in the preparation of alcoholic drinks (Walker and Arvidsson, 1953). Although other as yet unidentified factors may be involved, this high iron intake is almost certainly the primary cause of the varying degrees of iron overload so common in adult Bantu. Recent evidence suggests that such iron deposits may have deleterious effects. For example, there is a close correlation between severe hepatic siderosis and portal fibrosis (Higginson *et al.*, 1957 ; Gillman *et al.*, 1958), and this is especially so with iron concentrations above 2% dry weight (Bothwell and Bradlow, 1960). In addition a small proportion of subjects exhibit clinical and pathological features virtually indistinguishable from those found in idiopathic haemochromatosis (Seftel *et al.*, 1960, 1961 ; Isaacson *et al.*, 1961).

The present investigation was undertaken to define in more detail the extent of the problem in the Johannesburg area. This was done by estimating chemically the concentration of iron in the livers of several hundred male and female Bantu adults dying in hospital. The results in the two sexes were then correlated with the histological findings.

*In receipt of a grant from the Council for Scientific and Industrial Research, Pretoria, South Africa, and from the National Institutes of Health, U.S.A. (grant No. A-4912).

121/62

Methods

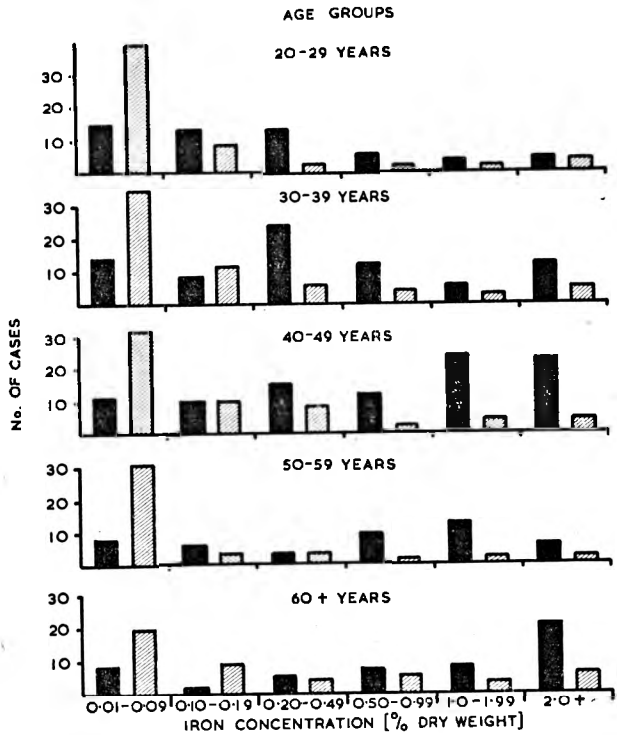
Liver specimens were examined from 318 males and 265 females dying in hospital over a two-year period. Aliquots were dry-ashed and the iron concentrations were estimated as described previously (Bothwell and Bradlow, 1960). Results were expressed as %/100 g. dry weight. In addition, sections were stained for connective tissue by Masson's method and for reticulum by the Gordon and Sweet silver impregnation technique. These were then examined for the presence of portal fibrosis or portal cirrhosis. Portal fibrosis was recorded as absent, as + (slight thickening of the portal tracts), as + + (moderate thickening of the portal tracts), or as + + + (marked thickening of the portal tracts, with early linkage of adjacent tracts in some areas but without distortion of the architectural pattern). Portal cirrhosis was regarded as being present only when there was a marked increase in portal fibrosis with linkage of adjacent portal tracts, distortion of the lobular pattern, and areas of nodular hyperplasia.

Results

Iron Concentrations in Liver Specimens (see Fig.).—Of the males, only 29.6% had iron concentration within the normal range (0.01 to 0.09%) or just above this range (0.09 to 0.19%), while 37.4% had severe siderosis (>1%). The degree of involvement tended to increase with age. For example, only 18.8% of subjects between the ages of 20 and 39 years had severe siderosis as compared with an incidence of 50% of those over the age of 40. The findings in females were far less striking, and 75.4% had hepatic iron concentrations within or just above the normal range. Although there was some tendency for concentrations to rise with age, this was not marked and severe siderosis was present in only 11.6% of cases.

Relationship Between Hepatic Siderosis and Portal Fibrosis.—The incidence of portal fibrosis and portal cirrhosis in the different groups is shown in the Table. In this comparison portal fibrosis was regarded as significant only when moderate or severe (+ + or + + +).

In males having hepatic iron concentration within or close to the normal range portal fibrosis was very uncommon at all age-groups. With higher concentrations the incidence increased, and in the 66 subjects with



Hepatic iron concentrations in males (black columns) and females (cross-hatched columns) at various ages.

concentrations above 2% portal fibrosis was present in 59% and portal cirrhosis in 16.6%. In females a similar trend was noted but the results were less striking, since most of the subjects had iron concentrations which were within or just above the normal range. In this group of 200 subjects portal fibrosis was found in 7% and portal cirrhosis in 2.5%. On the other hand, in the 19 subjects with concentrations of 2% or more, 12 showed portal fibrosis and two had portal cirrhosis.

Discussion

The findings in adult male Bantu dying in hospital are very similar to those obtained in a previous investigation in Johnnesburg on Bantu males dying from acute traumatism (Bothwell and Bradlow, 1960). The incidence of hepatic siderosis in both studies was extremely high and the severity tended to increase with age up to the forties. When the iron concentrations

Incidence of Portal Fibrosis and Portal Cirrhosis in Subjects with Different Iron Concentrations in the Liver

Iron Concentration (% Dry Weight)	Age-Group														
	20–29			30–39			40–49			50–59			60		
	No.	P.F.	P.C.	No.	P.F.	P.C.	No.	P.F.	P.C.	No.	P.F.	P.C.	No.	P.F.	P.C.
Males															
0·01–0·19	28	2	0	22	3	0	21	3	0	14	1	0	9	1	0
0·20–0·49	13	0	0	24	1	0	15	1	1	3	1	0	5	1	0
0·50–1·99	8	1	0	17	4	0	36	12	0	22	1	2	15	2	0
2 +	4	3	1	12	7	5	23	13	2	6	4	0	21	12	3
Females															
0·01–0·19	48	0	0	46	4	1	42	3	3	35	1	0	29	6	1
0·20–0·49	2	0	0	5	3	0	8	1	1	3	0	0	4	1	0
0·50–1·99	2	0	0	5	0	1	6	1	1	3	1	0	8	0	1
2 +	3	0	0	4	4	0	4	4	0	2	2	0	6	2	2

P.F. = Moderate or severe portal fibrosis. P.C. = Portal cirrhosis.

obtained in the present study were related to liver weight it was calculated that 53 subjects had between 5 and 10 g. in the liver and a further 35 had more than 10 g. Although there is no valid way of estimating the total body content in these subjects, it is probable that such figures are not very much higher, since iron is confined to the liver and reticuloendothelial system in the majority of subjects (Higginson *et al.*, 1953). It therefore seems unlikely that the total body content often exceeds 15 g. It should be noted that the disorder in iron balance necessary to produce this degree of iron overload is not a large one. An average absorption of 2 mg. daily between the ages of 20 and 50 would lead to a positive balance of approximately 1.4 mg. daily in the male, which is quite enough to account for deposits of this magnitude.

In females the findings were very different, since about 75% had iron concentrations in the liver which were normal or very close to normal, and although severe siderosis did occur it was uncommon. Of the whole group, nine had between 5 and 10 g. in the liver while only two had more than 10 g. The much lower incidence of iron overload is possibly explicable on the greater physiological losses which occur in females. It has been calculated that the average female loses up to 10 g. of iron through menstruation, pregnancies, and lactation during the reproductive period of life (Finch and Finch, 1955). On this basis it might be anticipated that the incidence and severity of iron overload would be less in females. Whether this is the only factor is, however, open to question. There is good evidence that the major source of iron in the Bantu diet is "kaffir beer" (Walker and Arvidsson, 1953), and females, on the whole, tend to drink less of this than do males. It is thus probable that the majority ingest less iron than do males.

The well-marked association between severe hepatic siderosis and significant portal fibrosis or actual portal cirrhosis, which has been noted in previous studies, was confirmed in the present investigation. This association was most striking with iron concentrations above 2% dry weight. The frequency with which such pathological changes develop in the siderotic liver suggests that they are the direct result of the iron deposits. However, it is perhaps noteworthy that 10 of the 35 subjects who were over the age of 50 and who had iron

concentrations above 2% dry weight showed no significant portal fibrosis. Discrepancies such as this underline the complexity of the problem and suggest that other as yet unidentified factors may be involved. It has been shown experimentally that the liver overloaded with iron is very vulnerable to toxic, nutritional, and metabolic hazards (Golberg and Smith, 1960), and it is possible that such factors may also potentiate the effects of iron deposits in the Bantu.

Whatever the exact relationship between iron overload and hepatic fibrosis there is no doubt that a proportion of subjects end up with histological findings in the liver which are indistinguishable from those in idiopathic haemochromatosis. The results of a previous study indicate that cases with severe siderosis and portal cirrhosis invariably have significant epithelial deposits elsewhere in the body (Isaacson *et al.*, 1961). These are particularly striking in the pancreas, and over 20% of such subjects have diabetes. It is therefore apparent that some Bantu do eventually develop both clinical and pathological findings very similar to those in idiopathic haemochromatosis. If those cases showing hepatic siderosis together with portal cirrhosis in the present study are regarded as examples of this entity, then the incidence of full-blown haemochromatosis in Bantu subjects coming to necropsy in Johannesburg is approximately 4% in males and 2% in females.

Summary

The concentration of iron in the livers of 318 Bantu males and 265 Bantu females dying in a Johannesburg hospital were estimated chemically. Of the Bantu males, 70% had significant hepatic siderosis, and in half of these subjects it was severe. In contrast 75% of females had iron concentrations in the liver which were within or just above the normal range.

Portal fibrosis or portal cirrhosis was present in the majority of subjects with hepatic iron concentrations above 2% dry weight. The fact that this was not invariably so suggests that there are other factors which potentiate the fibrogenic effects of excessive iron deposits.

We are grateful to Miss K. Mortimer and Mrs. R. Melrose for technical assistance, and to the Director of the South African Institute for Medical Research for granting facilities.

REFERENCES

Bothwell, T. H., and Bradlow, B. A. (1960). *Arch. Path. (Chicago)*, **70**, 279.

Finch, S. C., and Finch, C. A. (1955). *Medicine (Baltimore)*, **34**, 381.

Gillman, T., Hathorn, M., and Lamont, N. M. (1958). *S. Afr. J. med. Sci.*, **23**, 187.

Golberg, L., and Smith, J. P. (1960). *Amer. J. Path.*, **36**, 125.

Higginson, J., Gerritsen, Th., and Walker, A. R. P. (1953). Ibid., **29**, 779.

—— Grobbelaar, B. G., and Walker, A. R. P. (1957). Ibid., **33**, 29.

Isaacson, C., Seftel, H. C., Keeley, K. J., and Bothwell, T. H. (1961). *J. Lab. clin. Med.* In press.

Seftel, H. C., Isaacson, C., and Bothwell, T. H. (1960). *S. Afr. J. med. Sci.*, **25**, 89.

—— Keeley, K. J., Isaacson, C., and Bothwell, T. H. (1961). *J. Lab. clin. Med.* In press.

Walker, A. R. P., and Arvidsson, U. B. (1953). *Trans. roy. Soc. trop. Med. Hyg.*, **47**, 536.

Printed in Great Britain by Fisher, Knight and Co., Ltd., Gainsborough Press, St. Albans.

Iron Overload in Bantu Subjects

Studies on the Availability of Iron in Bantu Beer

T. H. Bothwell, M.D., M.R.C.P.,* H. Seftel, B.Sc., Dip. Med.,† P. Jacobs, M.B., B.Ch.,‡
J. D. Torrance, M.Sc.§ and N. Baumslag, M.B., B.Ch.‖

Varying degrees of iron overload occur in the majority of adult Bantu subjects living in South Africa.[1,2] Although it is accepted that this overload may be the result of an increased absorption from the gut, there is still controversy as to why this should occur. Originally it was postulated that there was an alteration in the behavior of the bowel mucosa induced by chronic malnutrition.[1] More recently, however, it has been shown that the diet of the Bantu population contains large amounts of iron, and it has been suggested that this is a more likely cause of the tissue siderosis.[3] Although the major foodstuffs consumed by the Bantu population contain moderate amounts of iron, most of the ingested iron is derived from the utensils which are used in cooking and in preparing fermented alcoholic beverages.[3]

The present study was undertaken to assess the amounts of iron currently being consumed by urban Bantu subjects of Johannesburg and to find out how much of this iron is absorbed by the gut. Since it was not feasible to label the whole diet, all absorption studies were carried out with Bantu beer since it appears to be the major source of iron.

From the Department of Medicine, University of the Witwatersrand Medical School and Baragwanath Hospital, Johannesburg, South Africa.

* Tutorial Physician; † Physician; ‡ Senior C.S.I.R. Bursar; § Lecturer; ‖ Research Fellow.

This work was supported in part by a grant (AM 04912-03) from the National Institutes of Health, U.S.A., and in part by grants from the Council for Scientific and Industrial Research, and the Atomic Energy Board, South Africa.

MATERIAL AND METHODS

Clinical Material

The Bantu subjects studied included healthy volunteers, patients in a paraplegic unit and convalescent surgical patients. All subjects were men, and none showed any evidence of active infection at the time of study. In addition a control group of white male volunteers was also studied. The mean age of the Bantu subjects was thirty-one years (range eighteen to sixty) and of the white subjects twenty-three years (range twenty-one to twenty-five).

Chemical Methods

The iron content of the stools was measured by leaving them for seven days in a 1:2 mixture of concentrated nitric and sulfuric acids. Each day the mixture was stirred. Finally, duplicate aliquots were wet ashed, and the iron content was measured using the thioglycolic acid method.[4]

The iron content of alcoholic beverages was measured by a modification of the method of Brückmann and Zondek[5] in which a 4 ml. aliquot was mixed with 4 ml. saturated sodium pyrophosphate and 4 ml. 20 per cent trichloroacetic acid. The sample was then maintained at 80°c. in a water bath for ten minutes. After cooling, the mixture was filtered, and estimation of iron content was carried out on an aliquot of the filtrate using the thioglycolic acid method. Since the beer was colored, each reading was made against a blank containing an aliquot of the sample and all reagents except thioglycolic acid. (Preliminary studies indicated that this method gave results which were in good agreement with those obtained by wet digestion of beer samples.)

Radioactive Measurements

Tracer amounts of ferric chloride labeled with Fe^{59} were added to samples of fermented Bantu beer

TABLE I
Iron Concentrations in Alcoholic Beverages

Name	Ingredients	No. of Samples Analyzed	Iron Concentration (mg./100 ml.)		Mean pH
			Average	Range	
Bantu beer (municipal)	Malt, degermed maize meal	50	1.9	1.1– 3.8	3.7
Bantu beer (home-brewed)	Malt, maize meal	14	8.2	0.8–15.0	3.8
Barberton	Sugar, malt, bread	29	4.5	0.5–15.8	3.9
Hops	Yeast, sugar, malt, hops	27	5.1	0.6–16.0	3.9
Pineapple	Yeast, sugar, oats, bread, pineapple	16	2.9	1.0–15.0	3.8
Shimeyana	Sugar, malt, potato	12	7.5	0.8–15.3	3.5
Chillies	Yeast sugar, malt, bread, ginger	8	5.3	1.0–10.5	4.1
Gin, Tot-Tot, etc.	Distillates made from other drinks	13	1.0	0.0– 5.0	5.9

(iron content 2.0 mg. per 100 ml.) prepared by the municipal authorities, and the beer was then allowed to stand overnight before being used. Although the label was not a physiologic one it was thought that valid results could be obtained by this method since preliminary studies showed that more than 80 per cent of the iron in the various types of alcoholic beverage consumed by the Bantu population is in the ionic form. In a second series of studies, carrier ferric chloride was added with the tracer to give a final iron concentration in the beer of 6.25 mg. per 100 ml.

Because of the well known variability of iron absorption from subject to subject a double isotope technic was used in which the availability of iron in Bantu beer was compared in each individual subject with that of a simple iron salt. On the first day 400 ml. of beer, labeled with 5 μc. Fe^{59}, was given, and on the second day a similar dose of iron, labeled with 75 μc. Fe^{55}, was administered either as ferric chloride or ferrous ascorbate. Blood samples collected two weeks later were digested, electroplated onto copper discs and differentially counted for the two isotopes as described previously.[2] The relative percentage absorptions of the two doses were then calculated on the assumption that 80 per cent of the iron absorbed from the gut was present in the red cell mass at two weeks and that the blood volume was 70 ml. per kg. Results obtained were, therefore, corrected by a factor of 10:8. (Although it has been established that approximately 80 per cent of iron entering the plasma is used for red cell production, the figure is somewhat lower in subjects with iron overload. It is, however, rarely below 60 per cent in Bantu subjects[6] so that levels of radioactivity in red cells should still provide a moderately accurate measure of the percentage of radioiron absorbed from the gut.)

