

BODY IRON EXCRETION

Ralph Green

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Ralph Green

Ralph Green
M.B., B.Ch. (Witwatersrand)

ABSTRACT

An attempt was made to document iron losses from the body as a whole, as well as from individual excretory routes using a combination of radioisotopic and chemical techniques. The purpose of this work was to gain a better understanding of external body iron exchange, and to resolve some of the existing controversies regarding the magnitude of daily iron losses. The basis for this controversy is extensively reviewed in the thesis.

In a long-term radioisotopic study using ^{55}Fe , iron excretion was measured in different groups of adult subjects, chosen in order to examine the effects of a number of variables on body iron losses. The calculated mean daily iron losses were: Johannesburg Negroes 2.42 mg (S.D. 1.09); Durban Negroes 2.01 mg (S.D. 0.94); Durban Indians 1.02 mg (S.D. 0.22). In two further groups which were included in a collaborative study undertaken at the same time as the work presented in this thesis, mean losses were: Seattle Whites 0.95 mg (S.D. 0.30); Venezuelan Mestizos 0.90 mg (S.D. 0.31). Higher excretion figures in the Negro subjects were ascribed to their greater than normal iron stores. The finding that Durban Indian subjects, who were working in an extremely hot and humid environment, did not lose greater amounts of iron than did the sedentary group of Seattle Whites who were living in a temperate climate, suggested that excessive sweating does not represent a major mechanism for iron excretion.

In studies on compartmental iron losses, the amounts of iron excreted through the gastrointestinal tract was measured by a radioisotopic technique. The results indicated a mean daily loss of 0.14 mg within desquamated mucosal cells and secretions, and 0.38 mg in blood. Mean biliary iron excretion was found to be 0.24 mg daily when measured chemically, but the calculated figure from radioisotopic data was only 0.04 mg per day. It was concluded that most of the iron in bile is derived from sources other than the plasma. Direct chemical

measurements on urine revealed a mean daily iron content of 0.11 mg, and this quantity did not appear to be influenced by the size of body iron stores. In a radioisotopic study, the amounts of iron taken up from the plasma by eccrine skin was positively correlated with the degree of saturation of transferrin. At low transferrin saturations little or no iron was taken up by the skin; at normal saturations, uptake was between 0.2 and 0.3 mg daily; at high saturations, the figure rose to between 0.6 and 0.7 mg. By estimating the size of the epidermal iron pool chemically, the mean calculated maximum figure for daily skin iron loss was 0.87 mg.

In a final analysis, the mean figures for calculated daily iron losses from individual body compartments were added together and compared with the figures obtained in the long-term total body iron excretion study. Agreement was close in Indian subjects with normal iron stores, but not in Negro subjects. Even when maximum figures for individual compartmental losses were used, the figures were still lower than those obtained for total excretion in Negro subjects. This suggests that iron overloaded subjects may lose iron in ways other than that examined in the present studies, such as the bile, or the shedding of iron-loaded reticuloendothelial cells into the lumen of the gastrointestinal tract.

It is concluded that external iron exchange in man is limited, but not fixed. Evidence was obtained that the state of the body iron stores does, at least in part, regulate the amounts of iron lost from the body. Iron losses may therefore be reduced in iron deficiency, and augmented in iron overload. Excluding menstrual losses, the possible range of iron losses lies between 0.5 and 4.0 mg per day. For a normal adult male to replace obligatory iron losses and remain in a state of iron balance, approximately one milligram of iron must be absorbed from the diet each day.

TO

MY WIFE IRENE,

MY MOTHER AND MY LATE FATHER

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CHAPTER I

INTRODUCTION

I. THE CONCEPT OF IRON BALANCE AND DAILY IRON REQUIREMENTS

Iron is an essential mineral nutrient for man. It forms an integral part of the haemoglobin molecule and other haem proteins, and also acts as a metal prosthetic group for a number of enzymes. Although the amount of iron necessary to fulfil these requirements is small, iron reserves are limited, and the body cannot tolerate much variation in its iron content which normally constitutes less than 0.01 per cent of body mass.

Total body iron varies between two and five grams in normal adults, depending on body size, haemoglobin level and sex. Approximately two-thirds of this iron is contained in the haemoglobin mass, and somewhat less than the remaining one-third is present in the form of storage iron (Drabkin, 1951). This storage component allows for a margin of safety when dietary iron supply is inadequate to meet the normal iron requirements. Storage iron therefore serves as a buffer which can offset a state of negative iron balance for a limited period and which mitigates against the development of iron deficiency.

Both iron deficiency and the converse condition of iron overload which represent the two major disorders of iron metabolism are reflected by an alteration in the amount of storage iron in the body, and result from disturbances in iron balance. The maintenance of normal iron balance is contingent on a nutritional supply of iron that is adequate to replace losses from the body.

II. ASSESSMENT OF THE DAILY IRON REQUIREMENT

Numerous attempts have been made to measure both the absorption and excretion of iron in man in order to arrive at a figure for the daily iron requirement. In 1937, McCance and Widdowson marshalled

compelling evidence on which they based their classical hypothesis on iron balance, that man has a limited capacity to excrete iron, and that variations in absorption are more important in determining iron status. Since then, most efforts have been directed at establishing the extent to which iron can be absorbed, and relatively less attention has been paid to the question of iron excretion. A considerable body of knowledge has accumulated on the absorption of a variety of inorganic iron salts, as well as on the mechanism of iron absorption and the factors which regulate it (Conrad, 1970). By contrast, little is known about the actual amounts of iron absorbed from the diet each day.

(a) Measurement of iron absorption

Accurate quantitation of the absorption of dietary iron in man is fraught with considerable technical and interpretive difficulties. In order to approach the problem of daily iron requirements in this way, it is necessary firstly to know how much food iron is ingested each day, and then to be able to measure how much of this iron is absorbed into the body. This can be achieved in one of two ways; either by chemical analysis or by the use of radioactive isotopes of iron.

(i) Chemical methods

Chemical methods involve the measurement of both the total iron intake and loss in the stool. The quantity absorbed is then calculated as the net difference between these two determinations in what amounts to a chemical balance study. Few carefully controlled balance studies have been carried out (Widdowson and McCance, 1942; Johnston, *et al.*, 1948; Schlaphoff and Johnston, 1949; McMillan and Johnston, 1951; Hussain and Patwardhan, 1959a; Moore, 1965), because the technique is beset by numerous problems. One drawback of the balance technique is that it does not differentiate between unabsorbed dietary iron and endogenous iron

excreted through the bowel lumen. Another is a technical drawback. Because of the ubiquitous occurrence of iron, the problem of chemical contamination makes long-term balance studies by quantitative analysis extremely difficult. This is amplified by the fact that the difference between oral intake and faecal loss may be small, and therefore difficult to measure with precision. Yet another difficulty is that estimates of the dietary iron content derived from food composition tables do not take into account the contribution which may be made from iron-containing cooking utensils (Moore, 1965) and there is evidence that a considerable fraction of the daily iron intake may be provided by this extraneous source with certain types of food and methods of food preparation (Monsen, *et al.*, 1967; de Bruin, *et al.*, 1968).

Finally, little attention has been paid to the appreciable quantities of iron which are present in certain alcoholic beverages such as wines (MacDonald, 1963; Anguissola, 1970) which may constitute a significant item of diet in some individuals.

Because of these formidable problems and pitfalls in assessing iron absorption by the chemical balance technique, this method has generally been abandoned in favour of using radioiron to measure the absorption of dietary iron.

(ii) Radioisotopic methods

In order to quantify total dietary iron absorption using a radioisotopic technique, it is necessary to label naturally-occurring food iron with a radioactive iron isotope. Retention of the radioiron may be measured using ^{59}Fe either directly using a whole-body counter or the unabsorbed tracer passed in the stool may be measured and the absorption calculated indirectly. A number of studies using either technique and a variety of labelled foodstuffs have been reported (Moore and Dubach, 1951; Chodos, *et al.*, 1957; Pirzio-Biroli, *et al.*, 1958; Price, *et al.*, 1962; Hussain, *et al.*, 1965; Moore, 1965; Callender and Warner, 1968; Elwood, *et al.*, 1968;

Layrisse, *et al.*, 1969).

The preparation of either meat or vegetable foodstuffs labelled with radioactive iron is laborious. In general, radioisotopic methods for assessing iron absorption share many, but not all, of the shortcomings of chemical balance techniques. At least two of the problems inherent in chemical techniques are obviated by the use of radioisotopes, namely, the problem of chemical contamination and the difficulty of differentiating unabsorbed iron from iron excreted through the bowel. On the other hand, the radioisotopic approach has additional drawbacks. Firstly, iron absorption can be measured only from those foods which it has been possible to label with radioiron so that such studies are limited to single foodstuffs, or artificially contrived rather than natural diets. Secondly, when the absorption of iron from labelled foodstuffs is measured, no account is taken of the effect on absorption by other foods which may normally be eaten at the same time. There is evidence that the interaction of different foods may be of considerable importance in determining iron absorption (Callender, 1970; Layrisse, *et al.*, 1973).

Recent experiments adding radioiron to food as an extrinsic tag have added a new dimension to measurement of the absorption of both haem and non-haem iron present in the diet (Cook, *et al.*, 1972; Layrisse and Martinez-Torres, 1972; Layrisse, *et al.*, 1973). These studies have demonstrated that radioiron added either in the form of labelled haem or in the form of inorganic salts are absorbed to the same extent as dietary haem iron and non-haem iron respectively. The absorption of both forms of iron may be measured simultaneously by the use of two different radioiron isotopes. It should be pointed out that even when a radioisotope technique is used to measure food iron absorption, it is essential to have accurate information on the total iron content of the diet consumed, in order to quantify dietary iron absorption in absolute terms.

Despite the problems in assessment of dietary iron absorption, current evidence from a number of studies using either chemical or radioisotopic methods have given some indication of the normal variation encountered on the positive side of iron balance. The general conclusion that may be drawn from such studies is that a normal individual absorbs between 5 and 10 per cent of the 10 to 18 mg iron present in an average, mixed, Western-type diet and that absorption may be increased up to threefold in states of iron deficiency (Moore, 1965; Bothwell and Charlton, 1970; Harris and Kellermeyer, 1970, p. 67). Expressed in quantitative terms, the estimated normal daily iron absorption is therefore between 1.0 and 1.5 mg. This may rise to between 3.0 and 4.5 mg in iron deficiency.

(b) Measurement of iron excretion

Because of the manifold difficulties in assessing dietary iron absorption, an alternative indirect method has been used in order to gain information on daily iron requirements, by measuring daily iron losses. Since the maintenance of normal iron balance depends upon the ability to replace from dietary sources the iron that is constantly being lost from the body, it is possible, by defining the extent of these losses, to arrive at an estimate for the daily iron requirement. This would be valid in normal individuals who are in a state of equilibrium insofar as external iron exchange is concerned. It may be reasonably anticipated that in such individuals, the amount of iron lost each day will be equal to that absorbed, and it may be argued that precise data on daily iron losses can be used as an indirect but accurate measure of the normal limits of iron absorption.

Like iron absorption, iron losses have been measured by both chemical and radioisotopic methods. Again, both approaches are associated with a number of methodological and interpretive difficulties. These will be elaborated upon in the following chapter which deals with a review of the literature on iron excretion.

CHAPTER 2

REVIEW OF THE LITERATURE ON IRON EXCRETION

I. HISTORICAL

The classical observations of McCance and Widdowson in 1937 marked a turning point in the development of an understanding of iron nutrition. Before that time, and despite some evidence to the contrary (Kobert and Koch, 1894) the prevailing view was that body iron balance was to a large extent controlled by excretion of iron through the bowel. This view was based largely on the demonstration of stainable iron in sections of bowel by histologists during the last century who had no means of distinguishing absorbed iron from excreted iron on purely morphological grounds. Before it was brought into question, the generally accepted concept on the regulation of iron balance was exemplified by the statement that: 'The site of absorption is the entire intestinal tract, although the duodenum and a portion of the upper small intestine probably play the major role Elimination is almost entirely by intestine, especially by the colon' (Robscheitt-Robbins, 1929).

McCance and Widdowson (1937a) challenged this view in the light of a number of established facts and observations concerning iron metabolism. They concluded from their critical analysis that the ability of the bowel to excrete iron had been exaggerated, and that its major role in the regulation of iron balance was to exercise control over iron absorption. The ultimate effect of their monumental observations, was to focus more attention on the study of iron absorption, but in the years immediately following the publication of their argument, McCance and Widdowson, as well as other workers carried out a number of investigations to confirm the hypothesis that the capacity of the body to excrete iron was indeed limited.

Shortly before the appearance in print of McCance and Widdowson's hypothesis, Welch and associates (1936), reported their observations on a patient with an ileostomy stoma and an isolated colon. They found that negligible amounts of iron were

excreted by the colon (less than 0.2 mg per day) even with an iron intake of more than 70 mg per day. McCance and Widdowson themselves produced a series of experimental observations to support their hypothesis. They carried out balance studies on four normal adults, before, during and after a period of very high iron intake (Widdowson and McCance, 1937). Although the net positive balance during the period of high iron intake amounted to between 1.5 and 5.0 g, the subjects returned to a state of iron equilibrium after changing to a low iron intake and increased amounts of iron were not detected in the stool.

In a further experiment, they showed that the daily intravenous injection of an amount of iron (7 mg) equal to the oral intake did not cause any increase in the amounts of iron present in the stools of six healthy subjects (McCance and Widdowson, 1938). They also studied a patient with polycythaemia rubra vera who was being treated with acetyl-phenylhydrazine (McCance and Widdowson, 1937b). During a period of one month, it was calculated that 6 330 mg iron were released from haemolysed red cells, yet the negative iron balance over this time was only 28 mg, representing less than 0.5 per cent of the liberated iron, and a net negative iron balance of approximately one milligram per day. In a similar clinical investigation, they found that a woman who had received 28 pints of blood over a period of 100 days for treatment of severe haemolytic anaemia was excreting less iron per day (stool and urine) than she was taking by mouth towards the end of this period (McCance and Widdowson, 1943).

Experimental data from other workers also supported McCance's and Widdowson's postulate. Brock and Hunter (1937) showed that patients with hypochromic anaemia treated with large doses of oral iron retained much more than the amount that was required for haemoglobin formation. Fowler and Barer (1937) showed no increase in the faecal iron excretion of subjects receiving parenteral iron injections. Maddock and Heath (1939),

studying dogs with colonic explants, found no evidence of iron excretion through the bowel after injection of iron.

Renewed interest in the question of iron excretion coincided with the introduction of radioactive isotopes of iron for clinical use. This provided a great boon to investigators in the field of iron metabolism, overcoming as it did the major problem of contamination which bedeviled earlier chemical techniques.

Radioiron was first used to study iron excretion by Hahn and his co-workers, who measured iron losses through the gastrointestinal and urinary tracts in dogs. Their work, which will be discussed in later sections in relation to biliary iron losses and animal studies, confirmed the concept that iron excretion is limited. Daily faecal iron loss was small, and urinary loss insignificant in normal dogs (Hahn, *et al.*, 1939). Dubach and her co-workers (1946), studying the reutilization of radioiron in dogs produced further indirect evidence that iron losses are small. Haemolysis was induced with acetyl-phenylhydrazine in dogs which had been made to incorporate large amounts of radioiron into their circulating haemoglobin by prior phlebotomy. Although the level of circulating radioiron dropped during haemolysis it almost quantitatively reappeared in the blood following recovery. This provided good evidence that body iron is avidly conserved, and by inference, that external losses are small.