RESULTS

Iron Concentrations in Alcoholic Beverages

Analyses were carried out on 169 samples of various types of alcoholic drink, most of which were home brewed. Although there was a considerable variation (0.5 to 16.0 mg. per 100 ml.) the mean concentration for the whole group was high (3.8 mg. per 100 ml.) (Table I). The lowest concentrations were found in beer prepared by the municipal authorities and in distilled liquors.

Drinking Habits of Urban Bantu Subjects

Two hundred Bantu adults attending an outpatient clinic were questioned on their drinking habits with special reference to the relative consumption of home-brewed beverages and those prepared by the municipal authorities. With a knowledge of the adult population served by the clinic and the amounts of municipally prepared beer being supplied to the area it was possible to make a rough estimate of the quantities of fermented beverages being consumed by the urban Bantu population. Results obtained in this way indicated a mean consumption in men of about 13 L. per week with a ratio of municipally prepared beer to home-brewed beer of 2 to 1. This would sug-

TABLE II

Comparison of Absorption of Iron in Bantu Beer and a Similar Dose of Iron given as Ferric Chloride

Subject	Dose of Iron (mg.)	% Absorption	
		Beer	Ferric Chloride
Paraplegic male Bantu patients	8	2.6	0.4
		2.8	0.9
		2.3	1.0
		2.5	3.2
		6.8	1.4
		8.0	0.9
	Mean.....	4.2	1.3
Healthy white male volunteer subjects	8	2.9	8.3
		17.8	16.6
		1.0	3.6
		1.6	1.6
	Mean.....	5.8	7.5
Convalescent Bantu male surgical patients	25	2.0	0.4
		1.1	0.1
		1.6	0.4
		1.9	2.6
		0.8	0.8
		1.2	4.3
		2.1	1.4
		0.5	0.3
		10.8	4.8
		1.6	1.0
		1.4	0.3
		3.1	1.0
	Mean.....	2.3	1.5
Healthy white male volunteer subjects	25	2.6	5.6
		17.5	31.5
		2.4	2.5
		13.0	16.5
	Mean.....	8.9	14.0

gest an average iron intake from beer of at least 50 mg. per day.

Iron Content of Stools from Bantu Subjects

Iron concentrations were measured in the stools of a random group of male Bantu adults attending an outpatient clinic. The mean concentration in stools collected on Monday mornings was 141 mg. per 100 gm. (range 28 to 330) as compared with 34 mg. per 100 gm. on Thursday mornings.

Availability of Iron in Bantu Beer

Fasting subjects were given 400 ml. of Bantu beer, containing either 8 or 25 mg. iron, and the percentage absorbed was compared in each subject with a similar dose of iron given as a simple ferric or ferrous salt. In both Bantu and white subjects the percentages absorbed were in the same range as those obtained with ferric chloride (Table II) and were about a third of those obtained with ferrous ascorbate (Table III). On the whole, Bantu subjects tended to absorb less than did the white volunteer subjects, and this was particularly so at the 25 mg. dosage level.

TABLE III

Comparison of the Absorption of Iron in Bantu Beer and a Similar Dose of Iron Given as Ferrous Ascorbate

Subject	Dose of Iron (mg.)	% Absorption	
		Beer	Ferrous Ascorbate
Healthy Bantu male volunteer subjects	8	2.8	7.4
		1.6	0.8
		2.3	6.3
		2.3	5.5
		10.3	27.5
	Mean.....	3.9	9.5
Healthy white male volunteer subjects	8	11.3	19.6
		4.3	18.8
		4.0	17.8
		1.3	9.9
	Mean.....	5.2	16.5
Male Bantu paraplegic patients	25	0.9	2.0
		1.2	3.6
		0.9	2.8
		0.9	1.9
		1.0	10.1
		2.7	4.0
		2.0	7.7
		0.1	3.5
	Mean.....	1.2	4.5
Healthy white male volunteer subjects	25	20.1	58.0
		6.5	15.3
		4.5	18.3
	Mean.....	10.4	30.5

COMMENTS

Although siderosis of varying degrees is common in Bantu adults only about 20 per cent show severe involvement, and most of these are middle-aged men.[7] Previous calculations based on chemical analyses of organs obtained

at autopsy suggest that the total quantity of iron in such subjects rarely exceeds 15 gm.[7] The disorder in iron balance necessary to produce this degree of iron overload is not a large one. A mean absorption of 2 mg. daily between the ages of twenty and fifty years would lead to a positive balance of approximately 1.4 mg. daily in the male subject, which is enough to account for deposits of this magnitude.

In the present study an attempt was made to assess the amounts of iron present in the diet of urban Bantu subjects and to measure how much of this iron is available for absorption. The importance of Bantu beer as the major source of iron in the diet was evident both from questioning Bantu subjects on their drinking habits and from direct chemical analyses on samples of various drinks. The fact that almost all the iron in these drinks is ionizable indicates that it is introduced from the containers used for brewing. In order to obtain more direct evidence of the high iron intakes in urban Bantu subjects, stools obtained from patients attending an outpatient clinic were analyzed for their iron content. Results were severalfold greater than would be expected in a comparable white population. In addition it was of interest that the mean concentrations of iron were much higher in stools collected from men on Mondays than in those collected on Thursdays (141 mg. per 100 gm. as compared with 34 mg. per 100 gm.). This difference probably reflects the weekend drinking habits of the subjects. From these various observations it was concluded that the mean iron intake of urban Bantu men is probably in the range of 50 to 100 mg. daily, although occasionally it may be a good deal higher than this. These estimates are in good agreement with previous calculations made by other workers.[3]

In assessing how much of the iron present in the Bantu diet is available for absorption Bantu beer was chosen since it not only represents the major source but also contains the iron in a readily assimilable form. The results indicated that this iron is absorbed to roughly the same degree as is a ferric salt. The relatively low pH of the fermented drinks does not seem to potentiate absorption nor do other constituents have any depressing effect. The fact that the Bantu subjects tended to absorb less than did white men is most probably due to the fact that the majority had some degree of iron overload since it has been shown that even a moderate increase in iron stores depresses absorption.[8] Another possible reason for the low absorptions in Bantu subjects was considered. At autopsy many Bantu subjects show deposits of iron in the mucosal cells of the upper portion of the gut and in the tunica propria. It therefore seemed feasible that a proportion of iron absorbed by siderotic Bantu subjects might be fixed in this intestinal pool for variable periods. To test this hypothesis a number of moribund Bantu patients were given 15 mg. iron as Fe^{59} chloride at various times before death, and the radioactivity present in the duodenum and upper part of the jejunum obtained at subsequent autopsy was measured in a counter composed of a ring of Geiger-Müller tubes.[9] The mean percentage present in the gut in twelve subjects who died between eight hours and twelve days after the administration of radioiron was 0.4 (range 0 to 1.1). It was, therefore, evident that no pool of significant dimensions exists in this site.

Although the absorptions in Bantu subjects were low the mean amount of iron retained from beer at the 25 mg. dosage level was 1.9 per cent. When these figures are related to a daily consumption of between 50 and 100 mg. it is apparent that the positive iron balance is of an order sufficient to account for the degrees of siderosis encountered in middle age. The fact that none of the Bantu subjects showed evidence of abnormally *high* absorption rates can be taken as good evidence that the siderosis so commonly found in these subjects is not the result of any intrinsic mucosal defect.

SUMMARY

A series of studies were carried out to assess the amounts of iron being consumed by urban male Bantu adults. It was found that the majority were taking between 50 and 100 mg. daily, most of which was present in fermented alcoholic beverages.

The availability of iron in Bantu beer was measured in Bantu and white subjects. In both groups it was absorbed to the same degree

as a ferric salt. On the whole the percentage absorption was lower in the Bantu subjects, and this was probably due to the fact that body stores are greater than normal in the majority of them.

From the data obtained it would appear that the degrees of siderosis present in middle-aged Bantu men can be explained on their high iron intake alone.

REFERENCES

1. GILLMAN, J. and GILLMAN, T. Perspectives in Human Malnutrition. New York, 1951. Grune & Stratton, Inc.
2. BOTHWELL, T. H. and FINCH, C. A. Iron Metabolism. Boston, 1962. Little, Brown & Co.
3. WALKER, A. R. P. and ARVIDSSON, U. B. Iron "overload" in the South African Bantu. *Tr. Roy. Soc. Trop. Med. & Hyg.*, 47: 536, 1953.
4. MCCANCE, R. A., WIDDOWSON, E. M. and SHACKLETON, L. R. B. The nutritional value of fruits, vegetables and nuts. In: Medical Research Council Special Report, Series 213, p. 21. London, 1936.
5. BRÜCKMANN, G. and ZONDEK, S. G. Iron, copper and manganese in the human organs at various ages. *Biochem. J.*, 33: 1845, 1939.
6. BOTHWELL, T. H. and KRAMER, S. Unpublished data.
7. BOTHWELL, T. H. and ISAACSON, C. Siderosis in the Bantu. A comparison of the incidence in males and females. *Brit. M. J.*, 1: 522, 1962.
8. PIRZIO-BIROLI, G. and FINCH, C. A. Iron absorption. III. The influence of iron stores on iron absorption in the normal subject. *J. Lab. & Clin. Med.*, 55: 216, 1960.
9. VEALL, N. and VETTER, H. An apparatus for the rapid estimation of the tracer quantities of radioactive isotopes in excrete. *Brit. J. Radiol.*, 25: 85, 1952.

Reprinted from THE AMERICAN JOURNAL OF CLINICAL NUTRITION
January issue, Vol. 14, No. 1, pp. 47–51, copyright 1964 and printed in the U.S.A.
Published by The Reuben H. Donnelley Corporation, 466 Lexington Avenue, N.Y. 10017

HEPATIC IRON STORES IN THREE DIFFERENT RACIAL GROUPS

by

F.G.H. MAYET and T.H. BOTHWELL*

Department of Medicine, University of Natal, Durban and C.S.I.R. Iron and Red Cell Metabolism Unit, Department of Medicine, University of the Witwatersrand, Johannesburg.

*This work was supported in part by a grant (AM04912-03) from the National Institutes of Health, U.S.A., and in part by a grant from the World Health Organisation.

In the body of a healthy adult male there is normally between 3 and 4g. iron (Bothwell and Finch, 1962). Two thirds of this iron is in compounds which are actively participating in metabolic functions, while the remaining fraction of approximately 1,000mg. is deposited in the stores of the body as ferritin and haemosiderin. The bulk of this iron is present in the liver and in the reticulum cells of the bone marrow.

The level of iron stores at any one time represents a useful measure of iron balance since stores are greater than normal when the iron content of the diet is high and are diminished or absent when the diet is inadequate. Storage iron serves as a buffer to protect the body against the effects of iron deficiency. Thus when a negative iron balance occurs, iron is removed from stores to supply red cell needs and anaemia only develops when the stores have been depleted (Coleman et al., 1953).

In clinical studies the level of iron stores is most easily gauged by assessing the amounts of haemosiderin present in bone marrow particles (Rath and Finch, 1948). In necropsy material more quantitative information can be obtained by estimating the concentrations of storage iron present in liver samples. Although this second approach might be expected to provide useful information on the iron status of different population groups it has not as yet been

- extensively -

extensively applied. The present study was therefore done to compare the pattern of iron storage in necropsy material obtained from groups of White, African and Indian subjects who had lived in South Africa.

MATERIAL AND METHODS

Necropsy Material: Liver specimens were obtained from 738 subjects at necropsy. There were 512 males and 226 females. The material, which was collected in Durban and Johannesburg, was obtained from subjects dying acute traumatic deaths (554 cases) and from patients dying in hospital (184 cases). All were over the age of 18 years.

Methods: All specimens were fixed in buffered 10% formal-saline. The concentrations of total and of haemoglobin iron were then estimated chemically by the method of Bothwell, Roos and Lifschitz (1964) and the storage iron concentrations were obtained by difference.

RESULTS (Table)

White Subjects. Median iron concentrations in males dying acute traumatic deaths were higher than in females (268 µg./g. wet weight as compared with 96 µg./g. in females). The difference was far less marked in subjects dying from natural causes. In this group the comparable figures were 251 and 193 µg./g. respectively. The reason for the significantly higher figures ($p < 0.01$) in hospital females was

not immediately apparent. Although the median age in these subjects was greater this could not be invoked as a cause since analysis of all the females revealed that the median age of the females with the 20 lowest values was the same as the females with the 20 highest values. Alternatively it was possible that some of the subjects dying in hospital may have been suffering from diseases which had increased the storage iron concentrations. However, if this were so it is surprising that a similar trend was not evident in White males.

African Subjects. Median iron concentrations in Bantu males dying acute traumatic deaths in Johannesburg and Durban were very similar (966 and 786 µg./g.respectively). A higher figure was obtained in older hospital subjects but this difference was probably not significant as the group was very small. Results in females from three different sources were remarkably uniform, with median iron concentrations of 268, 251 and 283 µg./g.respectively.

Indian Subjects. Median iron concentrations were higher in males than in females in both the subjects dying acute traumatic deaths (173 and 103 µg./g.respectively) and in patients dying from natural causes (206 and 126 µg./g. respectively).

Comparison of Range of Hepatic Concentrations of Storage Iron in Whites, Africans and Indians. For purposes of comparison all values in males and females from each of

the racial groups were considered together irrespective of the source of the material. The cumulative percentage incidence of different iron concentrations was then plotted graphically. Results in males (109 Whites, 327 Africans and 76 Indians) are shown in Figures 1A and 1B while Figures 2A and 2B show the comparable results in 47 White, 134 Africans and 45 Indian females.

DISCUSSION.

The results of the present study indicate that measurement of the hepatic concentrations of storage iron provide a useful index of the iron status in different populations. The fact that stores were uniformly lower in the females of each group can be taken as a good indication of the validity of the approach since there is abundant clinical evidence to show that iron deficiency is much commoner in females than in males (Bothwell and Finch, 1962).

Results in African males confirmed the high incidence of iron overload in this group. It was noteworthy that a small proportion of African females also accumulate large quantities of storage iron. In White subjects figures obtained were comparable to those reported previously (Morgan and Walters, 1963). Calculations based on iron concentrations and liver weights suggest a median storage iron content between 400 and 500 mg. in White males. Since

the liver contains about one-third of the storage iron in the body it is apparent that the total content is between 1200 and 1500 mg. This is in close agreement with figures calculated from the response of normal subjects to repeated phlebotomies (Haskins et al., 1952). Results in both Indian males and females revealed storage iron concentrations somewhat lower than in Whites, but the median figures obtained suggested that iron stores were adequate in the majority of subjects.

If meaningful information is to be obtained from a study of this type attention should be directed not only to the median iron concentrations but to the proportion of subjects in each group that might be expected to develop iron deficiency most readily. The percentage incidence of subjects with storage iron concentrations below 50 µg./g. was therefore calculated. The figures in males were as follows: White males 5.4%, African males 0.6% and Indian males 9.2%. Comparable figures in females were 6.7%, 4.5% and 17.8% respectively. These figures suggest that iron deficiency anaemia should be more common in Indian subjects, especially in females. Clinical experience in Durban indicates that this conclusion is a valid one (Mayet, 1964).

SUMMARY.

Storage iron concentrations were estimated in liver samples obtained from 109 White, 327 African and 76 Indian males, and from 47 White, 134 African and 45 Indian females.

The material was obtained in Durban and Johannesburg from subjects dying acute traumatic deaths and from patients dying in hospital. Median iron concentrations in µg/g. wet weight were as follows: White males 264, White females 152, African males 851, African females 270, Indian males 182, Indian females 118.

ACKNOWLEDGEMENTS

The authors are indebted to Miss A. Dorling, Miss N. Roos and Mrs. K. Hurd for technical assistance. They should like also to thank Professor J. Friedman, Professor B.J.P. Becker, the Government pathologists and the Department of Pathology at the University of Natal, for having made material available for study.

LEGENDS

Figure 1.

Cumulative percentage incidence of various concentrations of storage iron present in the livers of White, African and Indian males.

(A) Concentrations from 0 to 1,000µg/g. wet weight.
(B) Concentrations from 0 to 5,000µg/g. wet weight.

Figure 2.

Cumulative percentage incidence of various concentrations of storage iron present in the livers of White, African and Indian females.

(A) Concentrations from 0 to 1,000µg/g. wet weight.
(B) Concentrations from 0 to 5,000µg/g. wet weight.

- REFERENCES -

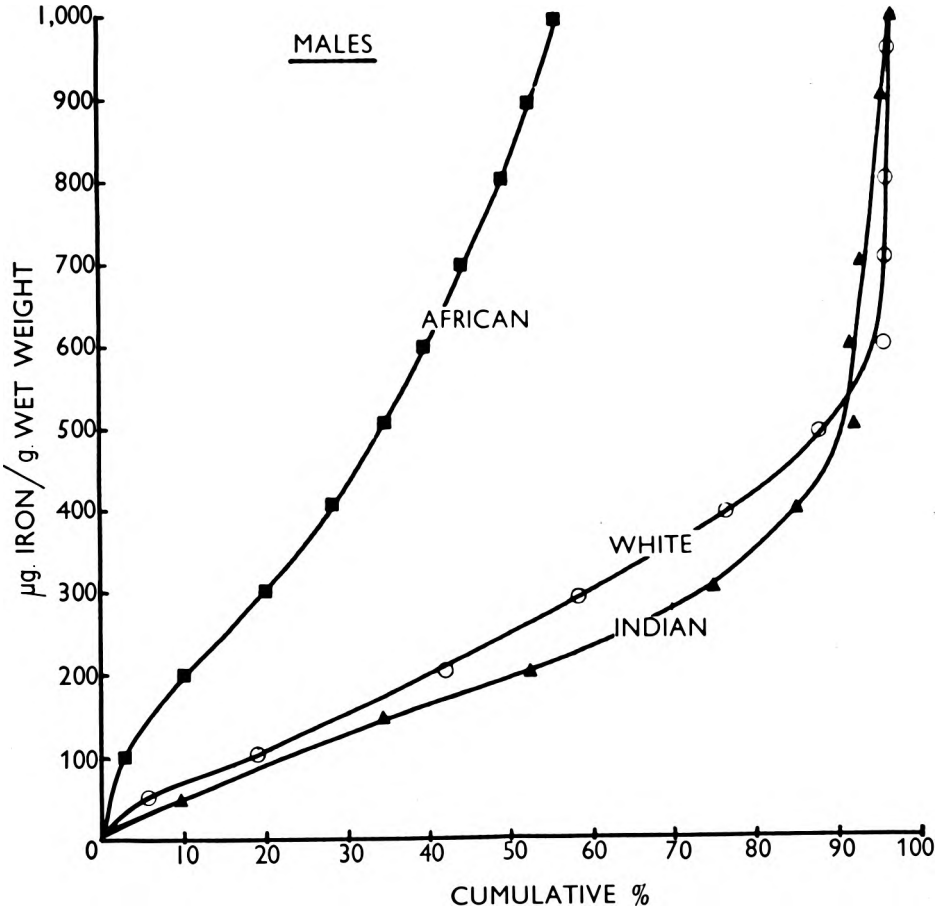


FIGURE I(A).

Cumulative percentage incidence of various concentrations of storage iron present in the livers of White, African and Indian males.

(Concentrations from 0 to 1,000µg./g. wet weight.)

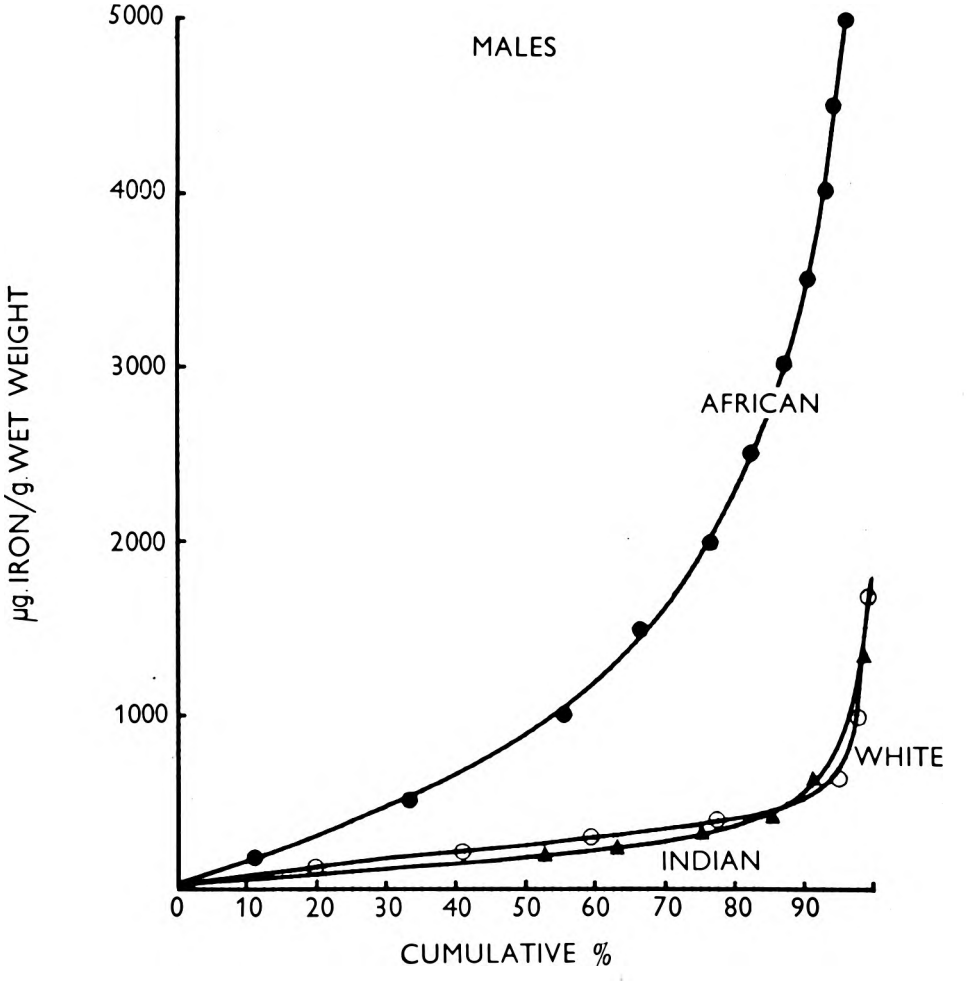


FIGURE I(B).

Cumulative percentage incidence of various concentrations of storage iron present in the livers of White, African and Indian males.

(Concentrations from 0 to 5,000µg./g. wet weight)

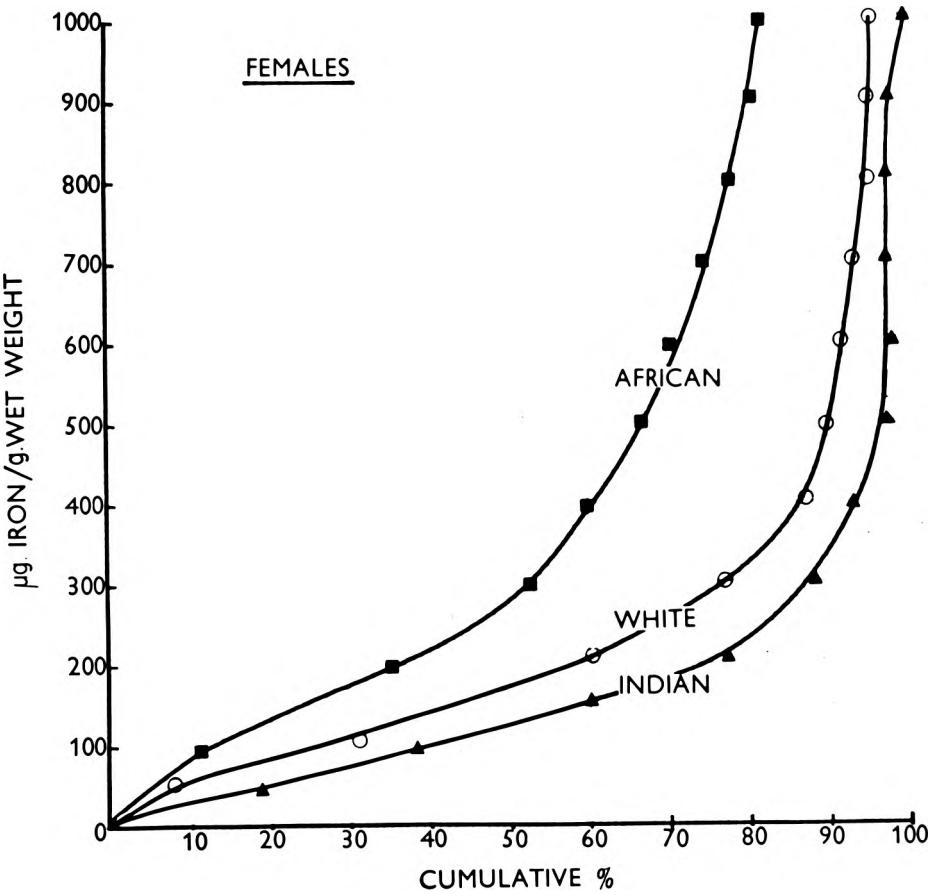


FIGURE 2(A).

Cumulative percentage incidence of various concentrations of storage iron present in the livers of White, African and Indian females.

(Concentrations from 0 to 1,000µg./g. wet weight.)

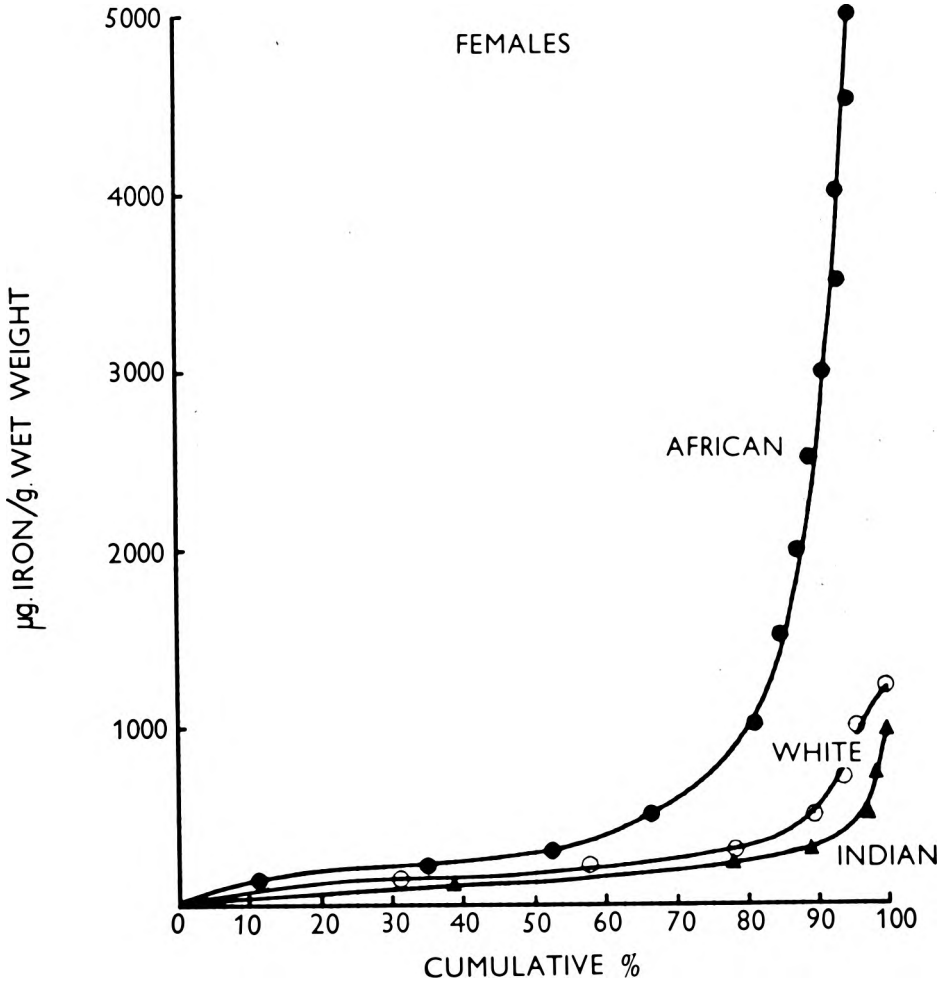


FIGURE 2(B).

Cumulative percentage incidence of various concentrations of storage iron present in the livers of White, African and Indian females.

(Concentrations from 0 to 5,000µg./g. wet weight.)

REFERENCES

TABLE: STORAGE IRON CONCENTRATIONS IN THE LIVERS OF DIFFERENT RACIAL GROUPS.

Type and Source of Clinical Material	Number of Cases	Median Age.	Median Storage Iron Concentrations including Range (µg./g. wet weight).	Median Liver Weights	Estimated Figures for Total Storage Iron in Livers (mg.).
(A) WHITES (Johannesburg & Durban)					
(i) Traumatic					
Males	85	39	268 (0 - 1448)	1810	485
Females	20	48	96 (3 - 378)	1570	150
(ii) Hospital					
Males	24	65	251 (33 - 1628)	1840	462
Females	27	71	193 (58 - 1136)	1510	291
(B) AFRICANS					
(i) Johannesburg (Traumatic)					
Males	81	30	966 (83 - 5741)	1500	1449
Females	16	35	268 (53 - 2931)	1390	372
(ii) Durban (Traumatic)					
Males	239	35	786 (18 - 11453)	1580	1241
Females	45	30	251 (36 - 7086)	1540	386
(iii) Durban (Hospital)					
Males	7	48	1763 (38 - 5333)	1420	2503
Females	73	43	283 (18 - 7578)	1330	376
(C) INDIANS					
(i) Durban (Traumatic)					
Males	53	40	173 (0 - 1781)	1400	242
Females	15	34	103 (31 - 981)	1300	134
(ii) Durban (Hospital)					
Males	23	51	206 (46 - 766)	1450	299
Females	30	46	126 (11 - 423)	1375	173

REFERENCES

BOTHWELL, T.H. and FINCH, C.A. Iron Metabolism. (Little, Brown and Co., Boston).

BOTHWELL, T.H., ROOS, N. and LIFSCHITZ, M.L. (1964). A method for measuring the storage iron content of formalinised tissues. South Afr. J. Med. Sci., in press.

COLEMAN, D.H., STEVENS, A.R. Jr. and FINCH, C.A. (1955). The treatment of iron deficiency anemia. Blood, 10: 567-581.

HASKINS, D., STEVENS, A.R. Jr., FINCH, S.C. and FINCH, C.A. (1952). Iron metabolism. Iron stores in man as measured by phlebotomy. J. clin. Invest., 31: 543-547.

MAYET, F.G.H. (1964). A comparative study of iron deficiency in Durban. Thesis presented for the degree of M.D. in the University of Natal.

MORGAN, E.H. and WALTERS, M.N.I. (1963). Iron storage in disease. Fractionation of hepatic and splenic iron into ferritin and haemosiderin with histochemical correlations. J. clin. Path., 16: 101-107.

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: 81-86.

ARCHIVES OF PATHOLOGY (1964) - IN PRESS

IDIOPATHIC AND BANTU HAEMOCHROMATOSIS: A COMPARATIVE HISTOLOGICAL STUDY.

+T.H. BOTHWELL, M.D., M.R.C.P.
*C. ABRAHAMS, M.Med.Path., M.C.Path., F.F.Path. (S.A.)
++B.A. BRADLOW, M.D., D.Path., M.C.Path., and
R.W. CHARLTON, M.D., M.R.C.P.E.

JOHANNESBURG, SOUTH AFRICA.

Departments of Medicine and Pathology, University of the Witwatersrand Medical School, Hospital Street, Johannesburg, and C.S.I.R. Iron and Red Cell Metabolism Unit.

This work was supported in part by a grant (AM04912-03) from the National Institutes of Health, and in part by a grant from the Council for Scientific and Industrial Research, South Africa.

+Senior Tutorial Physician
*Lecturer
++Senior Lecturer
**Tutorial Physician

In the past it has been customary to regard idiopathic hemochromatosis as the end result of an inborn metabolic disorder associated with the absorption of excessive amounts of iron from a normal diet[1]. In contrast, the iron overload so commonly noted in the Bantu of Southern Africa appears to be secondary to the high dietary content of iron, most of which is present in fermented alcoholic drinks[2,3]. Results of previous studies indicate that many male Bantu ingest between 50 and 100 mg. iron daily in these beverages, and heavy drinkers are exposed to even greater amounts[3]. Although the basic alcohol content of the drinks is only 3%, many of them are adulterated in a number of ways; distillates prepared from the brews and conventional liquors, such as brandy and gin, are also consumed. In the majority of these subjects the distribution of iron is different from that found in idiopathic hemochromatosis, since the iron deposits are confined to the liver and reticuloendothelial system[4,5]. However in those Bantu patients with severe overload and portal cirrhosis the findings have been found to be virtually indistinguishable from those in idiopathic hemochromatosis, with iron deposits prominent in epithelial tissues such as the pancreas, thyroid and adrenals[6,7].

In recent years evidence has accumulated which suggests that the Bantu are not the only group exposed to

- high -

high dietary intakes. It has been shown that a number of the wines of Europe and the United States contain significant amounts of iron, and it has been calculated that many wine-drinking alcoholic subjects have iron intakes several fold normal[8]. This finding is of special interest since many subjects with idiopathic hemochromatosis have a significant alcoholic background[9,10]. The question therefore arises as to whether the condition is no more than a variant of nutritional cirrhosis occurring in subjects exposed to a high dietary intake of iron[8,11,12,13]. If this proposal is correct then it might be anticipated that the histological findings in patients with classical idiopathic hemochromatosis would be indistinguishable from those in subjects with the hemochromatosis occurring after prolonged exposure to a diet containing an excess of alcohol and iron. The present study was undertaken to find out whether this is, in fact, so.

MATERIAL AND METHODS.