During the following three decades, numerous studies on iron excretion in man were reported in the literature. Iron excretion was studied in both normal and pathological states using a vast array of chemical and isotopic techniques. Some of these dealt with total body iron loss, but most were concerned with the excretion of iron through a particular route, such as the bowel, the urinary tract, or the skin. Many of these studies, notably those of Dubach, *et al.*, (1955), Finch (1959), Saito, *et al.*, (1964) and Moore (1965) confirmed and strengthened the concept that iron

excretion is normally limited. The results obtained by these workers suggested that normal daily iron losses fell within the fairly close range of 0.5 to 1.25 mg, which is in good agreement with estimates of daily iron absorption from an average mixed diet in normal subjects who are in a state of iron equilibrium (Moore, 1965; Bothwell and Charlton, 1970; Harris and Kellermeyer, 1970, p. 67).

However, some reports have been at variance with the view that iron excretion is limited, and have suggested that iron losses might be considerably greater than the narrow range cited above. Two types of approach, particularly, have yielded results which have not been in accord with the tenet of limited iron excretion. On the one hand, studies employing whole body counting techniques to measure radioiron retention have yielded results for daily iron losses which are several times greater than the generally accepted figure (Price, *et al.* 1962; Weintraub, *et al.*, 1965). On the other hand, a number workers beginning with Mitchell and Hamilton (1949) used chemical methods to measure iron loss from the skin through sweating, and have reported daily skin losses of several milligrams of iron. These studies will be considered under the appropriate section later in this chapter, and in the relevant discussion sections of subsequent chapters. In summary of this brief overview of the changing concepts regarding iron excretion during this century, it may be said that although there is much evidence to support the concept of limited iron excretion, there remains a considerable body of opinion that controverts this view. These areas of controversy will be dealt with in the following sections in which the literature on the subject of iron excretion will be considered in detail in relation to losses from various body compartments and from the body as a whole.

II. COMPARTMENTAL IRON LOSSES

Iron may be lost from the body through epithelial surfaces, either in cells which are desquamated from or extruded through these surfaces, or in fluids which are lost externally through secretion or excretion. The normal routes of iron loss are the gastrointestinal tract, the urinary tract and the skin. In addition, women of childbearing age incur further iron losses as a result of menstruation, pregnancy, childbirth and lactation. In some pathological conditions, iron losses through these various routes may be increased. In the consideration of each route of iron excretion, normal losses will be considered first, followed by the increased losses which may occur in certain disease states.

(a) Gastrointestinal tract

The belief that the gut was an important organ of iron excretion was abandoned following the work of McCance and others, referred to earlier in this chapter. However, subsequent work carried out in an attempt to quantify the actual amounts of iron lost through the gastrointestinal tract have indicated that while small, these losses are significant within the framework of a concept of limited external iron exchange.

(i) Chemical studies

Early attempts to measure iron loss from the bowel were carried out using chemical methods. This approach is identical to the chemical balance technique which has been referred to in Chapter 1, and suffers from the same drawbacks, namely iron contamination, and the inability to discriminate between excreted endogenous iron and unabsorbed dietary iron. However, at least two studies of gastrointestinal iron losses using chemical techniques merit further consideration. Ingalls and Johnston (1954), used a balance technique in which they gave female

volunteers diets containing three different levels of iron for eight-day periods. They then used a novel approach in an attempt to differentiate gastrointestinal iron from unabsorbed food iron by plotting faecal iron against the various levels of food iron intake and extending the faecal iron regression line to the hypothetical point of zero iron intake. From this they concluded that the amount of faecal iron contributed by losses into the intestinal tract was 0.2 mg per day.

Finch carried out a study in conjunction with the author of this thesis (Green, *et al.*, 1968) which was designed not to quantify the actual amounts of iron lost from the bowel, but to gain some idea of the maximum losses through this route. Three subjects of normal iron status and one with transfusional iron overload were given a special diet of low iron content (1.4 to 2.4 mg daily) for between 10 and 16 days. After a few days of adjustment to this diet, a balance study was carried out, and it was found that the three normal subjects had a mean daily iron balance of +0.4 mg, +0.4 mg, and -0.1 mg respectively, whereas a figure of -1.2 mg was obtained in the siderotic patient. Since a proportion of the dietary iron was almost certainly absorbed, it is not possible to express the balance results in terms of absolute excretion rates, but since only a small proportion of food iron is normally available for absorption, the balance figure calculated from the difference between intake and loss does provide an indication of the magnitude of endogenous iron losses from the bowel which could not be obtained from studies using higher iron intakes. Even making the extreme and unlikely assumption that all the dietary iron was absorbed, the results obtained would support the view that iron excretion via the bowel is limited, even when iron overload is present.

(ii) Radioisotopic studies

Whilst obviating the dual problems of contamination and of distinguishing unabsorbed from excreted iron, the use of radioiron to measure gastrointestinal iron losses has its own inherent drawbacks. These are of an interpretive nature. Radioiron losses can be measured and related to an administered dose of tracer, but to express this in quantitative terms, it is then necessary to apply the fraction excreted to some figure for the total body iron. To do this, an estimate of the size of the body iron pool must be made, and sufficient time must be allowed to elapse for complete mixing to take place between this pool and the injected tracer. These requirements apply to any method which employs radioiron, but in the case of the gastrointestinal tract, they assume particular significance because a number of sources contribute to the composite iron loss through this route. Normally iron is lost from the bowel in red cells which escape through the mucosa, in epithelial cells which are desquamated from the surface of the gastrointestinal tract, and in secretions, notably bile. A proportion of the iron that enters the bowel lumen from each of these sources at, or above, the level of the proximal portion of the duodenum which is the site of maximal iron absorption in man (Wheby, 1966), is probably available for reabsorption.

Various attempts have been made to measure how much iron may be lost by these mechanisms. It has also been suggested that small amounts of iron may be lost by exudation of transferrin-bound plasma iron through the normal mucosa (Singh and Loehry, 1968) and that iron may also be lost in macrophages which migrate into the gut lumen (Thirayothin and Crosby, 1962). These additional postulated mechanisms for gastrointestinal iron excretion are based on animal experiments, and have not been shown to operate in man.

The first attempt to measure iron losses in humans using radioiron was carried out by Dubach and her co-workers (1955). They measured the excretion of ^{59}Fe radioiron in the stools of four normal subjects for a period of 140 days and found that the average daily excretion was 0.01 per cent per day of the injected dose. By relating this figure to an estimate of the total body iron, they calculated that this was equivalent to a daily iron loss from the bowel of between 0.33 and 0.52 mg. Three iron deficient women excreted about one-third of this amount, and the daily losses of radioiron in a patient with a haemolytic anaemia were several-fold greater than normal. Although these figures represented the total amount of iron in the faeces, and did not indicate their source, three significant observations were made. Firstly, the excretion of radioiron during the first 20 days was greater than in later periods. Secondly, no increase in excretion of the tracer iron was observed at, or near, the 120th day after injection, when destruction of the cohort of radioiron-labelled red cells could have been expected to take place. Thirdly, the differences in radioiron losses between normal and iron-deficient individuals revealed that iron excretion was a variable process which could be influenced by the degree of body iron repletion.

The contribution to gastrointestinal iron losses made by various sources has been studied in a number of ways. Blood loss from the gastrointestinal tract has been measured using ^{51}Cr -tagged red cells, and average losses of 0.1 ml (Hughes Jones, 1958), 0.7 ml (Bannerman, 1957) and 1.2 ml (Roche, *et al.*, 1957a; Ebaugh, *et al.*, 1958) have been estimated in normal subjects. One gram of haemoglobin contains 3.4 mg iron (Bernhart and Skeggs, 1943), so that with a haemoglobin concentration of 14.7 g per 100 ml, one millilitre of blood would contain 0.5 mg iron. The above amounts of blood would therefore be equivalent to daily iron losses of approximately 0.05, 0.35 and 0.60 mg respectively. But, since the method measures the faecal losses of radiochromium rather than the iron contained

in red cells, possible sources of error must be taken into account. Firstly, some red cells will be digested in the gut and their haemoglobin iron reabsorbed independently of the ^{51}Cr tag. Conversely, a variable though small fraction of the ^{51}Cr is eluted from red cells in the bowel lumen, and this may be reabsorbed independently of blood (Hughes Jones, 1958).

Non-haemoglobin iron losses from the bowel occur as well, either through the loss of mucosal cells which are constantly being replaced throughout the gastrointestinal tract, or in secretions. Conrad, *et al.*, (1964) demonstrated that when transferrin-bound radioiron is given intravenously to rats, a fraction of the ^{59}Fe enters the newly formed epithelial cells in the crypts of the small intestine and migrates with the cells as they move to the villus tip from where the cells are lost. They also showed that the radioiron entered the gastric and colonic epithelial cells. These studies suggested that the mechanism by which plasma iron is taken up by mucosal cells and ultimately discharged into the intestinal lumen, constitutes a further avenue of gastrointestinal iron loss, and indicated an active excretory role for the mucosa.

Estimates made of the quantity of cells lost from the human gastrointestinal tract are in good agreement. Leblond and Walker, (1956) estimated a loss of 'about half a pound a day' of intestinal epithelial cells, and Croft (1970), calculated that the total weight of epithelial cells lost from the human gut is 287 g per 24 hours, based on studies in which DNA measurements were carried out in washings from the stomach, small intestine and large intestine (Croft, *et al.*, 1966; Croft, *et al.*, 1968b; Croft and Levitan, 1970). These workers also showed that the small intestine, which has both the largest surface area and the fastest turnover rate, contributes 90 per cent to the total cell loss from the gut. Many of the cells lost in this manner probably disintegrate in the gut, and a proportion of their contained iron is therefore probably reabsorbed.

Few attempts have been made to measure iron loss from the gastrointestinal tract through the desquamation of mucosal cells, or to differentiate this from blood and other sources of bowel iron loss. In one study, which forms part of the work presented in this thesis, faecal radioiron excretion data were analysed, by a method which will be detailed later (Chapter 5), in an attempt to distinguish haem from non-haem iron loss through the bowel. Mean daily haem and non-haem iron losses in a group of subjects were found to be 0.38 and 0.14 mg respectively (Green, *et al.*, 1968).

Recently, two studies have been carried out on gastrointestinal iron loss using a double isotope technique. The faecal losses of injected ^{59}Fe radioiron as well as ^{51}Cr -tagged red cells were measured simultaneously by a differential counting technique. The amount of ^{51}Cr excreted was used as an index of blood loss, and from this the haem-iron loss was calculated. Non-haem iron losses were computed by subtracting from the total faecal ^{59}Fe radioactivity the calculated amount due to ^{59}Fe -labelled haemoglobin which was derived from the figures for radiochromium losses. Using this approach, it was concluded that the major portion of faecal iron loss is of non-haem origin, caused by desquamation of the villus epithelium (Singh, 1970). In this study, the relative amounts of haem and non-haem iron lost in the faeces were compared, but no attempt was made to express the loss of non-haem iron in quantitative terms. In the other study 'free' (non-haem) iron was also found to exceed haem iron loss, but only slightly. The mean ($\pm$ SD) calculated daily 'free' faecal iron loss was 0.43 ± 0.08 mg compared with 0.32 ± 0.13 mg lost as haem iron (Baird, 1973).

(b) Biliary tract

Biliary iron losses have been measured both chemically and by the use of injected radioiron.

The results of most earlier workers indicated that considerable amounts of iron were excreted in the bile each day. One text cites figures ranging from one to 10 mg (Vannotti and Delachaux, 1949). Judd and Dry (1935) measured the chemical iron content of common bile duct or gall bladder bile and found a mean biliary iron concentration in 11 subjects of 0.306 mg per 100 ml (range 0.083 - 0.959). These studies were carried out prior to the observations of McCance and Widdowson (1937a), referred to previously, and unless the mechanism for biliary iron reabsorption is highly efficient, are not consistent with the concept of limited iron loss. Although it seems likely that chemical contamination might have played a part in such high estimations, these figures cannot be refuted without definite contradictory evidence from other studies. Such evidence is lacking, since no further attention seems to have been given to the question of biliary iron losses in man.

Dubach and her co-workers suggested, on the basis of their study on radioiron losses, that the bile might constitute an important avenue of iron loss through the gastrointestinal tract, (Dubach, *et al.*, 1955) but further investigations of this problem are limited to three reports in which the biliary excretion of intravenously administered radioiron was measured in patients with indwelling T-tubes in the common bile duct following gall-bladder surgery. In two of these reports, the results were expressed only as the percentage of injected radioiron which appeared in the bile. In one study, the measured daily losses in two patients were 0.0017 and 0.0012 per cent (Mekel, *et al.*, 1970). In the other study, results using the same approach were several-fold higher, being 0.007, 0.008, and 0.009 per cent in three patients (Singh, 1970). In neither study was any attempt made to express these losses in quantitative terms. In the third report, which forms part of the work presented in this thesis, biliary radioiron losses expressed in quantitative terms, amounted to an average

excretion of 0.035 mg per day in eight patients. The chemical iron content of bile was also measured directly in six of these subjects, and considerably greater values (mean, 0.282 mg) were found (Green, *et al.*, 1968). These findings will be detailed and discussed later (Chapter 5).

(c) Pathological iron loss through the gastrointestinal tract

(i) Blood loss

Bleeding from the gastrointestinal tract is the most common pathological cause of increased iron loss from the body. A variety of degenerative, inflammatory and neoplastic diseases, as well as congenital, vascular and haemostatic abnormalities can lead to abnormal blood loss from the bowel. In addition, pathological blood loss from the gastrointestinal tract is frequently the result of parasitic infestation, notably with hookworms, which, on a global basis, is the single most important cause of gastrointestinal bleeding. In a painstaking study, Roche and Layrisse (1966), investigated various aspects of hookworm blood loss. They showed that the average daily iron loss per worm is 0.03 mg for *Necator americanus*, 0.05 mg for *Ancylostoma caninum*, and 0.15 mg for *Ancylostoma duodenale*. Abnormal blood loss from the bowel may also be caused by drugs which produce gastrointestinal irritation, and may occur following certain operations, particularly gastrectomies. The various causes of pathological blood loss from the bowel have been well-documented in a number of standard texts on iron metabolism (Bothwell and Finch, 1962, p. 315; Fairbanks, *et al.*, 1971, p. 139) and in recent reviews on the aetiology of iron deficiency anaemia (Baird, 1973; Cowan and Bharucha, 1973). In the pathogenesis of negative iron balance resulting from gastrointestinal bleeding it is not only the volume of blood shed into the bowel lumen that is important, but also the site of the bleeding lesion. In general, lesions

situated distal to the upper small intestine are a more cogent cause of body iron loss, since the iron contained in shed red cells is then not available for reabsorption. Although any increase above normal in the amount of blood lost from the gastrointestinal tract would significantly alter daily iron needs, it would appear that pathologic blood loss via this route becomes important in the production of iron deficiency anaemia only when it exceeds 6 to 8 ml (equivalent to 3 to 4 mg iron) per day (Bothwell and Finch, 1962, p. 315). It may be noted that these figures are comparable to the 80 ml limit of monthly menstrual blood loss beyond which iron deficiency is likely to occur. A further noteworthy observation in relation to gastrointestinal blood loss is that the amount of blood lost in patients with hookworm infestation decreases as the haemoglobin content of the blood rises with iron therapy (Roche, *et al.*, 1957b). This situation may again be analogous to menstrual blood losses, where it has been suggested that treatment of the iron deficiency resulting from menorrhagia causes a reduction in the amount of menstrual blood loss (Taymor, *et al.*, 1960).