Clinical Material: Liver sections were examined from thirteen white subjects (11 males and 2 females) who had been diagnosed as suffering from idiopathic hemochromatosis[1]. All except one of them had been, or were still, under the clinical care of one of us (T.H.B.), and the material was unselected insofar as it represented all the subjects seen over several years in whom adequate pathological specimens of liver were available. The diagnosis was made on clinical grounds in the first instance. Skin pigmentation and firm

hepatomegaly were present in all subjects, and liver biopsy showed portal cirrhosis with heavy parenchymal iron deposits in each patient. Five were diabetic, and 4, all in their twenties, subsequently died of cardiac complications. The diagnosis was confirmed at necropsy in 7 instances. In addition to the findings in the livers, epithelial deposits of iron were prominent in a number of organs. In 3 subjects the hepatic iron concentrations were determined chemically, and were found to be 2.6, 3.0 and 3.3% dry weight. In other organs the iron concentrations were also within the range described by Sheldon[9]. The group as a whole included 3 siblings from one family and 2 from another. No patient gave a history of excessive consumption of alcohol, and careful questioning did not uncover any other obvious source of excessive dietary iron (two subjects had drunk well-water for a number of years but chemical analysis of samples from both wells gave negative results).

The 13 Bantu subjects (11 males and 2 females) all had advanced hepatic siderosis and portal cirrhosis. This was the only criterion for selection, since it has been shown that such subjects invariably have significant epithelial deposits elsewhere in the body[6,7]. In 9 subjects the diagnosis had been confirmed by liver biopsy and in the other 4 at necropsy. Although the clinical data on these subjects were not as adequate as in the patients

with idiopathic hemochromatosis, there is good evidence that Bantu with severe siderosis and cirrhosis always have a history of long exposure to the various local alcoholic beverages[14].

Methods: Liver sections were routinely stained with hematoxylin and eosin. Sections were also stained for hemosiderin using Perl's method without heating, and were counterstained with neutral red. Sections of spleen, which were available in 7 white subjects and 4 Bantu, were treated in the same way.

The histological grading of hepatic iron deposits was assessed separately in parenchymal cells, Kupffer cells, portal tract phagocytes and bile ducts. The criteria were as follows:

(a) Parenchymal Cells: Grade 0 = no stainable iron granules; Grade + = a few small fine granules of hemosiderin usually confined to the biliary pole in some, or most, of the liver cells, especially in the periportal regions; Grade ++ = numerous fine granules of hemosiderin in most of the cells, and Grade +++ = numerous coarse granules of hemosiderin in most of the liver cells.

(b) Kupffer Cells: Grade 0 = no stainable hemosiderin granules; Grade + = occasional fine hemosiderin granules in some of the Kupffer cells; Grade ++ = numerous fine hemosiderin granules in most of the Kupffer cells; and Grade +++ = numerous coarse hemosiderin granules in most of the Kupffer cells. (With Grade +++ the Kupffer cells were usually markedly

- enlarged.) -

enlarged.)

(c) Portal Tracts: Grade 0 = no stainable hemosiderin granules; Grade + = occasional fine hemosiderin granules in portal tract macrophages; Grade ++ = small clumps of macrophages containing fine and coarse hemosiderin granules; and Grade +++ = coarse, irregular, intra- and extra-cellular masses of hemosiderin.

(d) Bile Duct Epithelium: Grade 0 = no stainable hemosiderin granules: Grade + = occasional fine hemosiderin granules in some of the bile ducts; Grade ++ = numerous fine hemosiderin granules in most of the bile ducts; Grade +++ = numerous large, coarse hemosiderin granules in most of the bile ducts.

The histological grading of splenic sections was as follows: Grade 0 = no stainable hemosiderin granules; Grade + = occasional fine granules of hemosiderin in some of the pulp macrophages; Grade ++ = numerous fine and coarse hemosiderin granules in pulp macrophages; Grade +++ = numerous large masses of coarse intra- and extracellular hemosiderin in the splenic pulp and trabeculae. (Hemosiderin granules were also present in the venous endothelial cells and in the subcapsular region in the majority of specimens graded ++ or +++).

R E S U L T S

(a) Distribution of Iron in Liver: Although both groups showed significant deposits of iron in all 4 sites, there were differences in the distribution (see Table). In idiopathic hemochromatosis deposits were heaviest in

- parenchymal -

parenchymal cells, while moderate amounts were found in Kupffer cells and in the epithelium of the bile ducts. In contrast, little was present in the phagocytes of the portal tracts (Figure 1). In the livers obtained from Bantu subjects, on the other hand, the major deposits were present in portal tract phagocytes and Kupffer cells, with moderately heavy deposits in parenchymal cells and only scanty ones in the bile duct epithelium (Figure 2). The differences between the two groups were reflected by a comparison of the mean ratings for each part of the liver (Fig. 3). The validity of these differences was further tested by examining all samples blind to find out whether it was possible to place each in its correct group. When this was done by an independent pathologist complete separation into White and Bantu groups was obtained.

(b) Distribution of Iron in Spleen (Table): There was a striking difference between the two groups, with very little iron in the spleens of subjects with idiopathic hemochromatosis (a mean + rating of 0.57 in 7 subjects), and marked deposits in the Bantu (a mean + rating of 2.75 in 4 subjects).

DISCUSSION.

The histological findings in the various stages of iron overload in Bantu subjects have been studied previously by several workers[4,5,15,16]. These results indicate that although parenchymal deposits occur early in the liver, epithelial deposits elsewhere in the body are not a feature. In contrast, reticulo-endothelial involvement is prominent

at all stages, and with massive overload the concentrations in an organ such as the spleen may even be greater than those in the liver[4,5,6,7]. The distribution of iron found in the majority of Bantu with iron overload is therefore very different from that found in idiopathic hemochromatosis, but is similar to that reported in animals placed on deficient diets with heavy iron supplements[17,18]. Although this is the usual pattern of iron distribution found in Bantu with overload, a small proportion of subjects with severe involvement develop significant epithelial deposits in a number of organs. Such patients always give a long history of excessive consumption of the local alcoholic beverages[14] and portal cirrhosis is invariably present[6,7]. The etiology of the cirrhosis is still not clear, but may well relate to the heavy iron deposits and/or the effects of the alcohol with its associated malnutrition. The fact that fatty change is rare in these patients does not necessarily rule out alcohol as an etiological factor, since it is extremely uncommon even in alcohol-conditioned Bantu pellagrins[19]. Whatever the explanation for this anomaly, it is apparent that a small proportion of Bantu adults exhibit histological features at necropsy which closely resemble those reported in idiopathic hemochromatosis[6,7,20].

These various points have been raised since the whole concept of idiopathic hemochromatosis as a separate metabolic disorder has been questioned recently[8,11,12,13].

Evidence has been produced which suggests that the condition is only a variant of nutritional cirrhosis occurring in subjects also exposed to excessive iron. Support for this contention has been provided by the fact that many affected individuals have a significant alcoholic background, and by the observation that a large number of alcoholic drinks, and especially wines, contain significant amounts of iron[8].

The present study was undertaken in order to test this hypothesis, since the pathological findings in Bantu subjects with fully developed hemochromatosis might be expected to provide a prototype of the iron distribution resulting from prolonged exposure to a diet containing excessive iron and alcohol. Two points of difference between the distribution of iron in these patients and in white patients with idiopathic hemochromatosis were found to be sufficiently marked to allow for accurate histological diagnosis. Firstly, the results confirmed the fact that splenic iron concentrations are much greater in the Bantu than in the white subjects. In the second place it was apparent that the distribution of iron in the liver is different in the two conditions. In subjects regarded clinically as having idiopathic hemochromatosis the major impact was on parenchymal cells, with lesser amounts in Kupffer cells and bile ducts, and very little in portal tract phagocytes. In contrast, deposits in the Bantu were particularly prominent in Kupffer cells and portal

tract phagocytes, while deposits in bile duct epithelium were almost invariably insignificant. These various differences were striking enough to allow for correct categorisation of all the 26 cases in the study without previous knowledge of the source of each.

It therefore seems that prolonged ingestion of alcohol with a high iron content, together with a diet possibly suboptimal in certain respects, results in a hemochromatosis which is morphologically distinct from idiopathic hemochromatosis. While not conclusive, these observations are therefore compatible with the contention that idiopathic hemochromatosis is a metabolic entity, and not merely one end of a spectrum which ranges on the one hand from minimal siderosis, with or without cirrhosis, to the fullblown disease with extensive epithelial deposits on the other. This evidence may be added to a number of previous observations which suggest the existence of a specific inherited disorder characterised by the excessive absorption of iron from a normal diet. These observations include the familial incidence of the disease, and the finding of an abnormally high absorption of radioiron in some patients with the fully developed condition[1]. This has been found to be especially so in young subjects, and the failure to demonstrate increased absorption in some older patients may well be due to the inhibitory effect

exerted by the enormous iron stores. Support for this contention has been provided by the finding of very high absorption rates in such patients after reduction of stores by repeated phlebotomy[21].

Whether the different distribution of iron in the idiopathic disease represents some clue as to the fundamental defect in this condition is still not clear. The fact that reticulo-endothelial deposits are not conspicuous in the liver and spleen may point to some alteration in reticulo-endothelial function, but there is no assurance that this is of primary importance. It may, however, be noteworthy that the plasma iron level has been found to be high in some affected relatives at a time when iron stores have been normal or only moderately increased[22,23,24]. This observation has led to the suggestion that there may be an altered gradient between reticulo-endothelial cells and the plasma in the disorder[1].

While these speculations are of theoretical interest, it should be stressed that the present findings are open to other interpretations. For instance, the increased reticulo-endothelial deposits in the livers of Bantu subjects may only reflect a more active liver disease, perhaps related to alcohol and malnutrition, in which there is a constant movement of iron from necrosed parenchymal cells into the reticulo-endothelial system. The distribution of iron in

subjects with idiopathic hemochromatosis who consume excessive amounts of alcohol might therefore be expected to resemble the Bantu more closely. However, on this basis it is still difficult to understand why such large amounts of iron accumulate in the spleens of Bantu subjects.

Although there can be no final resolution of these morphological findings at the present time, they do emphasise the importance of cataloging the exact cellular localization of iron in different types of iron overload. In necropsy studies the chemical concentration of iron in the spleen should be measured, since very high values are almost invariably present in the iron overload secondary to a diet containing an excess of alcohol and iron. In clinical studies the differentiation is more difficult, but the present findings suggest that liver sections obtained by biopsy are adequate for categorising the type of overload present. It is only when such information is available that meaningful debates can occur between workers in different centers.

S U M M A R Y.

A study was done to find out whether it was possible to differentiate between the histological findings in the livers of 13 subjects regarded as suffering from idiopathic hemochromatosis, and those in the livers of 13 Bantu subjects with hemochromatosis. The first group had no obvious history of excessive dietary iron, while the Bantu had long-continued exposure to iron-containing alcoholic drinks.

There were clear-cut differences in the histological findings in the two groups. In idiopathic hemochromatosis the major deposits were in parenchymal cells, and little iron was present in portal tract phagocytes. In contrast, the Bantu showed maximal involvement in Kupffer cells and portal tract phagocytes. Splenic sections, available in some subjects, also showed differences, with far greater deposit present in the Bantu subjects.

- REFERENCES -

REFERENCES

1. Bothwell, T.H., and Finch, C.A.: Iron Metabolism, Boston, Little, Brown and Co., 1962.

2. Walker, A.R.P., and Arvidsson, U.B.: Iron "Overload" in the South African Bantu, Tr Roy Soc Trop Med & Hyg 47: 536, 1953.

3. Bothwell, T.H., Seftel, H., Jacobs, P., Torrance, J.D., and Baumslag, N.: Iron Overload in Bantu subjects. Studies on the Availability of Iron in Bantu beer, Amer J Clin Nutrit 14: 47, 1964.

4. Higginson, J., Gerritsen, T., and Walker, A.R.P.: Siderosis in the Bantu of Southern Africa, Amer J Path 29: 779, 1953.

5. Bothwell, T.H., and Bradlow, B.A.: Siderosis in the Bantu. A combined Histopathological and Chemical Study, Arch Path 70: 279, 1960.

6. Bradlow, B.A., Dunn, J.A., and Higginson, J.: The Effect of Cirrhosis on Iron Storage, Amer J Path 39: 221, 1961.

7. Isaacson, C., Seftel, H., Keeley, K.J., and Bothwell, T.H.: Siderosis in the Bantu. The Relationship between Iron Overload and Cirrhosis. J Lab Clin Med 58: 845, 1961.

8. MacDonald, R.A.: Idiopathic Hemochromatosis, Arch Int Med 112, 184, 1963.

9. Sheldon, J.H.: Haemochromatosis, London, Oxford University Press, 1935.

10. Finch, S.C., and Finch, C.A. Idiopathic Hemochromatosis, an Iron Storage Disease, Medicine 34: 381, 1955.

11. MacDonald, R.A., and Mallory, G.K.: Hemochromatosis and Hemosiderosis, Arch Int Med 105: 686, 1960.

12. MacDonald, R.A.: Idiopathic Hemochromatosis, a Variant of Portal Cirrhosis and Idiopathic Hemosiderosis, Arch Int Med 107: 606, 1961.

13. Pechet, G.S., and MacDonald, R.A.: Idiopathic Hemosiderosis. Relation to Idiopathic Hemochromatosis. New Eng J Med 267: 6, 1962.

14. Seftel, H., Isaacson, C., Keeley, K.J., and Bothwell, T.H.: Siderosis in the Bantu. The Clinical Incidence of Hemochromatosis in Diabetic Subjects, J Lab Clin Med 58: 837, 1961.

15. Gillman, J., and Gillman, T.: Perspectives in Human Malnutrition, New York, Grune and Stratton, Inc., 1951.

16. Wainwright, J.: Siderosis in the African, South Afr J Lab Clin Med 3: 1, 1957.

17. Kinney, T.D., Hegsted, D.M., and Finch, C.A.: The Influence of Diet on Iron Absorption. I. Pathology of Iron Excess, J Exper Med 90: 137, 1949.

18. Gillman, T., Hathorn, M., and Canham, P.A.S.: Experimental Dietary Siderosis, Amer J Path 35: 349, 1959.

19. Higginson, J., Grobbelaar, B.G., and Walker, A.R.P.: Hepatic Fibrosis and Cirrhosis in Man in Relation to Malnutrition, Amer J Path 33: 29, 1957.

20. Seftel, H.C., Isaacson, C., and Bothwell, T.H.: The relationship between Siderosis and Diabetes in the Bantu, South Afr J Med Sci 25: 89, 1960.

21. Pirzio-Biroli, G., Bothwell, T.H., and Finch, C.A.: Iron Absorption. II. The Absorption of radioiron administered with a Standard Meal in Man, J Lab Clin Med 51: 37, 1958.

22. Bothwell, T.H., Cohen, I., Abrahams, D.I., and Perold, S.M. A Familial Study in Idiopathic Hemochromatosis, Amer J Med 27: 730, 1959.

23. Williams, N., Scheuer, P.J. and Sherlock, S.: The Inheritance of Idiopathic Haemochromatosis: A Clinical and Liver Biopsy Study of 16 Families, Quart J Med 31: 249, 1962.

24. Harris, D.D.: Haemochromatosis: A Family Study, Med J Australia 49: 755, 1962.

- LEGENDS -

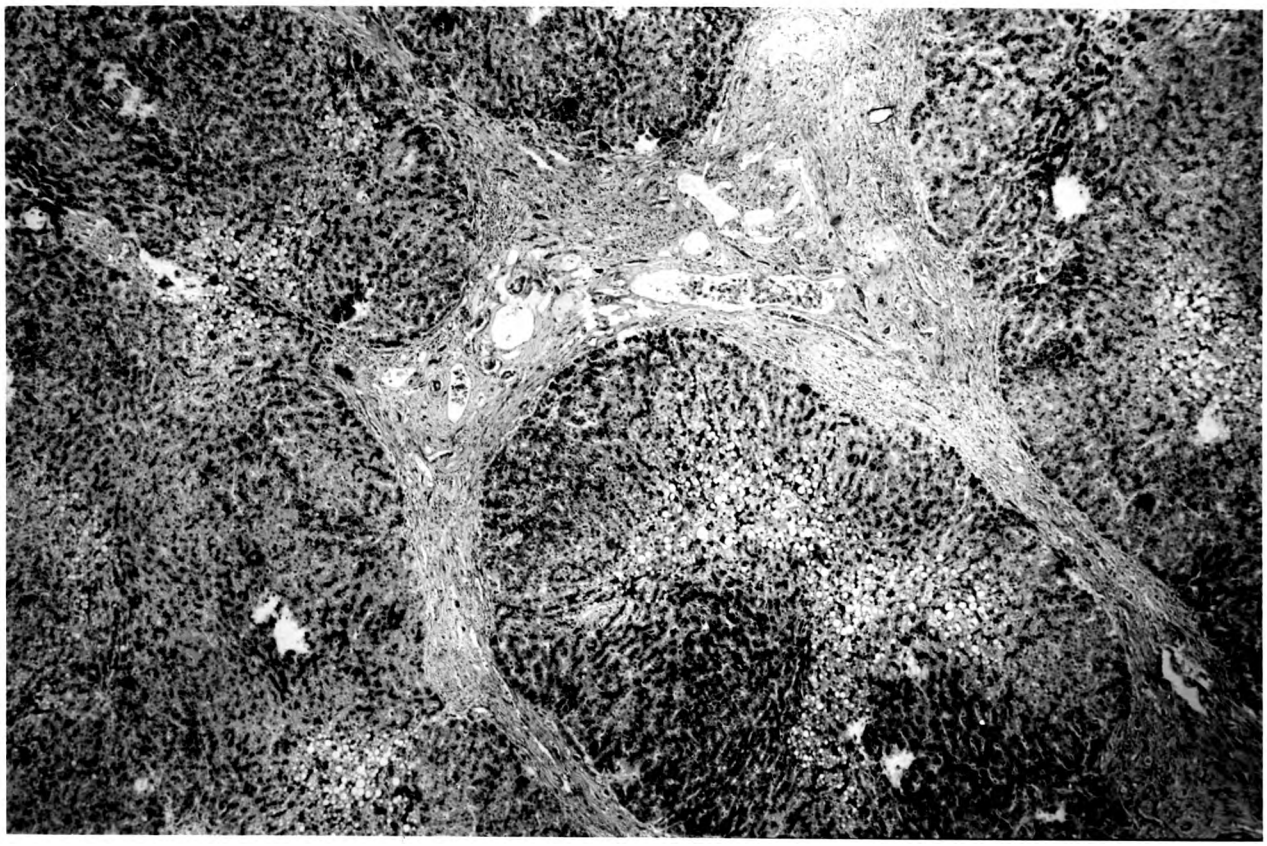


Figure 1: Section of liver from a patient with idiopathic hemochromatosis, showing a marked increase in hemosiderin granules in parenchymal cells, with relatively little iron in the portal tracts and fibrous tissue. Perl's stain x 30.