(ii) Non-haem iron loss

In addition to the numerous causes of abnormal blood loss from the bowel, certain diseases of the gastrointestinal tract may be associated with a significant increase in the amount of non-haemoglobin iron loss through this route. As mentioned above, small amounts of iron are normally lost through the process of desquamation of intestinal epithelial cells, and it might be expected that these losses would be greater in diseases associated with a more rapid rate of turnover of these cells. This has been confirmed in patients with simple atrophic gastritis and adult coeliac disease by use of the double isotope studies using ^{59}Fe and ^{51}Cr referred to previously (Croft, 1970; Singh, 1970; Sutton, *et al.*, 1970). Cell

loss in these conditions are increased from three- to eight-fold (Croft, *et al.*, 1966; Croft, *et al.*, 1968b) and corresponding two- to ten-fold increases in non-haem iron losses compared with normal subjects have been reported. Sutton, *et al.*, (1970) also noted a similar increase in non-haem iron loss in two-thirds of patients following partial gastrectomy. Confirmatory evidence that non-haem iron loss from the bowel is related to the rate of turnover of mucosal cells was obtained in experiments using ⁵⁹Fe in rats by Loehry and his co-workers (1969a and b). They found that compared with normal rats, the peak of faecal radioiron excretion due to mucosal cell exfoliation was considerably higher in rats infested with a nematode which causes mucosal changes similar to those seen in the coeliac syndrome.

Loss of plasma iron into the bowel lumen is normally prevented by its binding to the protein transferrin. There is some evidence that a small proportion of the plasma protein pool is normally catabolized or lost through the gastrointestinal tract, but owing to methodological difficulties, this has not been accurately quantified (Waldmann, 1966). However, in conditions characterised by an abnormally increased permeability of the gastrointestinal tract to large molecules, significant amounts of plasma protein, as well as transferrin-bound plasma iron may be lost into the bowel lumen. These conditions, analogous to the nephrotic syndrome, have been referred to as protein-losing enteropathies or gastro-enteropathies, and include a variety of inflammatory, degenerative and neoplastic diseases, affecting the gastrointestinal tract (Waldmann, 1966). An increased fractional catabolic rate of transferrin caused by loss into the bowel has been demonstrated in these conditions (Waldmann, 1966; Jensen, *et al.*, 1968; Jarnum and Jensen, 1972), and the occurrence of iron deficiency anaemia has been noted in these states (Ulstrom and Krivit, 1960).

(d) Urinary tract

Quantitative measurements of iron loss through the urinary tract in normal subjects have been confined to chemical determinations of urinary iron content. As with other avenues of iron loss from the body, earlier workers arrived at variable estimates of urinary iron excretion which in general were considerably higher than would be compatible with the concept of limited external iron exchange. Barer and Fowler (1937), reviewing twelve studies in the literature which preceded their own study, cited figures for daily urinary iron losses in normal subjects which ranged from zero to 1.26 mg. In their own study of 100 males and 100 females these workers, too, found a wide range for urinary iron excretion, which varied from 0.08 to 1.63 mg daily. The high figures obtained by earlier workers were probably due to contamination, since in a number of subsequent studies, also carried out using chemical methods, considerably lower values were found.

The technical problem of contamination notwithstanding, these more recent data are generally in good agreement, and suggest that urinary iron loss comprises but a small fraction of the total daily loss from the body. From the work of Cartwright, *et al.*, (1954), Gisinger and Puxkandl (1955), Dagg, *et al.*, (1966), Man and Wadsworth (1969), and from a study which forms part of this thesis (Green, *et al.*, 1968), it would appear that not more than 0.1 mg iron is lost in the urine each day, regardless of the state of the body iron stores. These lower estimates of urinary iron loss are supported by the results of a number of radioisotopic studies which, although not quantitative, showed a negligible loss of radioiron in the urine up to 10 days following injection (Dubach, *et al.*, 1955; Saito, *et al.*, 1964; Weintraub, *et al.*, 1965).

The source of urinary iron is uncertain, but there are a number of mechanisms whereby iron loss may arise through this route. The renal loss of plasma iron is normally prevented by

binding to transferrin and most of what enters the glomerular filtrate is reabsorbed. Small amounts of transferrin protein (less than 2.0 mg per day) have been measured in the urine of normal subjects (Finch, 1964; Man and Wadsworth, 1969). However, even assuming full saturation, on a stoichiometric basis, each milligram of urinary transferrin could bind a maximum of 1.5 μ g iron, since the molecular weight of transferrin is approximately 74 000 daltons, that of elemental iron 56 daltons, and each molecule of the protein has two ligand-binding sites for the metal (Harris and Kellermeyer 1970, p. 80). From this it may be concluded that in normal subjects only a very small proportion of urinary iron is transferrin-bound.

Another possible source of urinary iron is from cell loss through this route. The desquamation of cells lining the urinary tract would constitute a mechanism of iron loss analogous to that which occurs from the gut and other epithelial surfaces undergoing constant replacement. It seems probable that the major portion of urinary iron is derived from this source, but it is difficult to determine the actual amounts lost in this way, because iron originally located within cells may be released by the time that urine is voided. This could account for the finding that little iron was contained in the cellular debris separated from urine (Man and Wadsworth, 1969).

The physiological urinary loss of leucocytes and erythrocytes could also account for some of the iron present in urine, but the extent to which this occurs is normally limited, and based even on the accepted maximum urinary loss of 200 000 erythrocytes per hour (Gadeholt, 1964) approximately 0.5 μ g iron, at most, could arise from this source per day.

Although the normal mechanism of urinary iron loss has not been established, its magnitude, which is more important in relation to the question of iron balance and requirements, has

been well-defined. The importance of this route of iron loss is indeed the least controversial of all possible avenues of iron loss from the body, since the minor variations in urinary iron loss which have been reported during the last two decades are negligible in terms of an overall figure for external body iron exchange. Even the unconfirmed findings of Man and Wadsworth (1969), who reported that the amount of urinary iron was directly influenced by the level of dietary iron intake, do not seriously challenge the concept that urinary iron loss is an insignificant term in the iron balance equation. In contrast to an earlier report (Leverton, 1941) these workers found greater amounts of iron in the urine of two subjects as their dietary iron intake was increased over a three- to four-fold range. However, even on a high iron intake (35.7 and 24.8 mg per day) the urinary iron losses in these two subjects rose only from 51.8 to 138.5 μg and from 81.3 to 118.7 μg per day respectively. These differences would not materially influence the sum for total daily iron losses.

(e) Pathological iron loss through the urinary tract

Although iron excretion through the urinary tract is relatively small and insignificant in normal healthy subjects, appreciable losses of iron may occur in a number of disease states. In such conditions, there is an exaggeration of the mechanisms which are presumed to account for normal urinary iron loss, so that either abnormal amounts of transferrin-bound iron escape into the urine, or increased quantities of iron are deposited in cells which are sloughed from the linings of the urinary tract.

Increased losses of urinary iron have been reported in patients with proteinuria associated with the nephrotic syndrome (Cartwright, *et al.*, 1954; Rifkind, *et al.*, 1961; Dagg, *et al.*, 1966). This increased excretion of iron is

due to an abnormal renal leak of transferrin since a significant correlation was found between iron and beta-globulin output (Rifkind, *et al.*, 1961; Dagg, *et al.*, 1966). Direct estimations of the urinary iron content in the studies cited revealed that in patients with the nephrotic syndrome, up to one milligram of iron could be lost each day. This degree of iron loss could result in a state of negative iron balance, as evidenced by the frequent occurrence of iron deficiency anaemia in these patients. Transferrinuria may occur in glomerular as well as in tubular renal disease, and it is of interest that there is a relatively greater renal leak of intermediate sized proteins such as transferrin in diseases primarily affecting the glomerulus (Strober, *et al.*, 1973).

Certain haemolytic states are characterised by a considerable intravascular haemolytic component with the release of free haemoglobin into the circulation. In these conditions, which are exemplified by paroxysmal nocturnal haemoglobinuria and mechanical intravascular haemolysis resulting from intra-cardiac valvular prostheses or certain types of endocardial valve damage, the haemoglobin binding-capacity of plasma haptoglobin is saturated. As a result, haemoglobin and its iron-containing breakdown products enter the glomerular filtrate in excess of the amount that can be handled for reabsorption by the tubular epithelium. Consequently, renal tubular cells containing vastly increased amounts of haemosiderin and ferritin are desquamated, and figures of up to 14 mg of urinary non-haemoglobin iron loss have been reported (Myhre and Flatmark, 1962; Sears, *et al.*, 1966; Walsh, *et al.*, 1966; Kann, *et al.*, 1967; Reynolds, *et al.*, 1967; Eyster, *et al.*, 1968; Eyster, 1969; Halpern, *et al.*, 1971). In addition, large amounts of iron may be lost as a result of haemoglobinuria, and in one case, as much as 34 mg daily was lost in this way (Eyster, 1969).

Although it is not strictly a pathophysiological mechanism for iron loss, it is appropriate to include in the present discussion the iron depletion that has been described in patients with chronic renal failure who have undergone frequent extracorporeal haemodialysis (Adamson, *et al.*, 1968). The negative iron balance that may be incurred by these patients appears to be due to the loss of blood which remains trapped in dialysis coils (Lawson, *et al.*, 1971).

(f) Skin

Iron may be lost from the skin and its appendages either through the desquamation of epidermal cells, or in eccrine and apocrine sweat. Despite considerable disagreement on the actual magnitude of iron loss through this route, it is generally held that most of the integumentary loss of iron results from cell desquamation. Quantitation of skin iron excretion has been attempted by a number of workers, using either chemical or radioisotopic techniques and much disagreement on the magnitude of skin iron losses is evident in the literature, with estimates varying from a fraction of a milligram to several milligrams per day. Indeed, the most consistent challenge to the concept of limited external iron exchange has come from workers who have claimed that the skin constitutes a major avenue of iron loss.

(i) Chemical studies

Most of these claims have been based on the chemical determination of iron in sweat. This approach has yielded widely varying results, as is evident from Table 2.1 which summarizes, in chronological order, the literature on chemically measured sweat iron losses.

Mitchell and Hamilton (1949) reported a daily loss of iron from the skin of 6.5 mg under 'minimal' sweating conditions and suggested that this figure could increase several-fold when

Table 2.1. Iron content of sweat determined chemically (literature review modified after Coleman and Rowe, 1966)

AUTHOR	IRON CONCENTRATION ¹ (mg/litre)		Number of subjects ²	Source	SWEAT COLLECTION		Time (hours)	ESTIMATE OF DAILY IRON LOSS (mg)	REMARKS
	Cell-rich sweat	Cell-free sweat			Conditions temperature (°C)	relative humidity (per cent)			
Jackson & Hagan (1949)		40 (20-60)	5	whole body	38	59-67	2	0.02-0.06*	Assumed sweat loss of 1 litre/day in temperature zones.
Nichell & Hamilton (1949)	1778 (870-2380)		6	whole body	27-39	43-73	7.5	6.5*	For 'minimal' sweating conditions. Three to ten-fold increase suggested under 'profuse' sweating conditions.
Jefferson <i>et al.</i> (1950)		270 (220-450)	4	whole body excluding scalp	36-38	74-90	1	0.75†	Based on mean hourly sweat iron concentration and a 10-hour 'working' day.
Adams <i>et al.</i> (1950)	7050 (780-19500)	296 (0-2150)	25	forearm	thermal stimulus to opposite arm		1	6.0-10.0†	Extrapolated figure calculated by Foy and Kandi (1957).
Foy & Kandi (1957)	300-6000	100-200	not stated	whole body	"thermostatically controlled room"; conditions not stated		not stated	0.9-18.0*	Based on minimal sweat rate of 3 litres/day in the tropics, but evidence cited that sweat rate may rise to 11 litres/day (Kuno, 1956).
Hussain <i>et al.</i> (1960)	1610±165 (1210-2300)	440±56 (300-600)	6	(i) forearm and part of arm	thermal stimulus (42-43°C) to feet and opposite arm		1	4.8†	Extrapolating and assuming sweat rate of 3 litre/day (Foy and Kandi, 1957).
Ibid		0	17 (iron deficiency anaemia)	(ii) "	"		1	1.3†	As above. Iron content of cell-rich sweat in iron deficiency was significantly different from normal subjects.
Hussain & Patwardhan (1958b)	1150±52 (650-1880)	340±16 (150-530)	35	"	"		1	3.5†	As above. However authors point out that extrapolation is invalid because of anatomical differences in sweat rate.
Moore (1961)	400-1500	346 (from graph)	1	forearm	thermal stimulus to opposite arm		2-4	2-6*	Author states that extrapolation is not valid. Results presented together with data using radioiron which showed daily loss of 0.5-1.0 mg.
Ayie & Venkatachalam (1962)	350±76	190±13	16	(i) Upper extremity	Hot season (June) 35-35 with thermal stimulus to opposite arm	65-70	1		No attempt to extrapolate from these results to whole-body daily iron losses because:
Ibid	520±56	250±24	16	(ii) Lower extremity	"		1		(i) regional (anatomical) differences (head and trunk greater than extremities)
Ibid	1520±55		16	(iii) back	"		1		(ii) environmental climatic differences (losses in cold climate greater than warm climate)
Ibid	1440±292		16	(iv) forehead	"		1		
Ibid	622±32		6	(v) upper extremity	Cold season (November) 22-24 with thermal stimulus to opposite arm	70	1		

¹ Results given either as mean, mean ± S.D., mean ± S.D. (range), mean (range) or (range).² Normal subjects unless otherwise stated.

* Stated daily loss calculated by author.

† Daily loss calculated from data given in the reference.

Table 2.1. (continued) Iron content of sweat determined chemically (literature review modified after Colman and Rowe, 1966)

AUTHOR	IRON CONCENTRATION ¹ (mg/litre)		Number of subjects ²	Source	SWEAT COLLECTION		Time (hours)	ESTIMATE OF DAILY IRON LOSS (mg)	REMARKS
	Cell-rich sweat	Cell-free sweat			temperature (°C)	Conditions relative humidity (per cent)			
Apte (1963)	3240 ⁺ 1398 (850-15500)		10	whole body	30-34	65-70	1	0.6-3.2*	Extrapolated from hourly loss and assuming uniform loss over an 8-10 hour 'working' day.
Prasad <i>et al.</i> (1963)	1200 ⁺ 360	460 ⁺ 110	12	(i) upper extremity	direct sunlight 38-41		2-3	2.5-13.0*	Extrapolated figure based on range of sweat loss of 2 to 11 litres in tropics (Kuno, 1956).
<i>Ibid</i>	600 ⁺ 270	380 ⁺ 160	12 (iron deficiency anaemia)	(ii) "	"		2-3	1.2-6.5†	Significantly lower iron loss in cell-rich sweat from iron deficient subjects.
Consolazio <i>et al.</i> (1963)		(i) 364 ⁺ 212	3	Upper extremity	Environmental chamber 38	70 (1st 4 days)	7.5	1.0*	For 7.5 hour period in chamber, and based only on estimation of cell-free sweat. Concluded that iron excretion in sweat does not change appreciably with acclimatization.
<i>Ibid</i>		(ii) 352 ⁺ 160	"	"	"	(2nd 4 days)	"		
<i>Ibid</i>		(iii) 386 ⁺ 211	"	"	"	(3rd 4 days)	"		
<i>Ibid</i>		(iv) 250 ⁺ 209	"	"	"	(4th 4 days)	"		
Colman & Rowe (1966)		172 ⁺ 78 (20-370)	28	forearm	iontophoresis				No extrapolation to whole body iron loss attempted.
Velliar (1966)	412 (130-630)	298 (130-450)	23	whole body	Environmental chamber 40-45	80-90	1		No extrapolation attempted because extreme climatic conditions of experiment could not have been tolerated for protracted periods. Significant correlation between iron content of cell-free sweat and serum iron levels after sweat collection. Significant correlation between iron content of cell-rich sweat and body surface area.

¹ Results given either as mean, mean ⁺ S.D., mean ⁺ S.D. (range), mean (range) or (range).² Normal subjects unless otherwise stated.

* Stated daily loss calculated by author.