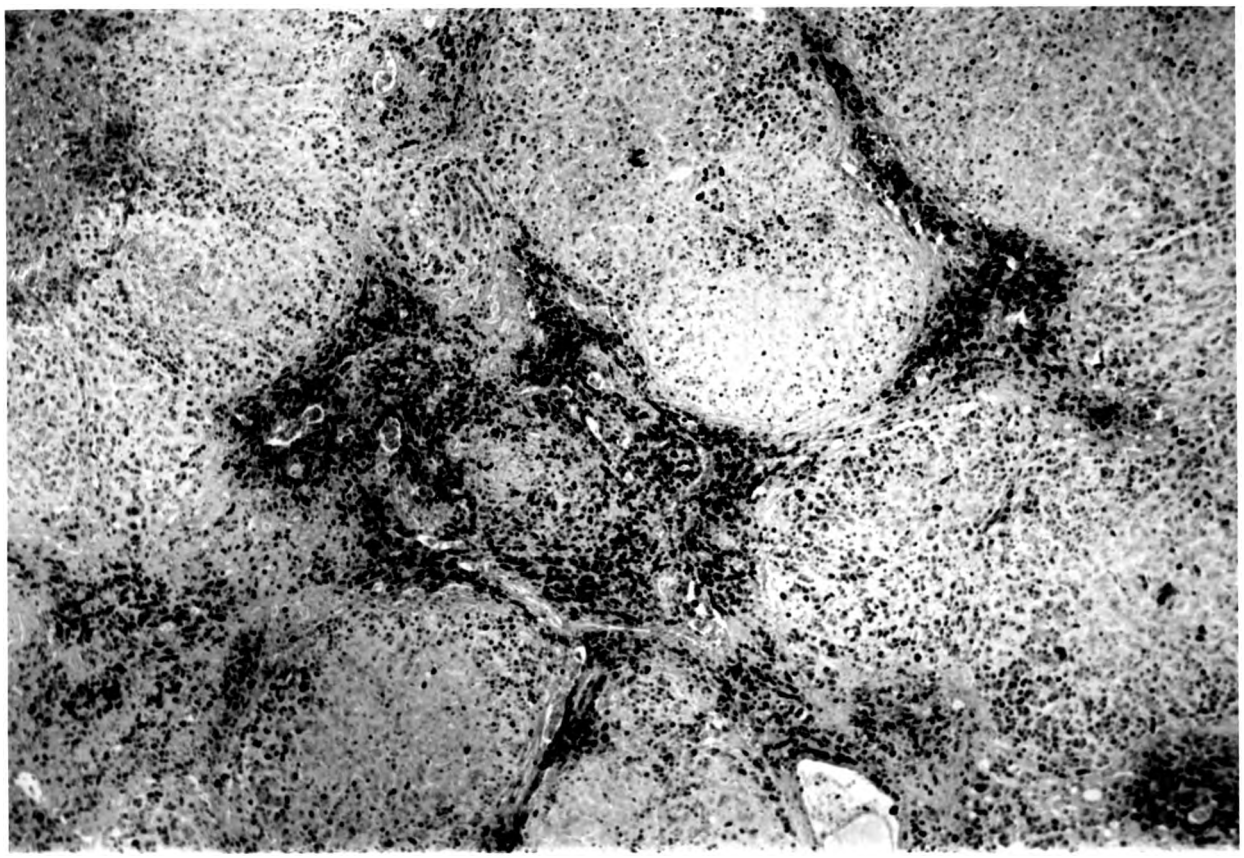


Figure 2: Section of liver from a Bantu patient with hemochromatosis, showing massive accumulation of hemosiderin granules in the portal tracts and fibrous tissue, and moderate amounts in parenchymal cells. Perl's stain x 30.

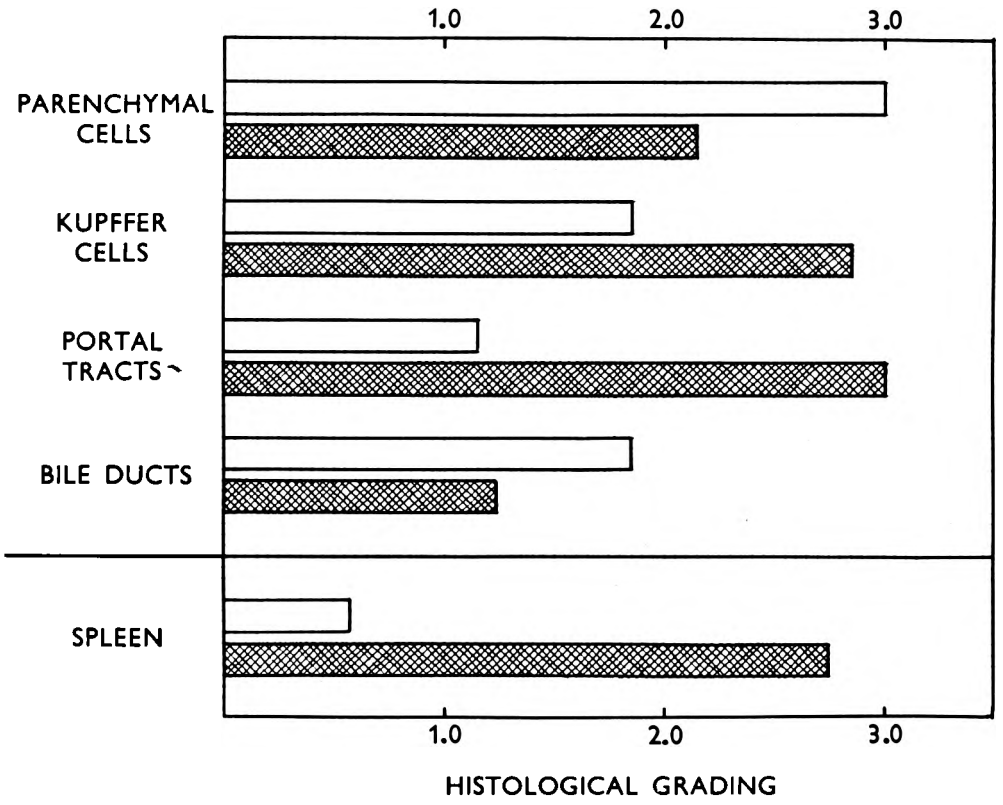


Figure 3: A comparison between the mean histological ratings of the iron deposits in the different parts of the livers and in the spleens of subjects with idiopathic hemochromatosis (white columns) and Bantu hemochromatosis (hatched columns).

	SEX	AGE	LIVER				SPLEEN
			PARENCHYMAL CELLS	KUPFFER CELL	PORTAL TRACT PHAGOCYTES	BILE DUCTS	
IDIOPATHIC HEMOCHROMATOSIS	M	19	+++	+++(++)	+	+++(++)	+
	M	24	+++	++	+	+	0
	M	55	+++	+(++)	+	++	+
	M	25	+++	+++(++)	+	+	+(++)
	M	43	+++	++(+++)	+(++)	+++	-
	M	52	+++	+	+(++)	+++	0
	F	28	+++	++	+	++(+)	+
	M	45	+++	++	+	++	-
	M	20	+++	+	+	+(o)	-
	M	48	+++	++	++	+++(++)	-
	F	28	+++	+	+	+	0
	M	39	+++	++	++(+)	+	-
	M	29	+++	++	+(++)	+(++)	-
BANTU HEMOCHROMATOSIS	M	41	++	+++	+++	+	-
	F	56	+++(++)	+++	+++	+	-
	M	49	+++	+++	+++	+	-
	M	46	++	++(++)	+++	+	-
	M	?	++	+(+++)	+++	+	+++
	M	28	+	+++	+++	+++	++
	M	65	+++	+++	+++	+	+++
	M	42	+	+++	+++	+(o)	+++
	M	?	++	+++	+++	+	-
	M	45	++	+++	+++	+	-
	M	45	+(++)	+++	+++	+	-
	M	30	+++	+++	+++	++	-
	F	50	+++(++)	+++	+++	+	-

TABLE

IRON DISTRIBUTION IN LIVERS AND SPLEENS OF 13 WHITE PATIENTS WITH IDIOPATHIC HEMOCHROMATOSIS AND 13 BANTU SUBJECTS WITH HEMOCHROMATOSIS

All sections were assessed independently by two observers. In those instances where the gradings differed both were included, the second observer's grading being given between brackets.

/DH.

REPRINT FROM

IRON METABOLISM

AN INTERNATIONAL SYMPOSIUM

CHAIRMEN

J.-C. DREYFUS AND G. SCHAPIRA · PARIS

EDITED BY

F. GROSS · BASLE

WITH THE ASSISTANCE OF S. R. NAEGELI AND H. D. PHILPS

SPRINGER-VERLAG / BERLIN · GÖTTINGEN · HEIDELBERG 1964

PRINTED IN GERMANY

IRON OVERLOAD IN THE BANTU

By

T. H. BOTHWELL

Iron overload in the Bantu

By

T. H. Bothwell

Over 30 years have passed since it was first noted that the majority of adult Bantu in South Africa have varying degrees of tissue siderosis (STRACHAN, 1929). These findings have been confirmed in a number of subsequent studies (GILLMAN and GILLMAN, 1951; BOTHWELL and FINCH, 1962), and there is evidence to indicate that iron overload is not only common in South Africa but also occurs in the Bantu of Rhodesia, Bechuanaland, Nyasaland, Moçambique (HIGGINSON et al., 1953), and Ghana (EDINGTON, 1959). In these various areas it is almost exclusively confined to the

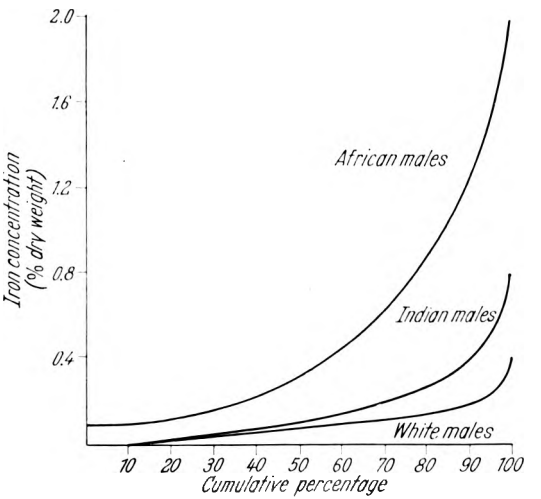


Fig. 1. The cumulative percentage incidence of various iron concentrations in the livers of 240 Bantu males (mean age 34 years), 40 Indian males (mean age 48 years), and 60 withe males (mean age 40 years). All subjects died acute traumatic deaths

Bantu; it is not found in white subjects and is rare in Indians (Fig. 1). Although the incidence and severity of the siderosis is variable, the usual pattern is a remarkably constant one with most of the excess iron confined to the liver and reticulo-endothelial system (HIGGINSON et al., 1953).

The condition first becomes manifest in late adolescence and reaches its greatest degree between the ages of 40 and 60 years (WAINWRIGHT, 1957; BOTHWELL and ISAACSON, 1962). It occurs in both sexes, but the most severe involvement is usually found in males. The extent of the problem in the Johannesburg area was recently defined by estimating the chemical concentrations of iron in the livers of several hundred Bantu adults dying in hospital

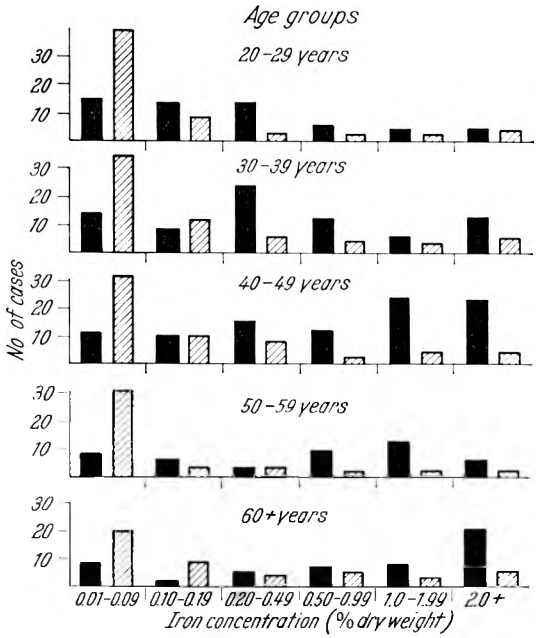


Fig. 2. Hepatic iron concentrations in Bantu males (black columns) and females (cross-hatched columns) at various ages. (From BOTHWELL and ISAACSON, 1962. Reprinted by courtesy of the Editor, Brit. Med. J.)

(BOTHWELL and ISAACSON, 1962). Of the males, only 30% had iron concentrations within the normal range, while 37% had severe siderosis with iron concentrations in the range of those found in idiopathic haemochromatosis. In the females, on the other hand, results were far less striking: 75% had hepatic iron concentrations within or just above the normal range, while only 12% showed severe involvement (Fig. 2).

Although it is accepted that the iron overload must be the result of increased absorption from the gut, there has been controversy in the past as to why this should occur. Originally it was

postulated that there was an alteration in the behaviour of the bowel mucosa induced by chronic malnutrition (GILLMAN and GILLMAN, 1951). More recently, however, it has been shown that the Bantu diet contains large amounts of iron (WALKER and ARVIDSSON, 1953), and there is evidence to indicate that this is a more likely cause of the tissue siderosis. Most of the iron is derived from the utensils used in cooking and in the preparation of fermented alcoholic beverages (WALKER and ARVIDSSON, 1953). Although it is difficult to estimate the actual quantities of iron being ingested, analyses of various alcoholic drinks have shown a mean iron concentration of about 4.0 mg./100 ml. (range: 0.5 to 15). Calculations from these figures suggest that the average Bantu male consumes between 50 and 100 mg. iron daily in beer alone (BOTHWELL et al., 1963). The high daily iron intake of the Bantu has also been shown by measuring the iron concentrations present in faeces. When this has been done, the values have been found to be several times greater than in a comparable white population. In one study, a mean figure of 144 mg./100 g. was found in faeces collected from males on Monday mornings as compared with a figure of 34 mg./100 g. on Thursday mornings. These variations almost certainly reflect the week-end drinking habits of urban Bantu. Over 80% of the iron present in drinks is in the ionisable form, and isotopic studies have demonstrated that it is absorbed to the same degree as simple ferric salts, and about one-third as well as ferrous salts (BOTHWELL et al., 1963) (Fig. 3). It is of interest that most Bantu have been found to have lesser percentage absorptions than white subjects. This can be ascribed to the fact that the majority have varying degrees of iron overload, as it has been shown that even a moderate increase in iron stores depresses absorption (PIRZIO-BIROLI and FINCH, 1960). It is, however, apparent that Bantu ingesting 100 mg. daily in alcoholic beverages absorb enough from this source (approximately 2–3 mg. daily) to account for the degrees of siderosis found in middle age. The fact that isotopic



Fig. 3. A comparison in Bantu and white subjects of the absorption of iron in Bantu beer with a similar dose of iron given as ferric chloride or ferrous ascorbate. (Data from BOTHWELL et al., 1963)

studies have not revealed any evidence of abnormally *high* absorption rates can be taken as good evidence that the siderosis so commonly found in the Bantu is not the result of any intrinsic mucosal defect.