† Daily loss calculated from data given in the reference.

sweating was profuse. This stimulated many workers to study the iron content of sweat, because of the possible bearing this could have on the important question of the aetiology of iron deficiency anaemia so prevalent in the tropics. However, subsequent studies on sweat iron losses produced widely discrepant results. Such discrepancies for both sweat iron concentration and estimates of the daily loss of iron through this route tend in themselves to cast doubt on the validity of this approach. It is not possible to selectively criticise the techniques used by one or other group of workers, since each appears to have given considerable attention to methodological details, particularly the risk of external contamination. Nonetheless, it may be stated that estimates of daily iron loss through the skin which are based on the measurement of sweat iron concentration are beset by numerous technical and interpretive problems, which are not possible to control adequately.

The risk of contamination associated with any chemical technique for measuring iron loss applies particularly in the case of the microscopically irregular external surface of the skin. Even if it were possible to completely remove all traces of exogenous iron contamination from the interstices of skin papillae and appendages by meticulous cleaning, it could then be argued that iron-containing epithelial cells might be removed by artificial abrasion. In other words it seems to be difficult, if not impossible, to clean the skin in such a way as to remove external iron contamination while at the same time not removing iron-containing cells prior to a sweat collection experiment.

Another major problem with the estimation of daily iron loss from the skin based on chemical determinations of iron in sweat is the need for extrapolation. From measurements of the iron concentration of sweat collected from one region of the body over a short time period, and under artificial conditions,

it is necessary to extrapolate to whole body loss over a 24-hour period under natural conditions. The measured iron concentration in sweat collected from say, one arm, over a one-hour period must therefore be multiplied both by the hourly sweat rate and by a factor of 24 in order to arrive at an estimate for daily sweat iron loss from the whole body. In this way, small technical inaccuracies or slight contamination in the initial determination could become amplified quite considerably. But apart from this obvious source of error, there is good evidence that such extrapolations are invalid. For example, it has been found that arm sweat differs both in its rate of production and its chemical composition from whole body sweat. Van Heyningen and Weiner (1952) reported that arm sweat is more concentrated, especially in unacclimatized individuals. Apte and Venkatachalam (1962), on the other hand, found that the iron concentration of cell-rich sweat collected from the upper extremity was considerably less than from other parts of the body. It has also been shown that sweat gland activity is rapidly depressed by enclosing the arm in a bag (Collins and Weiner, 1962), and that enclosure, by causing skin maceration, produces sweat which is artefactually rich in cells (Moore, 1961). Even when sweat from the whole body is collected, the normal diurnal fluctuations in the rate of sweating under natural conditions are not taken into account when sweat is collected over a short period in an artificial environment.

Vellar (personal communication, 1969) has found that the iron concentration of cell-rich sweat collected from the trunk falls considerably with time when serial collections are made on subjects kept in an environmental chamber. He has also demonstrated a secondary increase in sweat iron concentration when sweat collection is recommenced after an intervening period of cooling. For this reason Vellar concluded that the fall in sweat iron concentration with time was probably the result of an increase in sweat rate with the

progress of sweating rather than to any external contamination of the skin surface or sweat gland lumens and ducts.

A further indictment of the chemical method for measuring sweat iron losses stems from two reports in which sweat iron excretion was measured simultaneously in the same subjects by both chemical and radioisotopic techniques (Moore, 1961; Green *et al.*, 1968). In both studies, sweat iron loss measured chemically was several-fold greater than with the isotope technique. However it should be noted that proponents of the view that the skin is a major avenue of iron loss argue that this poor agreement reflects unfavourably on the radioisotopic technique for reasons which will be discussed below.

The general conclusion which may be drawn from reported studies on chemical determinations of sweat iron losses is that it is not possible to quantify daily iron excretion through the skin using this approach.

The idealised experiment to measure sweat iron losses would involve total sweat collection over an entire 24-hour period under natural conditions, yet without the attendant risk of contamination. For technical reasons, such an experiment seems unattainable.

Whereas studies on the chemical iron content of sweat have failed to quantify daily skin iron losses, they have provided some important information which is germane to the general understanding of how iron is lost through the skin, and the mechanisms which regulate this loss. Firstly, cell-rich sweat indisputably contains more iron than does cell-free sweat, indicating that cellular desquamation is quantitatively more important than the secretion of iron in sweat. Secondly, the iron concentration of cell-rich sweat is lower in iron deficient subjects compared with normals (Hussain and Patwardhan, 1959b; Prasad, *et al.*, 1963), presumably because of a lower iron content of desquamated cells.

Hussain and Patwardhan (1959b) also showed that the iron content of cell-free sweat was markedly diminished in iron-depleted individuals, which is in keeping with the observations of Vellar (1968) that the iron content of cell-free sweat was significantly correlated with the serum iron level. From these data, it may be inferred that iron loss through the skin may be regulated, in part at least, by the state of iron repleteness. Another significant observation was that the iron excretion in cell-free sweat did not change appreciably during acclimatization (Consolazio, *et al.*, 1963). Apte and Venkatachalam (1962) found greater iron losses in cell-rich sweat during the cold season than the warm season. Finally, Vellar (1968) reported a significant correlation between the iron content of cell-rich sweat and the body surface area, which implies that skin iron loss is proportional to body size.

(ii) Radioisotopic studies

The suggestion that the skin constitutes a major avenue of iron loss came not only from studies on the chemical content of sweat. In a study using radioiron, Weintraub *et al.* (1965) found that whole-body loss of radioactivity was significantly greater than could be accounted for in cumulative stool and urine collections. They also demonstrated by radioautography that radioiron injected into the skin which is selectively localized in the basal layers of the epidermis and in the glandular and ductular epithelium of the skin appendages, is subsequently lost externally. From their studies, although not quantitative, these workers concluded that the skin plays an active and significant role in iron excretion.

Quantitative radioisotopic studies have, however, given results which are considerably lower than chemical estimates of skin iron loss. Dubach, *et al.* (1955) studied subjects who had received radioiron by injection. They found only minute traces of the isotope in large volumes of sweat which were

collected from the whole body excluding the head during 0.5 to 2.5 hour periods under conditions of artificially induced heavy sweating. Collections were made at different times for as long as 320 days following injection. Based on a number of assumptions, estimates of maximum daily sweat iron losses were made. The assumptions were that there was complete mixing of radioiron with the body iron stores, a body iron content of 5 000 mg for males, 3 000 mg for females, and a constant rate of sweat production through a 24-hour period. On this basis, maximum mean sweat iron losses in three normal subjects were calculated to be 0.45, 0.92 and 1.48 mg per day, with a range of 0.07 to 1.50 mg per day. These workers rightly expressed caution in interpreting their calculated results, based as they were on so many assumptions. But, the assumptions which they made were more likely to have overestimated sweat iron losses, and yet their estimates were still considerably lower than most figures obtained by workers using chemical determinations.

In a study carried out by Finch which was published as part of a collaborative study with the author of this thesis (Green, *et al.*, 1968), a patient with chronic granulocytic leukaemia in remission was given an intravenous injection of 1 500 μ Ci high-specific activity ^{59}Fe bound to plasma. Chemical and radioactive measurements were carried out on the sediment and supernatant fractions of cell-rich sweat collected in a plastic bag during two-hour periods in a hot room at repeated intervals over 288 days. Very low levels of radioactivity were detected, almost all of which were present in exfoliated cells, and there was no evidence of a delayed peak in radioiron excretion at any time during the experiment. Assuming that complete mixing had occurred, and that the total body iron was 3 000 mg, the calculated amount of iron lost during each two-hour period of sweating was 0.075 mg. Using a chemical method, the iron content of this sweat was approximately 0.5 mg. Despite stringent precautions, the

discrepancy, similar to that reported by Moore (1961), between chemical and radioisotopic determinations could have resulted from contamination. It is equally possible that the radioisotopic determination could have been erroneous, based as it was on the assumptions detailed above. It may be argued that most of the iron in sweat is derived from a source other than the body's miscible labelled iron pool. The divergent results obtained in such studies do, however, illustrate the basis of the controversy that exists regarding quantitative skin iron losses.

(iii) Estimates of maximum possible skin iron losses

As an alternative to actually quantifying the daily loss of iron from the skin, some attempts have been made to measure the maximum possible losses that could occur through this route. Defining a figure for maximum possible losses could at least resolve the controversy that has arisen from the claims that the skin is a major route of iron excretion. Moore (1964), used a chemical technique to approach this problem. Chemical iron determinations were carried out on samples of epidermis stripped by heat. Epidermis from one gram of skin was found to contain a mean of 1.44 μg iron (range 0.6 to 2.9 μg). Since the skin comprises approximately 7 per cent of total body weight, it was estimated that there would be about 4 900 g of skin on a 70 kg man, which would contain 3 to 14 mg of iron. Based on this estimate of the total epidermal skin iron pool and a replacement rate of two to three weeks for epidermis, Moore concluded that not more than one milligram iron could be lost through the skin each day. Though this calculation is indirect, based as it is on an approximation and extrapolation, it nonetheless suggests a limit for daily skin iron loss, considering that most of the iron in sweat is derived from epidermal desquamation. Similar results were obtained in an autopsy study (Green, *et al.*, 1968)

which forms part of the work presented in this thesis. Another approach using radioiron, to estimate the upper limit of skin iron losses (Green, *et al.*, 1968) indicated that between 0.2 and 0.7 mg iron is taken up by the skin each day, depending on the degree of saturation of circulating transferrin. This work, too, forms part of the present thesis (Chapter 5).

Some interesting observations were made by Jacobs and his co-workers in a series of papers on iron kinetics in the skin (Beamish and Jacobs, 1968; Cavill, *et al.*, 1969; Cavill and Jacobs, 1970; Cavill and Jacobs, 1971; Cavill, *et al.*, 1972). Estimates of the maximum amounts of iron that can be lost from the skin based either on epidermal iron pool size or skin radioiron uptake data should be interpreted in the light of their findings. In these studies observations were made on the clearance of radioiron following its intradermal injection into a site on the forearm. Analysis of the disappearance curve of radioactivity revealed three separate exponential clearance phases. The first two phases were short, ($T_{1/2}$ of approximately 0.5 hours and one day) and were taken to represent the clearance of unbound and transferrin-bound radioiron respectively. The third, slower, phase had a $T_{1/2}$ of approximately 60 days, (range 43 to 100 days), which is considerably longer than the estimated turnover time of 26 days for epidermis (Rothberg, *et al.*, 1961). The long clearance of radioiron from the skin compared with the renewal rate of the cells that contain it, suggests that iron in the epidermis may be conserved by extrusion from the maturing cells and reabsorption into the basal layers through a mechanism analogous to the conservation of DNA in epithelia (Cutright and Bauer, 1967).

Further support for this hypothesis was provided by the observation that during the slow phase of clearance of radioiron from the skin, only a small fraction of the fall

in skin radioactivity could be recovered in squames collected from the site of injection (Cavill, *et al.*, 1969). This could imply a feedback of iron from the skin to the body iron pool, rather than its loss from the skin surface. It was also concluded from subsequent studies in this series that the amount of iron entering skin cells is decreased in iron deficiency anaemia (Cavill and Jacobs, 1971) and that the rate of iron exchange between epidermal cells and the interstitial fluid is increased in patients with iron overload (Cavill, *et al.*, 1972). The implications of these findings are that whatever the actual amounts of iron lost through the skin, this could be subject to a regulatory influence depending on the state of the body iron stores.

For completeness, other possible routes of normal iron loss from epidermal appendages such as the hair and nails should be mentioned. Because their magnitude is so small as to be negligible in relation to total external iron exchange, they are only of academic interest in this context. The two most recent studies on this question indicate that the yearly loss of iron is 1.22 mg from hair (Johnston, 1958) and 0.85 mg from the nails (Vellar 1970). Corresponding figures for daily losses would be 0.003 and 0.002 mg respectively. Jacobs and Jenkins, (1960) reported a lower than normal iron content in the finger nails of iron deficient subjects.

(g) Pathological iron loss from the skin

Since the major portion of dermal iron loss takes place through cellular desquamation from the skin surface, it might be anticipated that dermatological conditions associated with a more rapid turnover rate of the epidermis would give rise to excessive skin iron losses. This has in fact been alluded to or confirmed in a limited number of studies. The turnover time for epidermal cells in psoriasis has been shown

to be three to four days, against the normal of 26 to 28 days (Rothberg, *et al.*, 1961), and in a study on epithelial iron losses in psoriatic patients with generalised exfoliative dermatitis, it was found that peak losses of injected radioiron occurred between the second and fourth days (Reizenstein, *et al.*, 1968). Although no attempt was made to quantify iron losses in absolute terms, these authors suggested that the skin iron loss in psoriasis would be around seven-fold greater than normal. In another study on iron metabolism in skin disease Marks and Shuster (1968) noted that hypoferraemia was a common finding in a group of patients with dermatoses (eczema or psoriasis) involving more than 75 per cent of the skin surface. In patients with true iron deficiency (diminished iron stores), excessive loss of iron in skin scales was considered to be partly responsible for the iron depletion. Direct measurement of the iron content of skin scales in three patients gave values for daily skin iron excretion of 2.4, 2.7 and 7.3 mg.

A final point worthy of mention is the observation that whereas normal skin cells migrate towards the surface and lose their nuclei and other intracellular constituents before they are desquamated as scales, the situation in generalized exfoliative dermatitis is fundamentally different. In this condition, cells do not lose their intracellular constituents, but are lost from the body intact (Croft, *et al.*, 1968a). It is therefore unlikely that in psoriasis any iron, once it has entered epidermal cells, can be conserved by the body through the mechanism proposed by Cavill, *et al.*, (1969).

III. IRON LOSSES IN FEMALES OF CHILDBEARING AGE

In addition to the obligatory losses of iron that have been detailed above, and which occur in normal subjects of both sexes and all ages, females of childbearing age incur further physiological iron losses as a result of menstruation, pregnancy or lactation. These losses are quite considerable in relation to the amounts excreted through other avenues, and so constitute a significant term in the iron balance equation for women of fertile age. It is largely because of these extra iron losses that there is such a high prevalence of iron deficiency anaemia in females between the menarche and the menopause. Scott and Pritchard (1967) showed an absence of stainable iron in the bone marrows of 24 per cent of 114 college women, and a marked diminution in a further 42 per cent. In another study, Monsen and co-workers found a 10 per cent incidence of iron deficiency anaemia in menstruating women (Monsen, *et al.*, 1967). The incidence of iron deficiency anaemia during pregnancy is higher still. In one survey, it was found to vary from 10 to 20 per cent in communities with adequate levels of nutrition, to 50 per cent in poorly nourished groups (Committee on Iron Deficiency, 1968). Another report based on world-wide figures gave a range of incidence between 21 and 80 per cent, depending on the area (WHO Scientific Group on Nutritional Anaemias, 1968). However, in this report there was an even higher incidence (40 to 99 per cent) of iron depletion without anaemia, as evidenced by a transferrin saturation below 15 per cent.

Many studies have been carried out to define the iron losses which result from menstruation and pregnancy, and the subject has been dealt with in a number of recent symposia and excellent reviews (Hallberg, *et al.*, 1966a; Rybo, 1970; Pritchard and Scott, 1970; Rybo, 1973; Wallerstein, 1973). The range and the average iron loss incurred by females through normal menstruation and pregnancy has been well

documented. In the literature on this subject, serious dispute is conspicuously lacking, most published figures being in fairly good agreement. In the original work which is presented in this thesis, no attempt is made to examine the question of iron losses in women of childbearing age. However, for completeness, a brief review of knowledge in this subject is presented in this section.

(a) Normal menstrual losses

Menstrual iron losses have been measured either by chemical determination of iron or haemoglobin in the menses, or radioisotopically using ^{59}Fe or ^{51}Cr labelling techniques. Results with these various techniques are generally in good agreement, and have been reviewed by Hallberg, *et al.*, (1966a).

From the studies reported in the literature, it is apparent that there is great individual variation in menstrual losses. In one study (Frenchman and Johnston, 1949) losses varied between 2.3 and 79 mg iron per period.

The greatest contribution to our knowledge on the subject has come from a comprehensive series of studies carried out during the last decade by a group of Swedish investigators working with Hallberg. Their results agreed essentially with those from previously reported studies, but the thoroughness both of their investigations and analysis of data led them to a number of interesting conclusions concerning the factors influencing variations in menstrual iron loss. For this reason, it suffices to review mainly their data, and to allude only briefly to other studies in the voluminous literature on this topic.

The study which formed the basis for the analysis by the Swedish group was carried out on 476 randomly selected women (Hallberg, *et al.*, 1966a). Menstrual losses were measured after conversion of haemoglobin to alkaline haematin, and expressed as millilitres of blood by reference to the

circulating haemoglobin concentration. Iron contained in the menses is, apart from a small amount of non-haemoglobin iron present in the shedded endometrial lining, due to blood. The iron content of whole blood is approximately 0.5 mg per ml, so that figures for menstrual losses expressed in millilitres of blood may be readily converted, by halving them, into milligrams of iron. The mean menstrual blood loss for the entire group was 43.4 ml, but there was a marked skewness in distribution because of a relatively high number of individuals with heavy losses. After excluding all women who were subjectively either not healthy or did not have normal menses, as well as those with a haemoglobin concentration below 12 g per 100 ml, a mean corpuscular haemoglobin concentration (MCHC) below 30 per cent, or a plasma iron concentration below 80 μ g per 100 ml, a near normal distribution curve for menstrual blood losses was obtained for the 183 remaining women. The average loss for this group (mean $\pm$ SE) was 33.2 ± 1.6 ml which is equivalent to approximately 0.5 mg iron per day. However, 5 per cent of the women in this 'normal' group had losses which exceeded 76.4 ml per month (about 1.25 mg iron per day).

From this and other studies in their series, the Swedish group were also able to define various factors which influence menstrual blood loss. Despite the wide variation between subjects, in the same individual menstrual losses were remarkably constant from month to month (Hallberg and Nilsson, 1964; Hallberg, *et al.*, 1966b). Losses did not appear to be influenced by age, except that the oldest group (50 years) had a greater mean loss, probably because of their higher incidence of pelvic pathology. Increasing parity, on the other hand, was significantly correlated with greater menstrual losses (Rybo, 1966a). Both these observations were confirmed in a study by Cole, *et al.*, (1971), who also showed that menstrual losses were correlated with a woman's size. The amount of

blood lost was correlated with the duration of a period, but not with the interval between periods or the age of menarche (Rybo, 1966a). A comparison of monozygotic and dizygotic twins, revealed a much greater concordance in menstrual losses between the former, which suggests that the variation in normal blood losses is affected by genetic factors (Rybo and Hallberg, 1966).

Figures for menstrual iron loss such as those which have been cited are probably valid, although they are based on measurements of blood (haemoglobin) loss, rather than direct iron estimations. Some non-haemoglobin iron must be lost in cellular debris during menstruation. This must, however, be negligible, since studies in which menstrual losses were measured by chemical determination of iron (Schlapphoff and Johnston, 1949; Millis, 1951; Hytten, *et al.*, 1964) have in general given figures closely comparable to those obtained by haemoglobin measurement.

It may be observed that the range of published figures for the mean calculated daily iron loss resulting from menstruation fit in well with the radioisotopic study of total body iron loss carried out by Finch (1959). His finding, that menstruating women excreted 0.6 mg iron per day more than non-menstruating women, offers indirect support for the validity of results obtained by direct measurements of menstrual iron losses.

Oral contraceptive agents and contraceptive intra-uterine devices have been found to significantly alter the volume of menstrual blood loss, and because of their current widespread use, it is important to consider their effects on menstruation. In the study published by Cole, *et al.*, (1971), the mean menstrual blood loss in women using oral contraceptives was 12.7 ml, and in women with intra-uterine devices 56.3 ml, compared with 37.9 ml in women using neither form of contraception. It has also been noted that whereas the combined

type of oral contraceptives reduce menstrual blood losses, those of the sequential type increase the blood loss (Nilsson and Solvell, 1967).

(b) Excessive menstrual losses

A number of causes of uterine pathology, particularly fibroids, result in excessive menstrual blood loss. However, evidence of endometrial pathology is frequently lacking in menorrhagic patients (Rybo, 1973). Menorrhagia may be defined statistically from data on the distribution pattern of menstrual losses in the normal population. A more meaningful definition with reference to iron balance was arrived at by Rybo (1973), from a consideration of the amount of blood which could be lost each period without a significant increase in the risk of developing iron deficiency. He found that the haemoglobin concentration, MCHC and plasma iron concentration were all decreased when the range of blood loss was 60 to 80 ml per period or more compared with losses less than this. From this he concluded that the tolerance limit of menstrual blood loss is between 60 and 80 ml, and that loss exceeding this figure should be considered pathological. The figures given by Rybo provide a useful guideline, but must obviously be considered in relation to the iron intake of a population or a particular individual. On this basis, 11 per cent of the women whom he studied had menorrhagia.

When demonstrable uterine pathology is lacking, excessive menstrual blood loss is presumed to be related to hormonal factors. Oestrogenic overactivity results in endometrial hyperplasia and may presumably give rise to increased menstrual bleeding. Other than individuals with primary coagulation disorders, defects in the blood coagulation or fibrinolytic mechanisms are seldom found in patients with menorrhagia (Rybo, 1973). However, an interesting observation

has been made on the fibrinolytic activity of endometrial tissue from women with menorrhagia compared to a control group. In 50 per cent of the women with menorrhagia, levels of plasminogen activators were higher than normal. (Rybo, 1966b; Koutsky, *et al.*, 1969).

(c) The iron cost of pregnancy

Pregnancy constitutes a significant drain on the maternal iron stores. The iron 'cost' of pregnancy results from iron contributed to the foetus, iron contained in the placenta and cord, and from blood loss at the time of delivery. This is partly offset by absence of menstrual loss during, and for a time after pregnancy. An amount of iron is also required for expansion of the red cell mass, but since this is not lost from the body, it need not be taken into account in a consideration of external iron exchange during pregnancy.

The iron costs of pregnancy are not equally distributed in time, but increase during the latter half of pregnancy. Nonetheless, it is convenient to consider the total iron cost of pregnancy, and to calculate from this the average daily iron loss during pregnancy. Data on the average range of iron loss incurred as a result of pregnancy are shown in Table 2.2.

The iron conserved as a result of amenorrhoea, during, and for three months after pregnancy (in the non-lactating female), amounts to approximately 215 mg. This is based on an average of 13 missed periods and a mean menstrual loss of 33.2 ml blood (Hallberg, *et al.*, 1966a). The total net iron cost of a pregnancy therefore ranges from 120 to 577 mg, which amounts to a daily loss of between 0.4 and 2.0 mg over and above the normal iron losses which take place through other routes. These figures are based on observed ranges from independent studies carried out by different workers, and as such, are only approximations.

Table 2.2. Summary of the average iron 'cost' of a normal pregnancy (after Moore, 1964)

Source of iron deficit	Range of iron loss (mg)	Reference
Contribution to foetus	201-372	Widdowson and Spray (1951)
Lost in placenta and cord	34-170	McCoy, <i>et al.</i> , (1961)
Lost at delivery (On basis of 12 g haemoglobin per 100 ml)	100-250	Newton, <i>et al.</i> , (1961)
Total	335-792	

A number of variables will influence the loss incurred in an individual pregnancy. With respect to the contribution made to the foetus, birth weight and whether the pregnancy is single or multiple, determines the amount of iron loss. Blood loss at the time of delivery is highly variable. In one large study on 524 women, the average amount was 186 ml (75 mg iron based on 12 g haemoglobin per 100 ml), but individual losses varied from as little as 5 ml to as much as 1600 ml (Mathie and Snodgrass, 1967). Blood loss at delivery tends to decrease with increasing parity. The mean blood loss in 74 multiparae was 280 ml, compared to 507 ml in 26 primiparae (Newton, *et al.*, 1961). Operative procedures during labour such as episiotomy, and manual removal of the placenta increase the quantity of blood loss. In uncomplicated Caesarean sections, Wilcox, *et al.*, (1959) and Pritchard, *et al.*, (1962) found an average blood loss of about one litre.

In quantitative terms, iron losses attributable to menstruation and pregnancy would appear to constitute the single most important drain on body iron in normal women of fertile age. These mechanisms of iron loss are of considerable importance in the pathogenesis of iron deficiency amongst females, even when falling within the physiological 'normal range', but more particularly when they become excessive with menorrhagia, or repeated pregnancies.

(d) Lactation

Published figures on the iron concentration of human milk have varied widely. Feuillen and Plumier (1952) found between 270 and 1210 (mean 580) μg per litre. In other studies, the reported range was 1720 to 2100 μg /litre (Karmarkar and Ramakrishnan, 1960), and 182 to 268 μg per litre (Vestermarck and Andersen, 1966). Based on the figures of Feuillen and Plumier, which appear to be the most widely accepted, and a daily volume of milk secretion of 800 ml, lactational iron losses could range from 0.2 to 1.7 mg per day, with a mean of 0.5 mg per day. Although lactational iron loss may be partly offset by continuation of amenorrhoea, the menstrual pattern is often re-established when lactation is prolonged, as is often the case in poorly nourished developing populations. In this situation the iron balance of the mother, already taxed by a recent pregnancy, is often seriously jeopardized.

IV. BLOOD DONATION

Donation of blood, although neither physiological nor pathological, should not be overlooked in a consideration of possible routes of iron loss from the body. Since one millilitre of blood contains about 0.5 mg iron, donation of one unit of blood

(approximately 400 ml) incurs an iron loss of around 200 mg. Regular donation of this amount twice yearly would therefore result in an additional average iron loss of 1.0 mg per day. Iron deficiency anaemia occurs in regular blood donors, particularly in women of child-bearing age, in whom the incidence has been reported to vary from 1.7 to 17.4 per cent in different centres (Frick, 1970).

V. TOTAL BODY IRON LOSS

The composite loss of iron from the body, represents the sum of all the iron lost from the individual compartments detailed above. Several attempts have been made to measure composite body iron loss by using various radioactive iron tracer techniques. With any of these techniques, the derivation of quantitative figures for daily iron loss depends on at least three general assumptions. The first is that complete mixing takes place between the administered tracer and the body iron pool so that changes in the level of radioactive iron may be taken to reflect parallel changes in body iron as a whole. Secondly, it must be assumed that subjects are in a steady state, with a constant rate of absorption and excretion which balance each other. Thirdly, assumptions need to be made as to the size of the total body iron pool, in order to calculate absolute figures for iron loss from fractional changes in tracer levels. In addition, particular assumptions must be made depending on the technique employed.

Radioiron was first used to assess total body iron losses by Dubach and associates (1955). They injected ^{59}Fe intravenously in a group of subjects, and measured the radioactivity excreted in the stools, urine and sweat over a period of 140 days. Assuming a steady state, and by relating the percentage of injected radioiron which was lost in excreta to estimates of the total body iron, they calculated a normal obligatory loss

of iron from the body of 0.5 to 1.0 mg each day.

A number of workers have estimated composite iron loss from the body by measuring the rate of radioiron loss in a whole-body counter following the oral or intravenous injection of ^{59}Fe . The results obtained by different workers have shown considerable variation, are summarized in Table 2.3.

Table 2.3. Composite body iron loss measured in a whole-body counter
(Literature review modified after Will and Boddy, 1967)

Author	Duration of study (days)	No. of subjects ^x	Daily radioiron loss (per cent per day)	Daily iron loss (mg) [†]
Bonnet, <i>et al.</i> , (1960)	160	1	0.14	4.9
Price, <i>et al.</i> , (1962)	100	3	0.110-0.182	3.9-6.4
Ibid	"	2 (aplastic anaemia)	0.103-0.173	
Ibid	"	14 (iron deficient polycythaemia vera)	0.009-0.044	0.3-1.5
Saito, <i>et al.</i> , (1964)	240	12	0.030	1.0
McKee, <i>et al.</i> , (1965)	60-270	3	0.06-0.24	2.1-8.4
Reizenstein & Brann, (1965)	60-194	6	0.193	6.8
Schiffer, <i>et al.</i> , (1966)	105-210	16	0.186	6.5
	"	10 (aregenerative anaemia)	0.112	
Weintraub, <i>et al.</i> , (1966)	100	6	0.22	7.7
Will & Boddy, (1967)	80-156	5	0.04-0.26	1.4-9.1
Holt, <i>et al.</i> , (1967)	100	4	0.031	1.1
McKee, <i>et al.</i> , (1974)	400	8	0.011-0.110	0.4-3.9

^x Subjects were either males or non-menstruating females, and normal, unless otherwise stated.

[†] Calculated on an assumed total body iron content of 3.5 g (see text).

Reported figures for the percentage of injected radioiron lost from the body in normal subjects ranged from 0.01 to 0.26 per cent per day, with a scatter of results lying between these extremes. This twenty-fold difference represents a serious discrepancy, regardless of the assumed size of the body iron pool to which these figures for fractional rates of loss are applied in order to calculate absolute daily iron losses.

In general, the fractional rate of loss of radioiron from the body has been derived in the following manner. Whole-body radioactivity is measured at regular intervals after the administration of radioiron. The whole-body count rate falls fairly steeply during the first 10 to 20 days. This fall is more marked following oral administration of the tracer (Price, *et al.*, 1962) than when the tracer is given by injection (Saito, *et al.*, 1964) because of faecal excretion of unabsorbed radioiron. After the initial drop, body radioactivity rises again to approximately 97 per cent (in the case of injected radioiron) of the value recorded immediately after tracer administration (Saito, *et al.*, 1964). The fall and subsequent rise in whole-body radioactivity has been interpreted to represent internal redistribution of the tracer, most of which is cleared from the plasma to the bone marrow, and then reappears in the blood as newly formed red cells are released into the circulation. Beyond the time of reappearance of radioiron in this generation of newly formed red cells, the fall in whole-body radioactivity can be described by a single exponential function, and from the exponent of this function, the rate of radioiron loss from the body has been calculated.

This general approach is open to criticism on a number of grounds. Most of the radioiron (80 to 90 per cent) is contained in circulating haemoglobin during the approximately 120-day lifespan of the cohort of labelled red cells, and is therefore not available for internal exchange or external loss

apart from physiologic blood loss through the gastrointestinal tract. Only a small fraction (less than 20 per cent) of the tracer is present in the non-haemoglobin iron pool, from which a proportion of total body iron loss is known to occur, mainly through epithelial desquamation.

Price, *et al.*, (1962) concluded that the fall in body radioactivity between 20 and 100 days following radioiron administration represented only the loss from iron compartments other than circulating haemoglobin. This was based on the observation that there was no detectable fall in radioactivity in blood sampled during this period. Daily gastrointestinal blood loss of 1.0 ml per day (Ebaugh, *et al.*, 1958; Hughes Jones, 1958), whilst significant in terms of iron balance, would result in an overall fall of only about 2 per cent of circulating radioactivity during 80 days, assuming a total blood volume of 4.5 litres. The counting time used for blood samples was not stated in the study by Price, *et al.*, (1962), but unless this was long enough to achieve an error of less than 2 per cent, the small anticipated fall in circulating radioactivity could have gone undetected. It is of interest, however, that the rates of ^{59}Fe loss from the body in two patients with negligible incorporation of radioiron into red cells caused by aplastic anaemia were 0.103 and 0.173 per cent per day. These were similar to the rates found in three normal subjects (0.110, 0.110 and 0.182 per cent daily). In the study conducted by Schiffer, *et al.*, (1966) patients with aregenerative anaemia had a significantly lower mean rate of radioiron loss (0.112 per cent per day) compared with normal subjects (0.186 per cent per day).