In the earliest phases of iron overload, the iron can be detected as haemosiderin granules in the parenchymal cells of the liver, particularly in the periportal areas (BOTHWELL and BRADLOW,

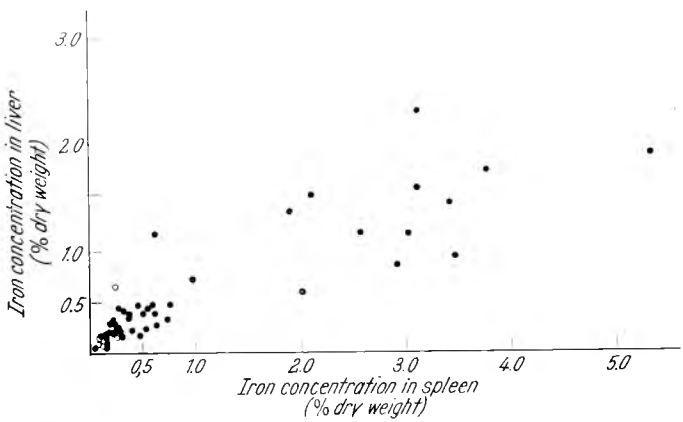


Fig. 4. A comparison of the iron concentrations in the livers and spleens of Bantu adults. The closed circles represent males and the open circles females. (From BOTHWELL and BRADLOW, 1960. Reprinted by courtesy of the Editor, Arch. Path.)

1960). As the condition progresses, the iron granules become dispersed throughout the hepatic lobules, and iron is also visible in the Kupffer cells. When the iron concentration reaches levels between 5 and 10 times normal, increasing amounts of haemosiderin are present in parenchymal and Kupffer cells, and at this stage the portal tracts also show involvement. With concentrations over 20 times normal, heavy deposits are usually present in all three sites. Although it is evident that Bantu siderosis initially manifests itself in the liver as parenchymal deposits, it is interesting to note that the splenic concentration rises at the same time as the liver concentration, and that with increasing levels of iron the splenic concentrations are often greater than those in the liver (Fig. 4). Deposits in the reticulum cells of the bone marrow are also a feature, and the results of a recent *in vivo* study using an isotopic dilution technique indicate that as much as 10 g. may be stored in this site (GALE et al., 1963). Epithelial involvement of significant degree is uncommon in Bantu siderosis, and the iron is usually

confined to the liver and reticulo-endothelial system, even when marked overload is present (Higginson et al., 1953; Isaacson et al., 1961). The pattern in the majority of subjects is, therefore, different from that in idiopathic haemochromatosis but is very similar to the distribution described in animals placed on deficient diets with heavy iron supplements (Kinney, et al., 1949).

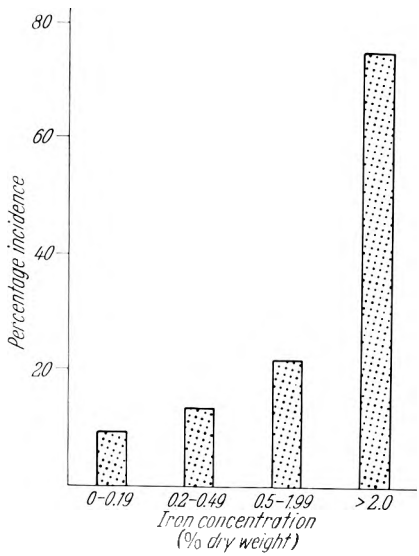


Fig. 5. The combined incidence of moderately severe portal fibrosis and portal cirrhosis in Bantu subjects with different concentrations of iron in the liver. (Data from Bothwell and Bradlow, 1960; and Bothwell and Isaacson, 1962)

In assessing the possible relationship between iron overload in the Bantu and tissue damage, most attention has been paid to the liver, since a major portion of the excess iron is located in this site. The results of several studies have shown a close relationship between the degree of overload and the presence of portal fibrosis or cirrhosis (Higginson et al., 1957; Gillman et al., 1958). For example, moderately severe portal fibrosis or frank cirrhosis has been found in the great majority of livers in which the concentration of iron was more than 20 times normal (Bothwell and Bradlow, 1960; Bothwell and Isaacson, 1962) (Fig. 5). As a corollary to this it has been noted that subjects exhibiting portal cirrhosis at autopsy almost invariably have heavy siderosis (Isaacson et al., 1961) (Fig. 6). This is in marked contrast to the findings in post-necrotic cirrhosis, where it has not been possible to demonstrate any relationship with iron overload (Isaacson et al., 1961).

These various observations all suggest that there is a close relationship between the presence of heavy hepatic deposits of iron and the development of a fibrous reaction in the liver. This is, however, not the only point of interest that has emerged from these studies. Another feature of note in cases of portal cirrhosis has been the finding of significant epithelial deposits in a number of organs (Bradlow et al., 1961; Isaacson et al., 1961). This is especially so in the pancreas, thyroid, adrenals, pituitary, and myocardium. The microscopic findings in such cases are, therefore, similar to

those in idiopathic haemochromatosis, and chemical analyses of various organs have demonstrated iron concentrations in the same

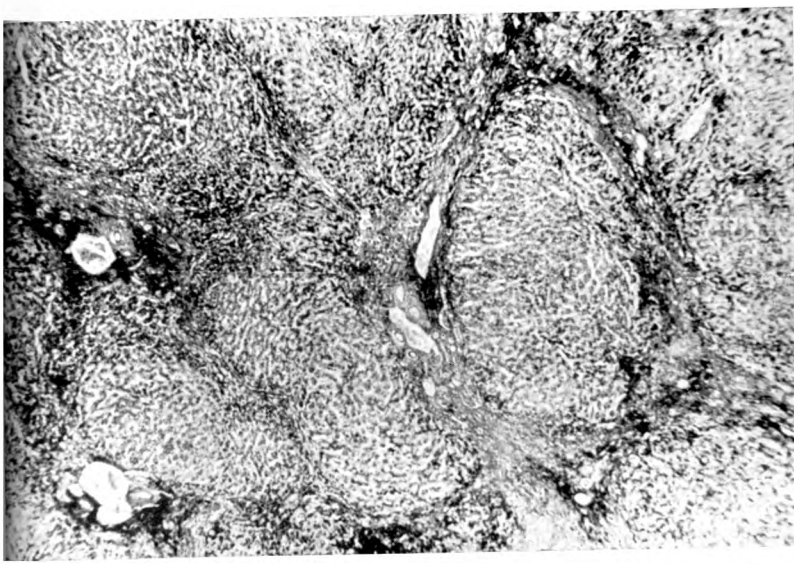


Fig. 6. A section of the liver from a Bantu adult with fully developed haemochromatosis. PERLS' stain. Original magnification 30 (From ISAACSON et al., 1961. Reprinted by courtesy of the Editor, J. Laborat. Clin. Med.)

range as are found in this classic iron-storage disease (Fig. 7). Why the development of cirrhosis should be associated with an

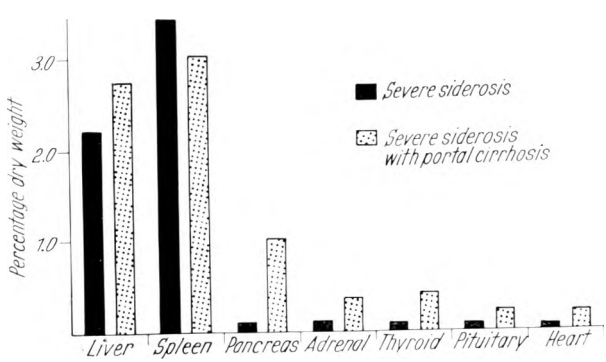


Fig. 7. A comparison of the concentrations of iron in various organs in 20 Bantu subjects with severe siderosis and 20 Bantu subjects with severe siderosis plus portal cirrhosis. (Data from ISAACSON et al., 1961)

iron distribution so different from that found in the majority of Bantu is not clear. It may, however, be related to the percentage saturation of circulating transferrin. In Bantu with siderosis but no cirrhosis, the plasma iron level is higher than normal, but there is, in addition, an increased amount of circulating transferrin with the result that the percentage saturation is normal or only moderately raised (HATHORN et al., 1960). On the other hand, when cirrhosis is present, transferrin levels tend to be lower, and the percentage saturation is very high (SEFTEL et al., 1961). It has been shown experimentally that iron is taken up more readily by tissues under such circumstances (JANDL et al., 1959), and it is therefore possible that this mechanism may be of importance in the transfer of iron to epithelial cells in cirrhotic subjects.

Whatever the explanation for these findings, there is no doubt that a small proportion of adult Bantu exhibit pathological findings indistinguishable from those in idiopathic haemochromatosis. The similarity between the two conditions is further underlined by the clinical manifestations (SEFTEL et al., 1960). Analyses of clinical records have shown that more than 20% of Bantu with pathological evidence of haemochromatosis at autopsy were diabetic prior to death (ISAACSON et al., 1961), while a recent survey of living diabetics has revealed that approximately 7% were suffering from fully developed haemochromatosis (SEFTEL et al., 1961). The clinical picture in such subjects is a fairly characteristic one. The condition is commoner in males (male to female ratio 2 : 1), and the majority of subjects are between the ages of 40 and 60 years. All give a history of excessive consumption of alcoholic beverages. They are almost invariably underweight, a firm hepatomegaly is always present, and insulin is needed for control of the diabetes. These features serve to distinguish the condition from the common form of late-onset diabetes found in the Bantu, since this usually occurs in middle-aged, obese females and can often be controlled by diet or by oral hypoglycaemic agents (SEFTEL et al., 1961). The prognosis in Bantu subjects with haemochromatosis is poor, and death usually results from liver failure and/or portal hypertension.

Although these various findings support the concept that long-term oral iron overload in the Bantu can cause severe damage to body tissues, it is important to stress that there are still certain puzzling discrepancies. While it is clear that the majority of subjects with marked hepatic siderosis develop portal fibrosis or cirrhosis, an appreciable percentage do not do so. For example, in one study 30% of subjects over the age of 50 years with hepatic iron concentrations above 2.0% dry weight showed no significant portal fibrosis (BOTH-

WELL and ISAACSON, 1962). A finding such as this raises the possibility that iron is only a low-grade fibrogenic agent and that other potentiating factors are often present. It is perhaps noteworthy that the major source of iron in the Bantu is an alcoholic drink which is adulterated in a number of ways. There is, therefore, the added insult of alcohol on the liver together with the possible harmful effects of the unidentified adulterants and the associated malnutrition. A concept such as this would fit in with the experimental observation that the siderotic liver is particularly vulnerable to toxic, metabolic, and nutritional hazards (GOLBERG and SMITH, 1960).

The preceding discussion has been primarily concerned with the pattern of iron overload in Bantu adults and with the possible effects exerted locally on tissues by the iron deposits when they have been present in high concentrations for long periods. While most attention has been directed to this aspect, there are certain interesting associations between iron overload in the Bantu and other diseases. These include porphyria cutanea tarda, scurvy, and vertebral collapse. Although the incidence of porphyria cutanea tarda is higher in subjects with siderosis (LAMONT and HATHORN, 1960), there is no evidence that its occurrence is directly related to the iron deposits *per se,* and it seems more reasonable to suppose that the major link is the high intake of adulterated alcoholic drinks. The relationship with scurvy is a more direct one. Severe siderosis is almost invariably present in scorbutic Bantu (BOTHWELL et al., 1963), and the results of one recent study suggest that it is extremely difficult to saturate such subjects with ascorbic acid even after adequate therapy (SCHULZ and SWANEPOEL, 1962). These observations have led to the intriguing suggestion that persons living on a borderline diet may be precipitated into clinical scurvy as a result of irreversible oxidation of available ascorbic acid by the excessive deposits of ferric iron (SCHULZ and SWANEPOEL, 1962). The last association is perhaps the most interesting one. It has been recognised for several years that collapse of lumbar vertebrae secondary to gross demineralisation of bone occurs commonly in middle-aged Bantu males (GRUSIN and SAMUEL, 1957) (Fig. 8). Current studies indicate that all such subjects have severe siderosis and give a history of excessive consumption of brewed alcoholic beverages (SEFTEL and BOTHWELL, 1963). Although the cause of the vertebral collapse has not yet been fully elucidated, it is noteworthy that the calcium content of the drinks is low, while the iron content is high. There is some experimental evidence to show that iron and calcium compete for active transport in the

duodenum (Manis and Schachter, 1962), and it therefore seems possible that the presence of excessive iron in a diet which contains suboptimal amounts of calcium may be of critical importance in promoting a negative calcium balance. Whatever the significance of the association between iron overload and vertebral deformities,

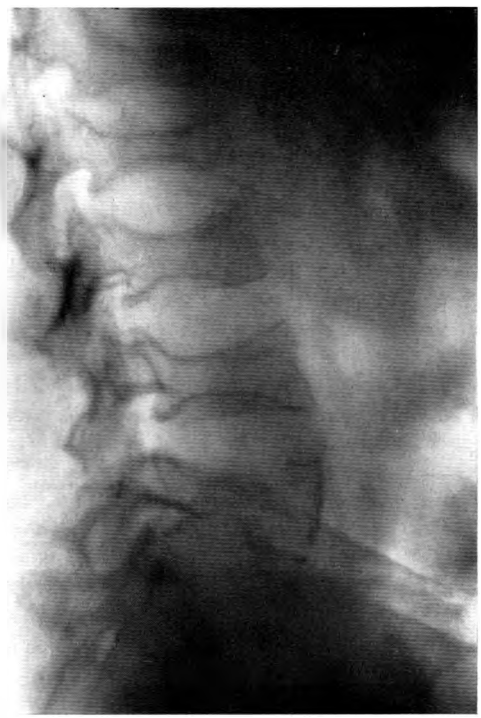


Fig. 8. An X-ray plate of the lumbar spine of a Bantu male with severe siderosis, showing a marked diminution in bone density and vertebral collapse

it is of interest to note that the present observations are not new. A syndrome with similar features was described almost a century ago in Manchurian subjects (Kaschin, as cited by Hiyeda, 1939). These people seem to derive the excess iron from well-water and at autopsy exhibit pathological findings similar to those in the Bantu (Hiyeda, 1939).

Current evidence, derived from a number of different studies over the years, indicates that a small proportion of Bantu adults suffer both directly and indirectly from the effects of long-term oral

iron overload. At the same time it must be appreciated that the majority exhibit only mild to moderate degrees of siderosis, and there is no evidence that these deposits exert deleterious effects. In fact, under special circumstances they may well be beneficial. This is especially so in pregnancy. The mean hae noglobin level at term in untreated Bantu females has been found to be 13 g./100 ml. (GERRITSEN and WALKER, 1954; EDELSTEIN et al., 1963), which must be quite unique for a population subsisting on a diet that is in many ways suboptimal.

Summary

The Bantu of Southern Africa consume a diet with a high iron content, and the majority of adults at autopsy show varying degrees of tissue siderosis. Isotopic studies suggest that the iron overload is directly related to the high intake, and there is no evidence to indicate any abnormality in gut behaviour.

In most Bantu the iron deposits are confined to the liver and reticulo-endothelial system. The distribution is, therefore, different from that found in idiopathic haemochromatosis. In the liver there is a fairly close correlation between the degree of siderosis and the presence of significant portal fibrosis or portal cirrhosis. This is especially so when the iron concentration is above 2.0% dry weight. When portal cirrhosis is present, the distribution of iron is different from that normally found, since epithelial deposits in a number of organs are invariably present. In this small group of cases, the histological findings and chemical concentrations of iron in organs such as the liver, pancreas, adrenals, thyroid, pituitary, and heart are very similar to those found in idiopathic haemochromatosis. In addition, clinical manifestations are also similar, and insulin-dependent diabetes is often present.

Although the findings in severe Bantu siderosis support the concept that long-term oral iron overload is noxious to tissues, there are enough exceptions to suggest that added factors, such as alcoholism and malnutrition, may be important aggravating factors.

Zusammenfassung

Die Nahrung der südafrikanischen Bantus ist sehr reich an Eisen, und die Mehrzahl der zur Sektion gelangenden Erwachsenen zeigt verschiedene Grade von Gewebssiderose. Untersuchungen mit radioaktivem Eisen sprechen dafür, daß die Überladung des Organismus mit Eisen in Beziehung zur hohen Einnahme steht und nicht auf einer abnormen enteralen Resorption beruht.

In den meisten Fällen beschränken sich die Eisendepots auf die Leber und auf das retikulo-endotheliale System. Die Verteilung unterscheidet sich somit von der bei der idiopathischen Haemochromatose gefundenen. In der Leber besteht eine recht enge Korrelation zwischen dem Grad der Siderose und dem Vorliegen einer deutlichen portalen Fibrose oder Zirrhose. Dies ist besonders der Fall, wenn der Eisengehalt der Nahrung über 2% ihres Trockengewichtes liegt. Ist eine portale Zirrhose vorhanden, so unterscheidet sich die Verteilung von Eisen von der normalerweise gefundenen dadurch, daß regelmäßig Ablagerungen in den Epithelien verschiedener Organe auftreten. Bei dieser kleinen Gruppe von Patienten sind die histologischen und biochemischen Befunde in verschiedenen Organen — Leber, Pankreas, Neben-

nieren, Schilddrüse, Hypophyse und Herz — sehr ähnlich wie bei der idiopathischen Haemochromatose. Auch die klinischen Erscheinungen sind vergleichbar, und oft ist ein Insulin-empfindlicher Diabetes vorhanden.