Regardless of the relative contributions made by blood loss and by other non-haemoglobin sources to total iron losses, it is evident that the rate of loss of radioiron from the body cannot be used as a valid index of whole-body iron excretion

until all body iron pools have become uniformly labelled. This requirement is not met for some considerable time following the administration of radioiron, as the erythron, which has a high avidity for iron introduced into the plasma compartment, acquires a higher specific activity than other body iron pools. Labelling of the erythron is gradually diminished only through successive cycles through reticulo-endothelial cells, as the iron is released from effete red cells at the end of their lifespan.

It has been shown in normal subjects that complete mixing of radioiron with the total exchangeable body iron pool takes place only after approximately one year, equivalent to about three or four full red cell lifespans (Finch, 1959). With the destruction of each successive generation of red cells, and the consequent release of a wave of radioiron, some further mixing of the tracer with the body iron pool occurs, although most of the radioiron is reutilized for new haemoglobin synthesis. This redistribution of the tracer continues to occur until all miscible body iron compartments attain the same specific activities.

In theory, therefore, it would be valid to use the rate of whole-body radioiron loss as an index of body iron excretion only after approximately one year, when complete equilibration has taken place between the tracer and unlabelled body iron. This is practically not feasible, because of the limitation imposed by the relatively short half-life of ^{59}Fe (45 days).

In practice, it has been claimed that the rate of radioiron loss from the body remains relatively unchanged through successive lifespans of labelled red cells, (Saito, *et al.*, 1964). Although troughs in the exponential function representing falling whole-body radioactivity were observed at the anticipated times of destruction of cohorts of labelled red cells, the slope of the function remained the same between these troughs. Furthermore, Dubach, *et al.*, (1955) found no increase in the amount of radioiron excreted in the stools, urine or sweat following destruction of

the labelled population of red cells. Such findings tend to vindicate the use of ^{59}Fe in relatively short term experiments (less than one year) to measure total body iron losses. But, the wide reported range of ^{59}Fe loss-rates (0.01 to 0.26 per cent per day : Table 2.3) in normal subjects attests to the inherent inaccuracies of the technique.

Most workers who have used a single exponential function derived from whole-body counting to measure total iron losses acknowledge that the method is inaccurate, but contend that it offers the best approximation which is available. Saito, *et al.*, (1964) in calculating the rate of whole-body radioiron loss, took into account the effect of tracer redistribution which occurs at the completion of each lifespan of labelled red cells. This was done by subtracting the percentage fall due to radioisotope redistribution following destruction of a generation of labelled red cells, from the slope of falling whole-body radioactivity extending over one complete red cell lifespan. Their results for the rate of whole-body iron loss are consequently lower than those obtained by most other workers, but since they eliminate a potential source of interpretive error, perhaps provide a more accurate index of the rate of iron loss.

The results of studies measuring iron loss by whole-body counting are generally given in terms of the rate of fall in retained body radioactivity. In order to express these data in terms of absolute iron losses, assumptions must be made on the body iron pool size from which iron loss takes place. For this reason, many workers have chosen not to express their results in direct figures for daily iron loss. However, for such results to be meaningful in terms of quantitative external iron exchange, some attempt must be made to calculate the actual amounts of iron which the radioiron loss-rates may represent.

The estimated total body iron content of a normal adult male is approximately 3.5 g (Drabkin, 1951; Bothwell and Finch, 1962, p. 4; Fairbanks, *et al.*, 1971, p. 47). If one applies the published data for daily radioiron loss-rate to this figure, calculated iron excretion values are obtained which vary from 0.4 to 8.4 mg iron per day in normal subjects (Table 2.3). The data from most workers, with the exceptions of Saito, *et al.*, (1964) and Holt, *et al.*, (1967), give calculated daily iron losses which are greatly in excess of amounts that would be compatible with the concept of limited external iron exchange.

More precise translation of ^{59}Fe whole-body excretion data into absolute figures for daily body iron loss must await the application of such data to a valid theoretical model describing tracer redistribution in a multicompartment system which is analogous to body iron pools. A number of different mathematical models of iron metabolism which fit the results of ferrokinetic studies have been described (reviewed by Cavill and Ricketts, 1974). This illustrates the non-uniqueness of such models, and suggests that the figures that would be obtained for body iron loss would differ according to the model chosen.

Although quantitative data on body iron excretion derived from whole-body counting data should be interpreted with reserve, some useful information has arisen from studies on the comparative rates of radioiron loss in subjects with differing degrees of iron repletion. Price, *et al.*, (1962) noted a significantly lower rate of loss in iron deficient patients with polycythaemia vera, compared to normal subjects (Table 2.3). On the other end of the spectrum, there are no published figures on the rate of radioiron loss in iron-overloaded individuals measured by whole-body counting. However, in a long-term iron excretion study, which forms a part of this thesis (Chapter 4), iron losses in subjects

with dietary iron overload were significantly higher than in subjects with normal iron stores (Green, *et al.*, (1968).

The major problem imposed by the use of the ^{59}Fe isotope of iron is its relatively short half-life in relation to the time span that is required to allow for adequate mixing of the tracer with the body iron pools. To overcome this difficulty, Finch (1959) developed a method to measure long-term body iron turnover using the beta-emitting iron isotope ^{55}Fe , which has a half-life of 2.94 years. Following the intravenous administration of ^{55}Fe , he measured the fall in radioactivity of circulating haemoglobin for periods of three to five years. A sharp fall in the radioactivity was noted during the first year, but after this the decline assumed a constant more gradual rate, which was exponential. The first slope was taken to represent equilibration between the radioiron and miscible body iron pools; the latter component to represent loss from the body. By relating the latter slope to calculated figures for the miscible body iron pools, estimates were made of the total iron losses in several groups of subjects. The mean calculated iron loss in six men was 0.61 mg per day, 0.64 mg per day in 12 non-menstruating women, and 1.22 mg per day in 12 menstruating women. These observations were extended, using the same approach, in order to evaluate the influence of certain physiologic variables on iron loss (Green, *et al.*, 1968), details of which form a part of this thesis (Chapter 4).

The results obtained by Finch (1959) agree well with those of some shorter-term studies using ^{59}Fe radioiron such as those reported by Dubach, *et al.*, (1955) and Saito, *et al.*, (1964).

In a recent study (McKee, *et al.*, 1974) long-term iron excretion was measured in the same subjects both by ^{59}Fe loss in a whole-body counter and by following the fall in ^{55}Fe radioactivity in red cells. These workers found a marked fluctuation in the pattern of circulating ^{55}Fe radioactivity over periods

extending for up to 1400 days, and attributed this to the lack of equilibration between radioiron and body iron pools. They concluded that both radioisotope techniques were unsuitable for quantifying daily iron losses.

VI. IRON LOSS IN INFANCY AND CHILDHOOD

There is little information about iron loss in children up to the age of puberty. This is due mainly to the ethical problem of obtaining informed consent for investigative procedures on normal children. However, the question of obligatory iron losses in this age group is important, because iron requirements for growth impose an additional burden which jeopardises the maintenance of adequate iron nutrition. The rate of growth in body size and haemoglobin mass is particularly marked during infancy, and in proportion to food intake, iron requirements are greater than at any other period of life (Lowe, *et al.*, 1969). As a result, iron deficiency anaemia amongst the paediatric age group is commonest at the end of the first and the beginning of the second year of life (Lahey, 1957; Betke, 1970). It may be calculated that from birth to the age of 17 years, in order to attain normal iron status, body iron must increase from 280 mg in the newborn, to between three and four grams. This would require an average daily positive iron balance of approximately 0.6 mg iron (Saddi and Schapira, 1970).

Quantitative estimates of iron loss in children have been confined to the gastrointestinal tract, an avenue which appears to constitute a significant route of iron loss, particularly during infancy. Cavell and Widdowson (1964), measured faecal iron losses in neonates, and found a high iron concentration in meconium (1.7 mg per 100 g). In breast-fed babies, they found that the faeces contained approximately ten times as much iron as the food during

the first week of life, and calculated that the mean iron loss during this period amounted to 3.6 mg per day. In another study Widdowson (1964) found that faecal iron loss exceeded food iron during the first two months of life, during which time total iron losses amounted to more than one mg per day. Using radioiron, Garby, *et al.*, (1964), measured faecal iron losses in three infants over 100 days. They found a daily faecal excretion rate of 0.03 mg per kg body weight which, on a weight for weight basis, is about five-fold greater than figures obtained in normal adults using the same technique (Dubach, *et al.*, 1955). Evidence of increased gastrointestinal blood loss of undetermined aetiology during infancy has also been reported by other workers (Hoag, *et al.*, 1961; Elian, *et al.*, 1966).

Although direct measurements have not been made, it has been proposed that iron loss through the skin may be greater in children than in adults, because of their relatively greater surface area. For this reason, Burman (1973) suggested that the calculated iron loss in childhood should be related to surface area rather than body weight, when based on adult figures. On this basis, he calculated daily iron losses at age one, three and seven years to be 0.25, 0.33 and 0.50 mg respectively. These figures are comparable to the estimate that iron losses rise progressively from 0.1 to 0.6 mg per day from infancy through to adolescence (Committee on Iron Deficiency, 1967).

Numerous pathological conditions may give rise to increased gastrointestinal blood and iron losses in the paediatric age group (Burman, 1973; Sjölin and Wranne, 1968; Betke, 1970). Of particular interest is the finding by Elian and associates (1966) of a significantly higher daily faecal blood loss in infants with acute diarrhoea (1.85 ml) compared with normal infants (0.43 ml). The

common occurrence of gastroenteritis; especially in poorly nourished infants, makes this an important factor to consider in the assessment of paediatric iron requirements. It has also been suggested that gastrointestinal intolerance to whole cow's milk may cause significant chronic blood loss (Lahey and Wilson, 1966), but this has been disputed (Diamond and Naiman, 1967).

VII. ANIMAL STUDIES

Iron excretion has been studied in the dog, rat and mouse. The quantitative results of these animal studies, although not directly applicable to man, are of interest from the comparative point of view, and give some insight into the factors which may regulate iron loss from the body. The literature on various aspects of iron excretion in animals which may be relevant to man will be reviewed chronologically.

The earliest reported studies using radioiron to measure iron excretion were carried out on dogs by Hahn and his co-workers (1939), who found negligible amounts of iron loss in the urine, compared with the bile and faeces. A significant finding was their observation that faecal and biliary radioiron losses increased markedly when haemolysis was induced in dogs by the administration of acetyl-phenylhydrazine. However, the iron loss in a plethoric dog was the same as in an anaemic dog. In an extension of these studies (Hawkins and Hahn, 1944) it was shown that although the normal biliary iron loss (0.2 mg per day) was increased ten-fold following acetyl-phenylhydrazine induced haemolysis, only 3 per cent of the iron released from broken-down red cells was excreted in the bile. Administration of large amounts of iron, either by mouth or by injection, did not result in increased biliary iron loss.

Stewart, *et al.*, (1950) failed to detect significant amounts of iron loss through the skin of dogs given ^{55}Fe by mouth or intravenous injection. They concluded that the skin was an insignificant route of iron excretion in this species, which is almost devoid of sweat glands. However, their experiments extended over seven days only following radioiron administration, which is probably too short to have detected radioiron loss through the desquamation of labelled epithelial cells.

Stevens, *et al.*, (1953) measured whole-body iron loss in normal and iron-loaded mice by sacrificing animals at various times up to 300 days following injection of ^{55}Fe and determining the retained radioactivity in the blood, liver, spleen and the remainder of the carcass. The rate of radioiron loss was similar from all sites, indicating that body iron compartments had become uniformly labelled, and suggesting that total body iron turnover could be monitored by sampling a single compartment such as circulating blood. The rate of radioiron loss was also similar in normal and iron-loaded mice (approximately 0.5 per cent per day). However, the same rate of tracer loss would represent a larger quantity of iron in an iron-loaded than a normal animal. The authors concluded that greater amounts of iron could be lost by iron-loaded animals, and that the size of the body iron stores therefore played a role in regulating iron excretion. These findings were corroborated and extended in a subsequent study by the same group (Chappele, *et al.*, 1955) by repeated monitoring of ^{59}Fe radioiron retention for 196 days following ^{59}Fe injection. By studying groups of mice with different levels of body iron, they again found that the amount of iron excreted was proportional to the body iron store. They also noted an increased iron excretion both in animals given acetylphenylhydrazine and others with turpentine abscesses. Since it is known that these conditions would tend to raise

and lower the serum iron concentration respectively, they inferred that serum iron levels have no direct bearing on the amount of iron excreted.

Ehrenstein and Hevesey (1959) injected ^{59}Fe into pregnant rats, and in this way were able to physiologically label the body iron of the offspring, which were sacrificed at various times up to 300 days after birth to determine radioiron retention. They found an average rate of radioiron loss of 0.2 per cent per day.

In another study on mice, Bonnet, *et al.*, (1960) recorded a rate of ^{59}Fe radioiron loss of 0.39 per cent per day over 151 days. Contrasting this with the rate of loss of 0.14 per cent per day which these workers found in man, they suggested that the more rapid loss-rate in the mouse was related to the shorter red cell lifespan in this species.

Kaufman, *et al.*, (1964) studied the effect of acetyl-phenylhydrazine haemolysis on iron loss in the rat. Using ^{59}Fe , they found that the rate of radioiron loss in animals given acetyl-phenylhydrazine (0.39 per cent per day) was higher than in control animals (0.23 per cent per day). However, measurement of total body iron content at the end of the experiment (105 days) revealed greater amounts of iron in the animals given acetyl-phenylhydrazine. This indicated that the haemolysis had increased iron absorption to a greater extent than it had iron excretion.

The rat has also been used as an experimental model to study the role of the gut in iron excretion. Thirayothin and Crosby (1962) adduced evidence for the existence of an excretory route for iron via the migration of iron-laden phagocytes from the serosal surface of the intestine into the gut lumen. They demonstrated ferritin in the small intestine of rats three days after the administration of

iron-dextran by intraperitoneal injection, which subsequently disappeared. Iron given intravenously did not accumulate in this fashion.

Conrad and co-workers also showed by autoradiographic studies that ^{59}Fe injected intravenously into iron-replete rats is selectively taken up by newly formed crypt cells in the small intestine which then migrate up the villi. The iron is lost when these cells desquamate from the villus tips (Conrad and Crosby, 1963; Conrad, *et al.*, 1964). These studies helped to elucidate the mechanisms whereby iron is lost through the gastrointestinal tract. Loehry, *et al.*, (1969a and b) studied the effect of mucosal villous atrophy on the rate of cell turnover and iron loss in rats. They measured the radioactivity of desquamated epithelial cells in small intestinal washings at various times after injection of ^{59}Fe , comparing normal rats with rats infested with a nematode which causes intestinal villous atrophy. Infested rats showed an earlier and considerably higher peak of radioiron loss, demonstrating greater epithelial iron losses in states of increased cellular turnover.

Bradford, *et al.*, (1969), examined the mechanism of biliary iron loss in iron-loaded rats in an elegant electron microscopic study. They demonstrated that a portion of the iron which is concentrated as ferritin within secondary lysosomes in hepatic parenchymal cells is discharged into the bile canaliculi by a process of reverse pinocytosis. Although this pathway does not account for all the iron that leaves the liver cell, it provides important evidence to explain the origin of at least some of the iron in bile, and a possible regulatory mechanism of iron excretion in states of iron overload.

CHAPTER 3

AIM OF THIS THESIS

From the preceding review of the literature, it is evident that there is considerable controversy regarding the quantitative aspects of iron loss from the body in man. This controversy applies both to the magnitude of total body iron excretion and to the contributions made by individual compartments. Although it has generally been accepted that the capacity for excreting iron from the body is limited, and that iron balance is dependent mainly on variations in absorption, this concept has been challenged by several groups of workers. In particular, radioiron studies using whole-body counters, and chemical determinations of iron loss from the skin have suggested that daily external iron losses may be considerably greater than previously supposed.