Obgleich die Beobachtungen bei den Bantus mit schwerer Siderose dafür sprechen, daß die langdauernde Zufuhr großer Mengen von Eisen in der Nahrung die Gewebe schädigt, gibt es genügend Ausnahmen, die darauf hinweisen, daß zusätzliche schädigende Faktoren wie Alkoholismus oder Mangelernährung ebenfalls von Bedeutung sind.

Résumé

Le régime des Bantous d'Afrique du Sud a une haute teneur en fer, et la majorité des adultes présente à l'autopsie une sidérose plus ou moins marquée des tissus. Des études radio-isotopiques suggèrent que la surcharge en fer est en relation directe avec l'apport élevé, et il n'y a aucune raison d'invoquer une anomalie dans le comportement de l'intestin.

Chez la plupart des Bantous les dépôts de fer sont localisés au foie et au système réticulo-endothélial. Leur distribution est donc différente de celle trouvée dans l'hémochromatose idiopathique. Dans le foie, il existe une relation assez étroite entre le degré de la sidérose et la présence d'une fibrose, ou d'une cirrhose portale importante. C'est en particulier le cas quand la concentration du fer dépasse 2,0% du poids sec. Quand il existe une cirrhose portale, la distribution du fer est différente de celle trouvée normalement, puisque des dépôts épithéliaux sont invariablement retrouvés dans un certain nombre d'organes. Dans ce petit groupe de cas, les constatations histologiques et les concentrations chimiques du fer dans des organes tels que le foie, le pancréas, les glandes surrénales, la thyroïde, l'hypophyse et le coeur sont très semblables à celles trouvées dans l'hémochromatose idiopathique. De plus, les manifestations cliniques sont également semblables, et un diabète insulino-sensible est souvent présent.

Bien que ces constatations dans la sidérose grave du Bantou soient en faveur de la conception selon laquelle la surcharge perorale de fer au long cours est nocive pour les tissus, il existe des exceptions, assez nombreuses pour que l'on soit amené à penser que des facteurs surajoutés, comme l'alcoolisme et la malnutrition, peuvent jouer un rôle aggravant important.

Acknowledgement

This work was supported by grants from the National Institutes of Health, U.S.A. (AM-04912-02) and from the Council for Scientific and Industrial Research, South Africa.

References

Bothwell, T. H., and B. A. Bradlow: Arch. Path. (U.S.A.) **70**, 279 (1960). — Bothwell, T. H., and C. A. Finch: Iron metabolism. Little, Brown & Co., Boston, 1962. — Bothwell, T. H., and C. Isaacson: Brit. Med. J. **1962**/I, 522. — Bothwell, T. H., H. Seftel, P. Jacobs, J. D. Torrance, and N. Baumslag: Amer. J. Clin. Nutr. (in press, 1963a). — Bothwell, T. H., B. A. Bradlow, P. Jacobs, K. J. Keeley, S. Kramer, H. Seftel, and S. Zail: Brit. J. Haemat. (in press, 1963b). — Bradlow, B. A., J. Dunn, and J. Higginson: Amer. J. Path. **39**, 221 (1961). — Edelstein, T., J. Metz, and T. H. Bothwell: Unpublished data (1963). — Edington, G. M.:

Centr. Afr. J. Med. **5**, 186 (1959). — GALE, E., J. TORRANCE, and T. H. BOTHWELL: J. Clin. Invest. (U.S.A.) (in press, 1963). — GERRITSEN, T., and A. R. P. WALKER: J. Clin. Invest. (U.S.A.) **33**, 23 (1954). — GILLMAN, J., and T. GILLMAN: Perspectives in Human Malnutrition. Grune & Stratton, New York, 1951. — GILLMAN, T., M. HATHORN, and N. M. LAMONT: S. Afr. J. Med. Sc. **23**, 187 (1958). — GOLBERG, L., and J. P. SMITH: Amer. J. Path. **36**, 125 (1960). — GRUSIN, H., and E. SAMUEL: Amer. J. Clin. Nutrit. **5**, 644 (1957). — HATHORN, M., T. GILLMAN, P. A. S. CANHAM, and N. M. LAMONT: Clin. Sc. (G.B.) **19**, 35 (1960). — HIGGINSON, J., T. GERRITSEN, and A. R. P. WALKER: Amer. J. Path. **29**, 779 (1953). — HIGGINSON, J., B. G. GROBBELAAR, and A. R. P. WALKER: Amer. J. Path. **33**, 29 (1957). — HIYEDA, K.: Jap. J. Med. Sc.**4**, 91 (1939). — ISAACSON, C., H. SEFTEL, K. J. KEELEY, and T. H. BOTHWELL: J. Laborat. Clin. Med. (U.S.A.) **58**, 845 (1961). — JANDL, J. H., J. K. INMAN, R. L. SIMMONS, and D. W. ALLEN: J. Clin. Invest. (U.S.A.) **38**, 161 (1959). — KINNEY, T. D., D. M. HEGSTED, and C. A. FINCH: J. Exper. Med. (U.S.A.) **90**, 137 (1949). — LAMONT, N. M., and M. HATHORN: S. Afr. Med. J. **34**, 279 (1960). — MANIS, J. G., and D. SCHACHTER: Amer. J. Physiol. **203**, 73 (1962). — PIRZIO-BIROLI, G., and C. A. FINCH: J. Laborat. Clin. Med. (U.S.A.) **55**, 216 (1960). — SCHULZ, E. J., and H. SWANEPOEL: S. Afr. Med. J. **36**, 367 (1962). — SEFTEL, H., and T. H. BOTHWELL: Unpublished data (1963). — SEFTEL, H., C. ISAACSON, and T. H. BOTHWELL: S. Afr. J. Med. Sc. **25**, 89 (1960). — SEFTEL, H., C. ISAACSON, K. J. KEELEY, and T. H. BOTHWELL: J. Laborat. Clin. Med. (U.S.A.) **58**, 837 (1961). — STRACHAN, A. S.: M. D. Thesis, Glasgow, 1929. — WAINWRIGHT, J.: S. Afr. J. Laborat. Clin. Med. **3**, 1 (1957). — WALKER, A. R. P., and U. B. ARVIDSSON: Transact. Roy. Soc. Trop. Med. (G.B.) **47**, 536 (1953).

Druck: Brühlsche Universitätsdruckerei Gießen



SPRINGER-VERLAG
Berlin · Göttingen · Heidelberg

Diurese und Diuretica
Diuresis and Diuretics

Ein Internationales Symposion
An International Symposium
Herrenchiemsee, 17.—20. Juni 1959
Veranstaltet mit Unterstützung der CIBA
Leitung: H. SCHWIEGK, München
Herausgegeben von E. BUCHBORN, München,
und K. D. BOCK, Basel
Mit 88 Abbildungen
XII, 382 Seiten (davon 92 Seiten in englischer Sprache) 8°. 1959
Ganzleinen DM 25,50

Essential Hypertension

An International Symposium
Berne, June 7th—10th, 1960
Sponsored by CIBA
Chairman: F. C. REUBI, Berne
Edited by K. D. BOCK, Basle, and P. T. COTTIER, Berne
In English. With 81 figures
VIII, 392 pages 8vo. 1960
Cloth DM 33,80

Essentielle Hypertonie

Ein Internationales Symposion
(deutsche Ausgabe)
Bern, 7.—10. Juni 1960
Veranstaltet mit Unterstützung der CIBA
Leitung: F. REUBI, Bern
Herausgegeben von
K. D. BOCK, Basel,
und P. COTTIER, Bern
Mit 81 Abbildungen
VIII, 421 Seiten 8°. 1960
Ganzleinen DM 33,80

Bitte Prospekte anfordern!

Shock

Pathogenesis and Therapy
An International Symposium
Stockholm, June 27th—30th, 1961
Sponsored by CIBA
Chairman: U. S. VON EULER, Stockholm
Edited by K. D. BOCK, Basle
In English. With 120 figures
VIII, 387 pages 8vo. 1962
Cloth DM 37,50
Die Verbreitungsrechte dieses Werkes für USA und Canada liegen bei Academic Press Inc., Publishers, New York

Schock

Pathogenese und Therapie
Ein Internationales Symposion
(deutsche Ausgabe)
Stockholm, 27.—30. Juni 1961
Veranstaltet mit Unterstützung der CIBA
Leitung: U. S. VON EULER, Stockholm
Herausgegeben von K. D. BOCK, Basel
Mit 120 Abbildungen
VIII, 434 Seiten 8°. 1962
Ganzleinen DM 37,50

Portein Metabolism

Influence of Growth Hormone Anabolic Steroids, and Nutrition in Health and Disease
An International Symposium
Leyden, 25th—29th June, 1962
Sponsored by CIBA
Chairman: A. QUERIDO, Leyden
Edited by F. GROSS, Basle
In English. With 159 figures
XII, 521 pages 8vo. 1962
Cloth DM 37,50

PROCEEDINGS OF Xth CONGRESS OF THE INTERNATIONAL SOCIETY OF HAEMATOLOGY (1964) - IN PRESS.

PATHOPHYSIOLOGICAL AND CLINICAL ASPECTS OF IRON OVERLOAD

by

T.H. BOTHWELL, M.D., M.R.C.P.*

C.S.I.R. Iron and Red Cell Metabolism Unit,
Department of Medicine,
University of the Witwatersrand,
Johannesburg, South Africa.

Running Title "IRON OVERLOAD".

*The author is indebted to the S.L. Sive Travelling Fellowship for financial assistance.

Iron exchange between man and his environment is normally very small in proportion to the total body iron content of between 3 and 4 grams. In the adult male the total loss from exfoliated cells and secretions is between a half and one milligram daily. These losses are matched by the absorption of iron from food, so that the total iron content of the body tends to remain fixed within relatively narrow limits. This delicate balance appears largely to depend on the behaviour of the absorbing cells of the upper gastrointestinal tract which can modify their behaviour according to the needs of the individual. Thus the absorption rate tends to rise when the body is depleted of iron and tends to fall when body stores are larger than normal. There are, however, certain situations in which normal iron balance becomes disturbed and an absolute increase in the total quantity in the body occurs.

Iron overload may arise in one of three ways. It is either due to excessive absorption from the gut or to the introduction of iron via the parenteral route or to a combination of both factors. The extra iron is quantitatively deposited in various tissues as the storage compounds ferritin and haemosiderin, and if the positive balance occurs for long enough the total body iron content may eventually reach figures of more than 10 times normal.

- In -

In assessing the significance of the various forms of iron overload it is first necessary to consider the part of entry of the iron since there is some evidence that this may influence not only its initial localisation but also its ultimate effects (Bothwell and Finch, 1962). Iron absorbed from the gut is delivered via the plasma of the portal system to the parenchymal cells of the liver whereas iron released from the breakdown of donor red cells or from injected colloidal complexes is released within reticuloendothelial cells. While it is clear that there is a subsequent redistribution of iron from its initial sites of deposition the association of oral overload with parenchymal deposits and parenteral overload with reticuloendothelial deposits represents a helpful dividing line between the two conditions.

Oral Iron Overload.

In any discussion of oral overload it is customary to consider first the classical iron storage disease, idiopathic haemochromatosis, since the wellknown pathological and clinical manifestations of the condition have been recognised and studied for many years. In the past, the condition has been regarded as a lifetime metabolic disorder associated with excessive absorption of iron from the gut (Finch and Finch, 1955). The extra iron is distributed in many tissues of the body and the clinical manifestations

such as portal cirrhosis, diabetes and cardiac failure have been regarded as being directly attributable to the heavy deposits of iron present in the liver, pancreas and heart. There are, however, certain disadvantages to considering idiopathic haemochromatosis as a prototype of the long range effects of increased absorption of iron from the gut since virtually nothing is known of the metabolic and pathologic findings in that long latent period, possibly stretching over half a lifetime, during which iron is accumulating in the body. It is because of these gaps in current knowledge that it is probably more profitable to consider first the siderosis which occurs so commonly in the adult Bantu of Southern Africa since it has been possible to study this condition at all stages of its development.

Iron Overload in the Bantu: The etiology of the condition seems moderately clearcut. It occurs as a result of long continued exposure to a diet containing too much iron. This iron is derived from cooking pots and, more important, from the drums used in the preparation of fermented alcoholic drinks (Walker and Arvidsson 1953; Bothwell et al, 1964). This means that those individuals with the most severe grades of siderosis tend also to be those with the largest alcohol intakes. Although it is difficult to calculate accurately the rate of which overload develops in these subjects, calculations based on the chemical

concentrations of iron present in the various tissues of different age groups suggest that the positive daily balance is not often more than 2 to 3 milligrams. At the same time it should be emphasised that such a modest daily increment is enough to explain deposits in late middle age as great as those found in idiopathic haemochromatosis.

From a pathological point of view the findings in Bantu are very constant (Bothwell and Bradlow 1960). In the earliest phases of iron overload haemosiderin granules can be detected in the parenchymal cells of the liver and in Kupffer cells. With concentrations between 5 and 10 times normal increasing amounts of haemosiderin are present in these sites and at this stage the portal tracts also show involvement. With concentrations over 20 times normal heavy deposits are present throughout the liver. Splenic deposits are also present from an early stage, and with increasing levels of iron the splenic concentrations are often higher than in the liver (Higginson et al. 1953; Bothwell and Bradlow 1960). Deposits in the reticulum cells of the bone marrow are also a feature and results of a recent in vivo isotopic study indicate that as much as 10 grams of iron may be stored in this site (Cole et al. 1963). Iron deposits elsewhere are not a feature, and the majority of subjects thus show a characteristic pattern, with the major impact on the parenchymal cells of the liver and on

the reticuloendothelial system. This parenchymal involvement of the liver is presumably due to the direct deposition of newly absorbed iron transported by the portal system from the gut. To explain the reticuloendothelial deposits is, however, more difficult. There seem to be two major possibilities. In the first, iron is delivered directly from plasma to reticuloendothelial cells. However, there is experimental evidence to show that these cells have only a very limited capacity to remove iron from plasma (Zail et al. 1964). The second possibility seems the more likely one. With increased amounts of iron entering the plasma from the gut and being taken up by red cell precursors in the bone marrow there is a slight decrease in the quantities of iron released each day by reticuloendothelial cells. In this way a steady build-up of iron derived from broken down red cells would be anticipated in the reticuloendothelial system.

In assessing the relationship between iron overload in the Bantu and tissue damage it is most helpful to consider the liver findings since this organ contains the largest deposits of iron. Several studies have shown a close relationship between the degree of overload and the presence of portal fibrosis or cirrhosis (Higginson et al. 1957; Gillman et al. 1958). For example, moderately severe portal fibrosis or frank cirrhosis has been found to be present in the majority of livers in which the concentration of iron is more than 20 times normal. (Bothwell and Bradlow 1960;

Bothwell and Isaacson 1962). In addition, it has been noted that subjects exhibiting portal cirrhosis at necropsy almost invariably have iron overload (Isaacson et al. 1961). Such patients show another interesting feature, and that is the presence of epithelial deposits in a number of organs. This is especially so in the pancreas but significant quantities are also present in the thyroid, adrenals, pituitary and myocardium. Why the development of cirrhosis should be associated with uptake of iron by epithelial tissues is not known but probably relates to the high percentage saturation of circulating transferrin at this stage of the condition, since there is some experimental evidence that this facilitates the uptake of iron by epithelial tissues. Of clinical interest is the fact that a proportion of such subjects suffer from insulin-dependant diabetes. Analysis of clinical records has shown that approximately 20 per cent of Bantu with pathological evidence of haemochromatosis at necropsy were diabetic prior to death (Isaacson et al. 1961), while a survey of living Bantu diabetics attending a hospital outpatients' department has revealed that approximately 7 per cent were suffering from haemochromatosis (Seftel et al. 1961). These findings are compatible with the thesis that the presence of excessive iron in epithelial tissues is of pathological signifance.

Idiopathic Haemochromatosis: When the various findings

in Bantu subjects with severe iron overload are related to those in idiopathic haemochromatosis there are obvious similarities, the most notable of which is the association of heavy parenchymal deposits in the liver and pancreas with portal cirrhosis and diabetes respectively. In fact, the similarity between the two conditions has raised the question as to whether idiopathic haemochromatosis is a specific metabolic disorder. Instead it has been suggested that it is no more than a variant of a nutritional cirrhosis occurring in subjects exposed to a large intake of iron and alcohol (MacDonald 1964). There are several points in favour of this thesis. In the first place it is known that more than 30% of patients with so-called idiopathic haemochromatosis admit to an increased alcohol intake (Finch and Finch, 1955), and in some series the figure has been much higher (MacDonald and Mallory 1960). Secondly, there is the recent observation that a large number of American and European wines contain significant amounts of iron (MacDonald 1963). On this basis it has been calculated that many alcoholics have an iron intake several times normal. Further evidence indicates that the alcohol itself may have a dual action. Apart from its wellknown relationship to the development of cirrhosis, current studies indicate that it increases the absorption of ferric iron markedly (Charlton et al. 1964). Even more support for the tie-up between increased consumption

of alcohol and iron overload is provided by the recent observation that subjects with chronic liver disease (Callender and Malpas 1963) or chronic pancreatitis (Davis and Badenoch 1962), both of which are common sequelae of alcoholism, absorb iron excessively from the gut.