The uncertainty regarding iron excretion from the body underlines a need to define in more detail, the limits of iron loss in different circumstances. Such information has a number of important applications to our understanding of iron metabolism in health and disease.

Firstly, in normal persons, who are in a state of equilibrium insofar as external iron exchange is concerned, precise data on daily iron losses may be used as a measure of the normal daily iron requirement. Such information would therefore also be useful for indicating the magnitude of iron absorption in normal subjects.

Secondly, knowledge of the limits of non-pathological iron excretion is essential to the understanding of contributory factors in the pathogenesis of iron deficiency in different parts of the world. Although excessive blood loss is the single most important cause of iron deficiency, iron balance is precarious in some regions, even in normal individuals. This may be attributed, to a large extent, to deficient dietary intake, and the relative unavailability of iron in staple foodstuffs, such as wheat, rice and corn. However,

it has been suggested that increased dermal losses of iron may be an important cause of iron deficiency in tropical climates. It is therefore important to assess to what extent loss through the skin, or any other route, may contribute to the causation of iron deficiency under various conditions.

Thirdly, it is of importance to determine the ability of the body to regulate iron losses. This would indicate whether, in states of iron deficiency, limited iron stores could be conserved, and conversely, whether excessive amounts of iron could be eliminated in states of iron overload.

The studies described in this thesis were undertaken in an attempt to quantify more accurately the range of iron excretion from the body under different circumstances. Special attention was directed to the relationship between climatic conditions and iron excretion, and to the effects of the size of body iron stores on the amounts of iron lost from the body. No single available technique for measuring iron loss is free of technical and interpretive difficulties, and for this reason, a number of methods, both chemical and radioisotopic, were used in combination. Using a multi-faceted approach, it was hoped that meaningful data could be obtained on the quantitative aspects of total and compartmental iron losses.

The experimental findings of this thesis are presented in two sections, the first dealing with total body iron losses, and the second with the relative importance of different excretory routes.

CHAPTER 4

TOTAL BODY IRON LOSSES

I. INTRODUCTION

In order to study total body iron losses, the long-term radioisotopic technique described and used successfully by Finch (1959) was employed. In this method, the beta-emitting radioisotope of iron, ^{55}Fe , which has the conveniently long half life of 2.94 years is used. High specific activity ^{55}Fe is injected intravenously and a variable but high proportion of this iron enters the marrow to become incorporated into the haemoglobin of newly formed red cells. After approximately one year, the injected tracer equilibrates with body iron. After this time, the fall in circulating red cell radioactivity is taken to represent the rate of external loss of radioiron.

II. MATERIAL AND METHODS

(a) Principle of method

The method is based on the principle of radioisotopic dilution, and its applicability to the problem of measuring iron loss depends upon a number of reasonable assumptions. The body iron pool is labelled by injecting radioactive iron, and after adequate mixing is allowed to take place, the rate of disappearance of this radioiron from a body iron compartment such as circulating red cells is assessed over a period of years. The red cell compartment is chosen because of its convenient ease of access for repeated sampling. In this way a measure of the rate of turnover of the body iron pool may be obtained which, if related to a figure for the actual size of the pool, gives an estimate of the amount of iron lost from the body. The validity of the method depends upon the premise that the subject remains in a state of reasonable iron equilibrium throughout the course of the study, so that the size of the

body iron pool remains constant over the period of observation. It may then be assumed that the amount of iron being lost from a uniformly labelled pool is being constantly replaced by a similar amount of nonradioactive iron. In this way, with progressive radioisotopic dilution, the specific activity of the body iron pool will fall exponentially with time.

It must further be assumed that the body iron pool becomes uniformly labelled. Then only can the fall in specific activity of one compartment, such as the circulating red cells, be regarded as an index of loss from the body iron pool as a whole. If labelling is not uniform, then fall in specific activity of one compartment may be due to further mixing or redistribution of the radioactive iron with the remainder of the body iron pool.

Finally, certain assumptions must be made as to the actual size of the body iron pool, in order to express figures for iron loss in quantitative terms. Because there is no satisfactory method of directly measuring body iron pool size, estimates must be made which are based on observations of tracer redistribution and on an assumed red cell mass for the subject's body-weight.

The use of such an approach to measure the rate of radioiron loss, and to calculate from that the amount of iron excreted has been shown to be generally valid in animals (Chappelle, *et al.*, 1955). The validity of these assumptions will be considered in further detail in later discussion.

(b) Subjects studied

All the subjects selected for study were healthy male volunteers ranging from 21 to 65 years of age. Specific questioning excluded any history of abnormal bleeding or anaemia in the subjects studied. Three groups of subjects

were studied in South Africa.* These groups came from climatically different areas, and were employed in either manual or sedentary occupations. Furthermore, the subjects belonged to two different ethnic groups which are known to differ with respect to their storage iron status (Mayet and Bothwell, 1964). Iron overload of varying degrees is present in the majority of adult male Bantu-speaking Negroes** in Southern Africa (Strachan, 1929; Bothwell and Finch, 1962, p. 394).

The groups were chosen primarily in order to determine whether the variables of body iron store size, physical activity and climatic conditions materially influence the quantities of iron excreted from the body.

Group I consisted of Negroes, aged between 29 and 60, who were employed as clerks and porters at the Baragwanath Hospital near Johannesburg. Their work was mainly of a sedentary nature, and involved only mild to moderate physical activity. Johannesburg has a temperate climate for most of the year.

Group II consisted also of Negro subjects who lived in Durban, which has a semi-tropical climate. All the subjects whose ages ranged from 25 to 40 years were engaged in strenuous manual labour in a factory.

* Two further groups which will be described below were studied together with co-workers in a collaborative study which has been published (Green, *et al.*, 1968).

** 'Bantu-speaking Negroes' is the preferred descriptive term for the ethnic group consisting of persons of Negro extraction indigenous to Southern Africa. However, for brevity, the term 'Negroes' will be used in this thesis to describe members of this group. This is synonymous with the previously used term 'Bantu' now widely regarded as inexact because the term refers to the family of languages spoken by these people.

Group III also resident in Durban, consisted of Indian subjects aged between 21 and 65 years who worked in a steam laundry.

The climatic conditions in which these three groups of subjects worked differed widely, and are given in Table 4.1.* The mean ages and weights of the subjects in each group were similar (Tables 4.2, 4.3 and 4.4).

Table 4.1. Climatic conditions for the working environment of groups of subjects in the long-term iron excretion study

	MEAN ANNUAL TEMPERATURE (°C)	MEAN RELATIVE HUMIDITY (per cent)
Group I (Johannesburg Negroes)	16.2	71
Group II (Durban Negroes)	25.3 (In factory)	73 (In factory)
Group III (Durban Indians)	31.3 (In laundry)	98 (In laundry)

* The two additional groups in the collaborative study referred to above were made up as follows: one group consisted of White subjects from Sedro Wooley, near Seattle Washington, U.S.A., whose ages ranged from 27 to 61 years. The subjects were involved in moderate physical activity, and the area has a temperate climate. Mean annual temperature 13.3°C, mean relative humidity 72%. The other group was composed of Mestizo subjects (interbred White, Indian and Negro stock) from Araya, Venezuela, whose ages ranged from 22 to 55 years. They were employed outdoors as heavy manual labourers. Although the mean annual temperature in Araya is 24°C, (relative humidity 74%) the subjects worked out in the sun, with the temperature during most of the year usually above 38°C between 10.00 and 14.00 hours.

Table 4.2. Johannesburg Negro subjects (Group I) in the long-term total body iron excretion study: description and results

SUBJECT	AGE AT BEGINNING OF STUDY	BODY WEIGHT (kg)	DURATION OF BLOOD SAMPLING CRT	MEAN HAEMATO- CRIT	MEAN HAEMATO- BIN	SERUM IRON (μ g per 100 ml)	TOTAL IRON BINDING CAPACITY (μ g per 100 ml)	TRANS- FERRIN SATURATION (per cent)	$T_1^{55}\text{Fe}$ ORDINATE INTERCEPT (Y) AT ZERO TIME	RED CELL TISSUE IRON	MISCIBLE IRON POOL	TOTAL IRON POOL	TURNOVER RATE per cent per year	mg per year	CALCULATED DAILY IRON LOSS	
J 1	39	82.3	53.8	46.0	15.7	82	324	25	6.03	93.2	2840	523	3363	11.5	387	1.06
J 4	38	120.0	38.0	50.0	16.5	73	277	26	2.16	99.6	4500	518	5018	32.1	1611	4.41
J 5	42	76.6	35.7	43.5	15.0	131	350	37	3.51	82.9	2500	793	3293	19.7	649	1.78
J 7	35	72.1	53.7	47.0	15.5	222	305	73	2.90	55.5	2542	2321	4863	23.9	1162	3.18
J 8	29	76.4	47.1	49.5	15.5	99	361	27	4.07	58.1	2826	2351	5177	17.0	880	2.41
J 10	26	86.4	42.3	52.5	17.8	139	323	43	2.61	75.9	3403	1458	4861	26.5	1288	3.53
J 11	45	57.4	47.1	48.0	16.1	101	317	32	3.74	88.9	2067	488	2555	18.5	473	1.30
J 13	29	76.6	37.3	48.0	15.6	143	434	33	4.29	86.7	2758	729	3487	16.2	565	1.55
J 15	39	62.3	46.2	47.0	17.4	51	215	24	3.27	66.7	2197	1340	3537	21.2	750	2.05
J 18	41	79.5	46.0	48.0	14.9	65	258	25	3.93	48.1	2863	3406	6269	17.6	1103	3.02
MEAN	35	79.0	44.7	48.0	16.0	111	316	35	3.65	75.6	2850	1393	4242	20.4	887	2.43
S.E.M.	2	5.3	2.0	0.8	0.3	16	19	5	0.34	5.6	218	316	364	1.9	125	0.34

Table 4.3. Durban Negro subjects (Group II) in the long-term total body iron excretion study : description and results

SUBJECT	AGE AT BEGINNING OF STUDY	BODY WEIGHT (kg)	DURATION OF BLOOD SAMPLING	MEAN HAEMATO- CRIT	SERUM IRON (μ g per 100 ml)	TOTAL IRON BINDING CAPACITY (μ g per 100 ml)	TRANS- FERRIN SATURATION (per cent)	T_1 ^{55}Fe INTERCEPT (Y) AT ZERO TIME	RED CELL IRON	MISCIBLE IRON POOL	TOTAL MISCIBLE IRON POOL	TURN-OVER RATE per cent per year	CALCULATED DAILY IRON LOSS			
D22	32	73.2	35.3	49.0	15.5	109	303	36	4.00	67.0	2525	1524	4049	17.3	700	1.92
D23	33	71.8	35.3	46.5	16.0	125	307	41	3.67	44.7	2350	3168	5518	18.9	1042	2.85
D24	30	61.4	35.3	50.0	16.3	90	308	29	5.11	77.4	2161	871	3032	13.6	411	1.13
D25	29	55.5	35.3	48.5	15.5	120	250	48	4.72	70.0	1895	1023	2918	14.7	428	1.17
D26	38	79.5	23.4	51.0	16.7	224	359	62	4.77	59.2	2854	2284	5138	14.5	747	2.05
D28	25	70.5	35.3	52.0	17.0	117	329	36	3.52	75.1	2581	1143	3724	19.7	734	2.01
D31	40	76.4	35.3	52.0	16.8	136	266	51	3.16	43.2	2797	3988	6785	22.0	1491	4.08
D36	32	68.2	35.3	46.0	14.8	184	319	58	4.56	57.5	2209	1878	4087	15.2	622	1.70
D37	36	72.7	35.3	42.0	13.4	106	317	33	5.51	58.2	2150	1783	3933	12.6	495	1.36
MEAN	33	69.9	34.0	48.6	15.8	135	306	44	4.34	61.4	2391	1962	4354	16.5	741	2.03
S.E.M.	2	2.5	1.3	1.1	0.4	14	11	4	0.26	4.1	107	346	415	1.0	114	0.31

Table 4.4. Durban Indian subjects (Group III) in the long-term total body iron excretion study: description and results

SUBJECT STUDY	AGE AT BEGINNING (years)	BODY WEIGHT (kg)	DURATION OF STUDY (months)	MEAN HEMATO-CRIT (per cent)	MEAN HEMOGLOBIN (g per 100 ml)	SERUM IRON (µg per 100 ml)	TOTAL IRON BINDING CAPACITY (µg per 100 ml)	TRANS-FERRIN SATURATION (per cent)	T ₁ ⁵⁵ Fe ORDNATE INTERCEPT (Y) AT ZERO TIME (years)	RED CELL IRON (mg)	MISCIBLE IRON POOL (mg)	TURNOVER RATE		CALCULATED DAILY IRON LOSS (mg)	
												per cent per year	mg per year		
D 1	43	74.1	35.3	47.0	15.9	92	350	26	3.75	2452	527	2979	18.5	551	1.51
D 3	28	57.7	35.3	47.5	16.3	85	323	26	4.11	1930	419	3249	16.9	396	1.09
D 5	49	70.9	35.3	46.0	16.4	77	317	24	4.78	100.0	2296	255	2551	370	1.01
D 6	42	55.9	35.3	54.0	18.4	112	433	26	5.92	87.8	2125	531	2656	311	0.85
D 7	21	50.0	35.3	48.0	16.6	114	343	33	4.56	80.4	1690	600	2290	348	0.95
D 9	25	65.0	35.3	53.0	18.0	178	458	39	5.28	97.0	2425	344	2769	364	1.00
D 10	41	64.1	19.5	45.0	15.4	64	318	20	3.95	91.7	2031	410	2441	428	1.17
D 11	38	69.1	35.3	48.0	15.8	139	380	37	5.79	95.9	2335	359	2694	322	0.88
D 12	29	52.3	35.3	53.5	18.0	93	302	31	6.72	83.2	1970	617	2587	267	0.73
D 13	65	70.9	35.3	47.0	14.2	99	386	26	6.29	96.1	2346	356	2702	298	0.82
D 14	49	84.5	35.3	53.5	18.3	134	404	33	5.61	74.9	3183	1421	4604	569	1.56
D 15	40	59.5	35.3	50.0	16.4	156	473	33	5.10	90.0	2094	466	2560	348	0.95
D 16	38	55.9	35.3	47.0	15.8	194	474	41	3.97	96.8	1850	267	2117	370	1.01
D 17	34	57.7	35.3	46.0	15.8	175	341	51	4.69	77.8	1869	741	2610	386	1.06
D 18	26	51.4	35.3	48.0	16.1	127	318	40	4.41	83.6	1737	534	2271	357	0.98
D 19	26	62.3	35.3	47.0	17.0	80	293	27	5.93	86.6	2061	548	2609	305	0.84
D 20	56	57.7	35.3	45.0	15.2	84	378	22	5.43	78.5	1828	704	2532	323	0.89
MEAN	38	62.3	34.4	48.6	16.4	118	370	31	5.08	88.3	2131	535	2666	371	1.02
S.E.M.	3	2.2	0.9	0.7	0.3	10	15	2	0.22	1.8	88	65	131	20	0.05

At the beginning of the study each group consisted of the following numbers of subjects:

Group I (Johannesburg Negroes) : 18.

Group II (Durban Negroes) : 20.

Group III (Durban Indians) : 20.

During the course of the study, however, which extended over three to four years, a number of subjects either changed their employment, or could otherwise not be traced. Only those subjects from whom blood samples had been collected for a sufficient length of time were included in the final analysis of data. Five subjects were also excluded on statistical grounds which are discussed below under 'calculation of results'. The final numbers in each group were:

Group I (Johannesburg Negroes) : 10.

Group II (Durban Negroes) : 9.