With all this impressive array of evidence marshalled against it the question arises as to whether idiopathic haemochromatosis survives as a nosological metabolic entity. There are several observations which suggest that it does. For example, there are clear-cut but as yet unexplained differences between the distribution of iron in subjects with idiopathic haemochromatosis and subjects such as the Bantu who are exposed to a diet containing an excess of iron and alcohol. As mentioned previously the Bantu show prominent reticuloendothelial deposits at all stages, and in those with fully developed haemochromatosis splenic deposits are striking (Higginson et al. 1953; Bothwell and Bradlow 1960; Isaacson et al. 1961). Similar findings to those in the Bantu have been reported in animals after long continued exposure to a deficient diet containing an excess of iron (Gillman et al. 1959). In contrast, reticuloendothelial deposits are not a striking feature in idiopathic haemochromatosis and this is reflected by the low concentrations of iron usually

- present -

present in the spleen at necropsy (MacDonald, 1964). A finding such as this raises the question as to whether there may not be some alteration in reticuloendothelial function in idiopathic haemochromatosis. This is, however, only speculative at the present time. Apart from the histological findings there are other features which suggest that idiopathic haemochromatosis is a specific disorder. Firstly, it should be emphasised that a large number of reported patients with the disease have not been alcoholic, and no other source of excessive dietary iron has been uncovered in these cases. Secondly, there are several family studies in which varying degrees of iron overload have been demonstrated in relatives of patients with haemochromatosis (Bothwell et al. 1959; Williams et al. 1962), thus supporting the concept of an inherited metabolic disorder. The fact that only a proportion of siblings living in the same household have shown increased stores can also be taken as evidence against the idea that some environmental factor, such as a high dietary intake, is essential in pathogenesis. Thirdly, it has been possible to show an abnormally high absorption of radioiron in some patients with the fully developed disease (Finch and Finch, 1955). This is especially so in young subjects, and the failure to demonstrate increased absorption in some older patients may well be due to the inhibitory effect exerted by the enormous iron stores. Support for this contention has been provided by the finding

of very high absorption rates in such individuals after reduction of stores by repeated phlebotomy (Pirzio-Biroli et al. 1958; Crosby et al. 1963). This has been noted at a time when the haemoglobin level was normal and the serum iron, normal or even raised.

While there are, therefore, several pointers to the existence of a specific metabolic disorder associated with increased absorption from the gut the nature of the underlying defect remains uncertain. Is there some change within the lumen? The fact that chronic pancreatic disease leads to increased absorption (Davis and Badenoch, 1962) is certainly compatible with this thesis as is the observation that the volume of pancreatic juice after secretin stimulation is abnormally increased in the disorder (Marks and Bank 1963; Perman and Bonera 1964). In addition, there is suggestive evidence that increased iron absorption in affected individuals can be reduced by the administration of pancreatic extract (Bigge and Davis 1963). A second possible site for the defect in idiopathic haemochromatosis is the mucosa of the upper gastrointestinal tract. Recent work from several laboratories has given some insight into the way in which absorption is regulated at the mucosal cell level (Wheby and Crosby 1963; Charlton et al. 1963). There appear to be at least two phases in the absorptive process. Some of the iron taken up moves rapidly across the cell, probably as a small molecular complex (Brown and Rother 1963). However variable

amounts are incorporated into ferritin within the absorbing mucosa and under certain circumstances this iron is not available to the body and is lost when the cells exfoliate. Deviation of iron into ferritin occurs especially when the body is replete. It thus represents a mechanism which serves to protect the body from excess iron. While it is not difficult to visualise how minor derangements in this mechanism could lead to progressive iron overload there is as yet no positive evidence to support this idea. If the defect in idiopathic haemochromatosis does not reside in the lumen of the gut or in mucosal cells could it perhaps lie in an abnormal avidity of other tissues in the body for iron? Since iron in transport is carried on a specific protein, transferrin, studies have been carried out to try to find out whether the protein is abnormal. This has been done using electrophoretic, salting out and immunoprecipitin techniques, but with negative results (Bothwell et al. 1962). Finally, the possibility exists that the liver, which is the chief storage organ for iron, has an abnormal affinity for the metal in the disorder and that the increased absorption from the gut is secondary to this. However, although minor derangements in hepatic iron kinetics have been noted in one study (Pollycove 1964), other evidence indicates that the liver has no abnormal propensity to take up iron in subjects with haemochromatosis after their stores

have been removed (Finch and Finch, 1955).

Iron Overload in Refractory Anaemias.

With all the confusion that surrounds the different forms of oral iron overload it is not surprising that there is some controversy as to the significance of the siderosis which has been noted in a number of refractory anaemias. The condition was first recognised as a consequence of the administration of repeated blood transfusions. Under these circumstances the major impact might be anticipated on the reticuloendothelial system since donor blood is eventually broken down in this site. In fact, an iron distribution of this type is often a feature in subjects with hypoplastic anaemias who have received many blood transfusions (Oliver 1959; Bothwell and Finch 1962). This can largely be ascribed to the fact that iron turnover through the plasma is diminished in such subjects with the result that redistribution of iron from the initial sites of deposition tends to be slow. Analysis of the reported findings in such subjects suggests that the reticuloendothelial system can tolerate large quantities of iron very well, and cirrhosis is extremely rare in patients with hypoplastic anaemias, even after massive transfusion therapy. This finding is also in agreement with experimental data in dogs which were followed for several years after the administration of large amounts of parenteral iron (Brown et al. 1957).

When interest had been aroused in so-called

transfusional siderosis it soon became apparent that a number of subjects with refractory anaemias had considerably more iron in their bodies at necropsy than could be accounted for by donor blood. Subsequent investigations have defined this group more clearly. It includes a large number of chronic anaemias in which the only common denominator is a hyperactive bone marrow (Bomford and Rhoads, 1941; Bothwell, 1953). Since the extra iron can only be derived from the gut it is apparent that iron absorption must be increased in such anaemias. Although this is consistent with the experimental observation that absorption is enhanced when the bone marrow is stimulated acutely (Bothwell et al. 1958), it is noteworthy that high absorption rates are not a feature in all chronic haemolytic states. Increased absorption only seems to occur in those refractory anaemias in which there is a large element of ineffective erythropoietic activity (Kent and Popper 1960). It is thus common in thalassaemia major and other refractory hypochromic anaemias (Erlandson et al. 1962). In subjects with such conditions absorption is not only increased but iron medication is often mistakenly given at one stage or another and this almost certainly contributes further to the iron overload (Wyatt 1956). The eventual distribution of iron in the bodies of such individuals is obviously a complex one since many also require blood transfusion therapy. As in other forms of

oral overload, a large portion of the iron absorbed from the gut is laid down in the parenchymal cells of the liver and in the reticuloendothelial system. Reticuloendothelial deposits are also increased as a result of the breakdown of donor blood in this site. In addition, epithelial deposits in organs such as the pancreas are a feature in the majority of subjects with severe siderosis. The off-loading of iron into such tissues is presumably a reflection of the fact that circulating transferrin is saturated with iron and the plasma iron turnover is increased. Although it is difficult to judge the percentage incidence of secondary organ damage in patients of this type reported in the literature it is noteworthy that cirrhosis appears to have been present in a large proportion (Bothwell and Finch, 1962). Since portal cirrhosis is such an uncommon feature in transfusional siderosis per se it seems reasonable to suppose that the oral route of entry of the iron, and hence its cellular localisation, is of primary importance in determining possible secondary effects (Kent and Popper 1960).

Summary of Relationship between Iron Overload and Tissue Damage.

From the available evidence it would appear that iron overload of severe degree is potentially harmful to tissues. This relationship is, however, only a feature when the iron is present in parenchymal cells. A

distribution of this type implies that the excessive iron has been derived from the plasma, and it is therefore most prominent in all those situations where absorption from the gut is increased. This is best exemplified in the disease idiopathic haemochromatosis in which very characteristic clinical and pathological findings eventually develop. Similar findings are found in a proportion of Bantu who consume a diet with a high iron content and in subjects with refractory anaemias who have hyperactive, but ineffective erythroid marrow activity. In all these conditions uptake of iron by epithelial tissues appears to be facilitated by a high percentage saturation of circulating transferrin.

In spite of these various associations there are still unexplained discrepancies. For example, there is no doubt that a proportion of Bantu with massive hepatic deposits show little or no tissue damage. On available evidence it is therefore necessary to postulate that other factors may influence the rate at which such changes develop. Of such factors, alcoholism with its associated malnutrition, is certainly the most important, but others such as viral hepatitis, may possibly contribute under certain circumstances.

Approach to Therapy: Rational therapy is directed towards the removal of the excessive iron (Finch and Finch, 1955). In idiopathic haemochromatosis this is most effectively achieved by repeated phlebotomy. Such therapy is, however,

not usually feasible in patients with refractory anaemias and alternative methods must therefore be tried. At present the most effective of these is the chelate desferrioxamine (Moeschlin and Schnider, 1964). On such therapy it is usually possible to mobilise between 10 and 20 mg. daily.

To what extent the removal of excess iron alters the prognosis in the various forms of iron overload is not yet clear. While there is a growing body of evidence that phlebotomy regimes ameliorate the course of idiopathic haemochromatosis there is as yet no reported study in which it has been unequivocally shown that the life span of affected individuals is increased (Bothwell and Finch, 1962). In spite of this, current results are promising enough to indicate that affected individuals should be advised strongly to undergo such therapy.

REFERENCES.

1. Biggs, J.C., and Davis, A.E. (1963) "Relationship of diminished pancreatic secretion to haemochromatosis." Lancet, 2 : 814.

2. Bomford, B.F., and Rhoads, C.P. (1941) "Refractory anemia; clinical and pathological aspects." Quart. J. Med. 10 : 175 - 234.

3. Bothwell, T.H. (1953) "The relationship of transfusional haemosiderosis to idiopathic haemochromatosis." South African J. clin. Sc. 4 : 53 - 70.

4. Bothwell, T.H., Pirzio-Biroli, G., and Finch, C.A. (1958) "Iron absorption. I factors influencing absorption." J. Lab. & clin. Med. 51 : 24 - 36.

5. Bothwell, T.H., Cohen, I., Abrahams, O.L., and Perold, S.M. (1959) "A familial study in idiopathic hemochromatosis." Am. J. Med. 27 : 730 - 738.

6. Bothwell, T.H., and Bradlow, B.A. (1960) "Siderosis in the Bantu. A combined histopathological and chemical study." Arch. Path. 70 : 279 - 292.

7. Bothwell, T.H., and Finch, A.C. (1962) Iron Metabolism. Boston. Little, Brown and Company.

8. Bothwell, T.H., Jacobs, P., and Torrance, J.D. (1962) "Studies on the behaviour of transferrin in idiopathic haemochromatosis." S. Afr. J. med. Sci. 27 : 35 - 39.

9. Bothwell, T.H., and Isaacson, C. (1962) "Siderosis in the Bantu. A comparison of incidence in males and females." Brit. M. J. 1 : 522 - 524.

10. Bothwell, T.H., Seftel, H., Jacobs, P., Torrance, J.D., and Baumslag, N. (1964) "Iron overload in Bantu subjects." Amer. J. clin. Nutrit. 14 : 47 - 51.

11. Brown, E.B., Jr., Dubach, R., Smith, D.E., Reynafarje, C., and Moore, C.V. (1957) "Studies in iron transportation and metabolism. X. Longterm iron overload in dogs." J. Lab. & clin. Med. 50 : 862 - 893.

12. Brown, E.B., and Rother, M.L. (1963) "Studies of the mechanism of iron absorption. I. Iron uptake by the normal rat." J. Lab. clin. Med. 62 : 357.

13. Callender, S.T., and Malpas, J.S. (1963) "Absorption of Iron in Cirrhosis of Liver." Brit. Med. J. 2 : 1516 - 1518.

14. Charlton, R.W., Jacobs, P., Torrance, J.D., and Bothwell, T.H. (1963) "The role of ferritin in iron absorption." Lancet, 2 : 762 - 764.

15. Charlton, R.W., Jacobs, P., Seftel, H., and Bothwell, T.H. "The effect of alcohol on iron absorption." Brit. Med. J. In Press.

16. Crosby, W.H., Conrad, M.E., Jr., and Wheby, M.S. (1963) "The rate of iron accumulation in iron storage disease." Blood 22 : 429 - 440.

17. Davis, A.E., and Badenoch, J. (1962) "Iron absorption in pancreatic disease." Lancet, 2 : 6 - 8.

18. Erlandson, M.E., Walden, R., Stein, G., Hilgartner, W., Welman, J., and Smith, C.H. (1962) "Studies on congenital hemolytic syndromes. IV. Gastrointestinal absorption of iron." Blood 19 : 359 - 378.

19. Finch, S.C., and Finch, C.A. (1955) "Idiopathic hemochromatosis, an iron storage disease." Medicine 34 : 381 - 430.

20. Gale, E., Torrance, J., and Bothwell, T. (1963) "The quantitative estimation of total iron stores in human bone marrow." J. clin. Invest. 42 : 1076 - 1082.

21. Gillman, T., Hathorn, M., and Lamont, N.M. (1950) "Liver disease in Durban Africans. Histopathological findings in biopsies." South African J. Med. Sc. 23 : 187 - 200.

22. Gillman, T., Hathorn, M., and Canham, P.A.S. (1959) "Experimental dietary siderosis." Am. J. Path. 35 : 349 - 367.

23. Higginson, J., Gerritsen, T., and Walker, A.R.P. (1953) "Siderosis in the Bantu of Southern Africa." Am. J. Path. 29 : 779 - 815.

24. Higginson, J., Grobbelaar, B.G., and Walker, A.R.P. (1957) "Hepatic fibrosis and cirrhosis in man in relation to malnutrition." Am. J. Path. 33 : 29 - 53.

25. Isaacson, C., Seftel, H., Keeley, K.J., and Bothwell, T.H. (1961) "Siderosis in the Bantu. The relationship between iron overload and cirrhosis." J. Lab. & clin. Med. 58 : 845 - 853.

26. Kent, G., and Popper, H. (1960) "Secondary hemochromatosis: Its association with anemia." Arch. Path. 70 : 623 - 639.

27. MacDonald, R.A., and Mallory, G.K. (1960) "Hemochromatosis and hemosiderosis. A study of 211 autopsied cases." Arch. Int. Med. 105 : 686 - 700.

28. MacDonald, R.A. (1963) "Idiopathic hemochromatosis; Genetic or acquired." A.M.A. Arch. Int. Med. 112 : 184 - 190.

29. MacDonald, R.A. (1964) "Hemochromatosis and Hemosiderosis." Charles C. Thomas. Springfield, Illinois.

30. Marks, N., and Bank, S. (1963) "The aetiology, clinical features and diagnosis of pancreatitis in the South Western Cape." South African J. Med. 37: 1039 - 1053.

31. Moeschlin, S., and Schnider, U. (1964) "Treatment of primary and secondary haemochromatosis and acute iron poisoning with a new, potent iron-eliminating agent (desferrioxamine B)." in Iron Metabolism. Springer-Verlag, Berlin.

32. Oliver, R.A. (1959) "Siderosis following transfusions of blood." J. Path. and Bact. 77 : 171 - 194.

33. Perman, G. and Bonera, E. (1964) "The secretin test in patients with haemochromatosis." Acta Med. Scandinav. 175 : 787 - 790.

34. Pirzio-Biroli, G., Bothwell, T.H.; and Finch, C.A. (1958) "Iron absorption. II. The absorption of radioiron administered with a standard meal in man." J.Lab. and clin. Med. 51 : 37 - 48.

35. Pollycove, M. (1964) "Iron Kinetics." in Iron Metabolism. Springer-Verlag, Berlin.

36. Seftel, H.C., Isaacson, C., and Bothwell, T.H. (1961) "Siderosis in the Bantu. The clinical incidence of hemochromatosis in diabetic subjects." J. Lab. and clin. Med. 58 : 837 - 844.

37. Walker, A.R.P., and Arvidsson, U.B. (1953) "Iron overload in the South African Bantu." Tr. Roy. Soc. Trop. Med. & Hyg. 47 : 536 - 548.

38. Wheby, M.S., and Crosby, W.H. (1963) "The gastrointestinal tract and iron absorption." Blood 22 : 416 - 428.

39. Williams, R., Scheuer, P.J., and Sherlock, S. (1962) "The

- inheritance -

inheritance of idiopathic haemochromatosis; a clinical and liver biopsy study of 16 families." Quart. J. Med. 31 : 249 - 265.

40. Wyatt, J.P. (1956) "Patterns of pathological iron storage; exogenic siderosis in chronic anaemia due to prolonged oral iron medication." Arch. Path. 61 ; 56 - 61.

41. Zail, S.S., Charlton, R.W., Torrance, J.D., and Bothwell, T.H. (1964) "Studies on the formation of ferritin in red cell precursors." J. clin. Invest. 43 ; 670 - 680.