Group III (Durban Indians) : 17.*

• (c) Haematological measurements

In this, and other sections of the thesis, the following haematological methods were employed: Haemoglobin concentration was measured by the cyanmethaemoglobin method. The haematocrit was measured by the microhaematocrit method (Dacie and Lewis, 1968). The serum iron concentration was measured by the method of Bothwell and Mallett (1955) and the serum unsaturated iron binding capacity by the method of Bothwell, *et al.*, (1959).

* Twelve subjects each remained at the end of the study in the Seattle and the Venezuelan groups.

The serum iron concentration and unsaturated iron binding capacity was measured in all subjects at the beginning and end of the long-term iron excretion study.

(d) Radioisotopic measurements

Each subject was given 150 microcuries (μCi) of high specific activity $^{55}\text{Fe}^*$ (ca 2.5 μCi per μg) intravenously as the citrate salt (Loeffler, *et al.*, 1955). Blood samples were then withdrawn after 90 days, at which time erythrocyte utilization of the radioactive iron would be at a maximum. Thereafter, further blood samples were taken from all subjects at four- to eight-monthly intervals over a total period which varied between 19 and 54 months (mean 37 months). The number of samples taken from each subject ranged from four to nine (average, seven). On each occasion 15 to 20 ml of blood were collected and placed into bottles containing dried ammonium and potassium oxalate (2 mg per ml blood) as anticoagulant (Dacie and Lewis, 1968). After thorough, gentle mixing, two 5 millilitre quantities of whole blood were accurately pipetted into separate containers using bulb pipettes. The haemoglobin concentration and haematocrit were determined on the remaining volume of blood. The duplicate blood samples which were in glass vials with aluminium foil-lined plastic screw-caps were stored at -4°C until the end of the study, when all samples on any particular subject were processed and counted at the same time. This obviated the need to apply any corrections for isotopic decay.

* This is within the safe limit laid down by the ICRP Committee (1960). The maximum permissible burden for ^{55}Fe is 1000 μCi for the spleen which is the critical organ.

At the end of the study, the stored five millilitre samples were transferred into one-litre Kjehldahl digestion flasks in order to extract and count all the radioiron in each sample. Complete transfer was achieved in the following manner. The vials containing each blood sample were allowed to thaw, and the contents poured into a digestion flask. The vial was then rinsed out twice with distilled water, and each wash was also poured into the flask. The method described by Katz, *et al.*, (1964) was used for the processing of samples and determination of radioactivity. In this method, samples are wet-ashed using sulphuric, nitric and perchloric acids. The amount of iron present in each five millilitres of blood may be calculated from the haemoglobin concentration. It is assumed that one gram of haemoglobin contains 3.4 mg of iron (Bernhart and Skeggs, 1943), so that the calculation:

$$\text{haemoglobin concentration (g per 100 ml)} \times 3.4 \times \frac{5}{100}$$

gives the iron content of each sample in milligrams. The total amount of iron in each sample is then brought to 10 mg by the addition of a suitable volume of a ferric chloride solution containing one milligram iron per millilitre.

After wet-ashing, the iron in each sample is precipitated in a counting vial as ferric hydroxide, and this is dissolved in hydrochloric acid containing ascorbate, so that the iron goes into solution in the form of ferrous chloride. This solution is emulsified in a thixotropic gel containing powdered silicon dioxide, and PPO (diphenyloxazole) and POPOP (1,4-bis (2,5-phenyloxazolyl) benzene) as scintillant fluors.

The samples were counted in a liquid scintillation spectrometer.* At optimal gain and window settings, a 3 to 4 per cent counting efficiency was obtained for ^{55}Fe which emits a very low energy (0.0059 Mev) K-X-ray. Although other methods for counting ^{55}Fe had been described, when this study was carried out most methods involved the electroplating of iron onto copper discs.** These methods consist of lengthy procedures, and are therefore not ideally suited to the analysis of large numbers of samples. Liquid scintillation counting techniques are technically preferable for processing many samples simultaneously.

One of the drawbacks of liquid scintillation counting is that a variable degree of loss of counting efficiency may occur in each sample as a result of quenching. Liquid scintillation counting depends on the conversion of the energetic particles released by disintegration of a radioisotope into light energy, or photons, which may be detected by photomultiplier tubes. This is achieved by placing the sample in a 'liquid scintillator' which consists of a fluor

* Packard Tri-Carb (model 3002). All samples, in this and other studies which formed part of this thesis were counted for a sufficient length of time to ensure a maximum of 5 per cent error on statistical grounds.

** In the collaborative study referred to earlier, (Green, *et al.*, 1968) samples on the subjects from Washington and Venezuela were analysed in the laboratory of Dr. Clement Finch in Seattle, where the method described by Peacock and his associates (1946) was used. In this method, the extracted radioiron is electroplated onto copper discs, and counted in a Geiger end-window counter. In order to compare the results obtained in Seattle with those obtained on the South African groups described in this thesis, it was necessary to ensure that the different methods used gave the same results when applied to the same samples. For this reason, a number of samples were exchanged between the two laboratories in which the samples were analysed. In all cases, agreement was good. (See Figure A.1, appendix).

dissolved in a solvent. Bombardment of this fluor results in the emission of photons in proportion to the energy of the disintegrations. A number of factors may interfere with the passage of photons through the solvent resulting in fewer photons reaching the photomultiplier tubes. This process, known as quenching, may be produced by a number of mechanisms, the most important of which are colouring of the sample, chemical quenching and dilution of the scintillator.

Of these, colour quenching was the only one which was certainly encountered in the present study. This was evidenced by a yellowish-brown discolouration in occasional samples. The sporadic occurrence of this discolouration could not be explained. Each sample was treated in an identical fashion, so chemical and diluent quenching, although present, were not likely to have differed between samples. Even so, the degree of quenching in each sample was monitored using the technique of channels ratio based on the principle of pulse-height shift which was described by Baillie (1960). The method is based on the observation that for certain radioisotopes all quenching, regardless of the cause, results in a diminution of the photon output in a particular scintillant. This diminution is proportional to the fall in efficiency of counting for a particular radioisotope.

Because quenching causes a downward shift of the pulse-height spectrum which is proportional to the degree of quench, it is possible to assess the degree of quench by the extent of shift of the pulse-height spectrum. In practice, this is achieved by simultaneously counting two portions of the spectrum. The liquid scintillation spectrometer used in these studies was equipped with two separate channels, a 'red' and a 'green', each having its own scaler and two discriminators. The discriminators on one channel (red) were set to count the full range of pulse-heights produced

by ^{55}Fe disintegration; the other (green) channel had its discriminators set to count only the higher energy pulses in the upper half of the spectrum. The ratio of counts recorded in the red channel to those recorded in the green was used as the index of quenching. This ratio was approximately 2.00 in the least quenched samples. With greater degrees of quench, fewer counts were recorded in the green channel resulting in a greater red/green channels ratio.

In preliminary experiments, it was demonstrated that chemical quenching due to chloroform or acetone produced the same alteration in channels ratio for reduction in ^{55}Fe counting efficiency as did colour quenching due to methyl orange. A quench correction curve relating counting efficiency to red/green channels ratio was therefore constructed using acetone as the quenching agent. This curve was linear over the range of quench encountered in most samples.

Each sample was counted using the same settings on both channels as those used for construction of the quench correction curve. An appropriate correction factor was then applied to the counts in each sample depending on the channels ratio figure. For a few samples the channels ratio figure was outside the range over which the quench curve was linear. These samples were excluded from the final analysis.

(e) Calculation of results

After the counts recorded in each processed five millilitre blood sample had been suitably corrected for quenching, the mean number of counts for each set of duplicate tubes was calculated. To make allowance for the minor variations in haematocrit which occurred from time to time in each subject, these counts were further corrected by a factor related to the recorded haematocrit for each specimen in the following manner:

$$\frac{\text{mean counts per sample}}{5} \times \frac{100}{\text{haematocrit (per cent)}}$$

In this way, the counts present in one millilitre of packed red cells was obtained enabling the comparison of radioactivity between samples of unit volume from the same compartment. The mean red cell lifespan in normal subjects is 120 days, with a coefficient of variation in the order of ± 5 per cent (Harris and Kellermeyer, 1970, p. 520). Thus, the radioactivity present in the sample obtained 90 days after injection of the ^{55}Fe would represent maximal erythrocyte utilization of the radioiron. For this reason, the counts in this sample were used as a reference standard for later samples, and all subsequent levels of red cell radioactivity were expressed as a percentage of the 90 day value. When these percentages were plotted against time, a curve representing the rate of fall in the level of circulating radioactivity was obtained. The form of this curve was similar in all the subjects studied. After the first few months, the rate of fall in radioactivity was exponential in that a straight line was obtained when results were plotted on semilogarithmic graph paper. Over the first few months, the decline in radioactivity was more steep, and did not follow the linearity of the subsequent rate of fall. (Figure 4.1).

The initial rapid drop in circulating radioiron was interpreted to represent mixing of the tracer with other body compartments of iron upon its release from the first few generations of labelled erythrocytes. This was based on the following considerations: when the red cells in the first labelled cohort were destroyed at the end of their lifespan, a large proportion of their contained radioiron would have been reutilized for new haemoglobin formation. The remainder of the radioiron would have entered and mixed with other body compartments of iron. In this way, a

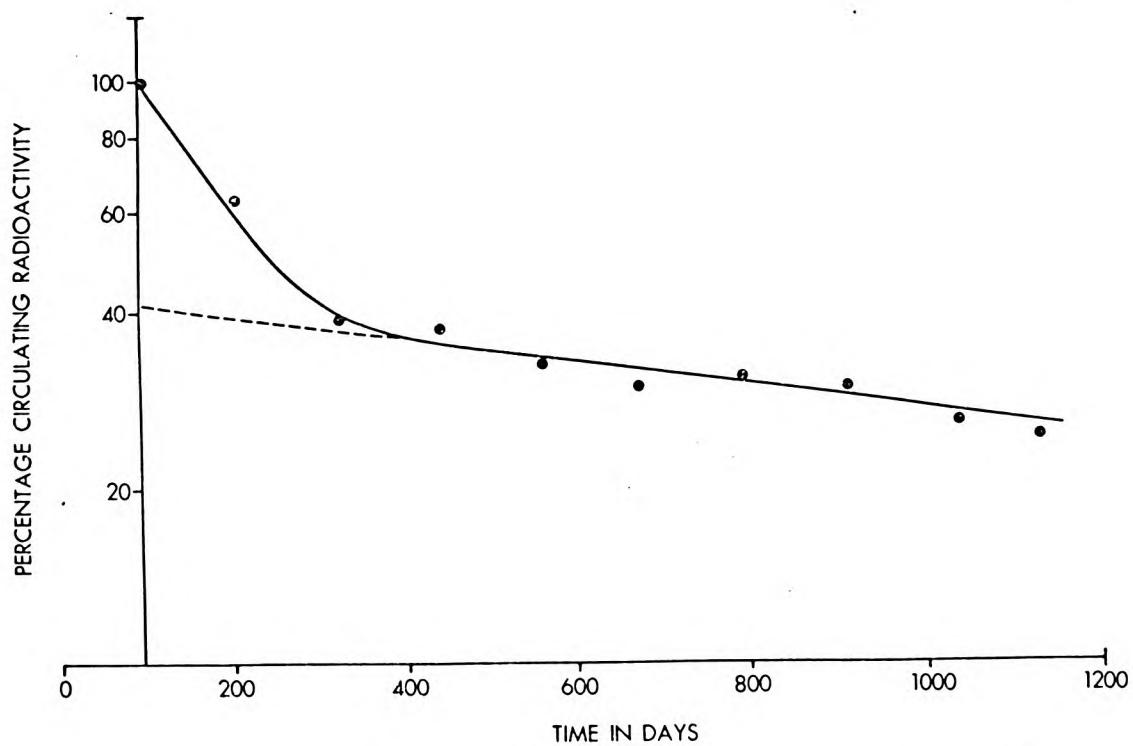


FIGURE 4.1. Representative curve showing the change in blood ^{55}Fe specific activity in a Negro subject in the long-term iron excretion study. Circulating radioactivity is expressed on a logarithmic scale as a percentage of the value 90 days after radio-iron injection, when there was maximum utilization.

stepwise diminution in the amount of radioiron remaining within the erythron would have occurred as each successive generation of labelled erythrocytes came to the end of its lifespan. This would have continued, until an equilibrium was reached between the specific activity of iron in the erythron and iron in other body compartments. Loss of further radioiron from the erythron would then have been offset by the entry from plasma of ^{55}Fe having the same specific activity. After complete mixing had taken place between erythrocytic and miscible non-erythrocytic body iron pools, there should have been no further fall in red cell specific activity resulting from radioiron redistribution.

The period of time taken for the curve of falling red cell radioactivity to become linear varied between subjects. This was related to the size of the body iron pool and was longer, the greater the pool size. For example, in a number of the Negro subjects with increased iron stores, the graph became linear only after the first year.

After equilibration had been established and the curve representing the disappearance of radioiron from the circulation had become linear, the slope of the line did not alter, and it could therefore reasonably be assumed that the rate of fall in red cell radioactivity reflected the continuing rate of loss of radioiron from the body as a whole. If there had been any further slow but appreciable internal redistribution of radioiron contributing to the exponential decrease in red cell radioactivity, then there should eventually have been a further change in the slope of the radioiron disappearance curve when equilibrium was finally attained. Once the curve became linear, there was no further alteration in its slope. It could therefore be assumed that the exponential fall in red cell radioactivity represented a situation of simple first-order kinetics, in which there

was a steady external loss of radioactive-labelled iron which was constantly being replaced by unlabelled iron absorbed from the diet. A regression line of y (ordinate) on x (abscissa) was fitted to each curve by the method of least squares for:

x = time from point of maximal red cell utilization
of radioiron
 y = percentage of maximum utilized radioactivity
remaining in red cells.

Only those values which were obtained after equilibration and linearity had been established were used to calculate the slope of the regression line.

The regression lines and their parameters and variances for all subjects were derived with the aid of an IBM 1620 Model 2 computer suitably programmed for the purpose.^x Additional information was also obtained from the computation and description of these regression lines. The value of y for $x = 0$ (the ordinate intercept) was calculated. This is equivalent to extrapolating each linear portion of the curve back to zero time as shown in Figure 4.1. The value of the ordinate intercept for each regression line was used to calculate the non-erythrocytic miscible body iron pool for each subject.

Each line representing the decline in red cell radioactivity was also tested by means of an analysis of variance for the significance of its linearity. When the calculated variance ratio for any regression line gave a level of

^x The programme and a sample of the printout format are given in the appendix.

significance below the 95 per cent confidence limit ($p > 0.05$) the data on that particular subject were excluded from any further consideration or analysis. Five of the 41 subjects in the study were excluded on this basis. (One from group I, two from group II and two from group III).

The confidence limits of the significance of linearity for the regression lines in the remaining 36 subjects was greater than 99.9 per cent ($p < 0.001$) in 24, greater than 99 per cent ($p < 0.01$) in 10, and greater than 95 per cent ($p < 0.05$) in two. Further calculations were carried out on the 36 subjects whose regression lines were significantly linear.

Since the slope of the line representing the decline in red cell radioactivity could be taken to reflect the rate of external loss of iron from the body, its biological half life ($T_{1/2}$) could be measured as the time taken for the red cell radioiron level to fall by one-half. This was computed for each regression line.

Using this figure for the $T_{1/2}$, it was then possible to calculate the turnover rate of radioiron tracer according to the formula:

$$\text{Turnover rate (per cent per year)} = \frac{0.693 \times 100}{T_{1/2} \text{ (years)}}$$

where 0.693 is the natural logarithm of 2 (Solomon, 1949).

In order to quantify the external losses of iron represented by the turnover rate, it was necessary to estimate the size of the total miscible body iron pool which consists of both erythrocytic and non-erythrocytic fractions. The erythrocytic fraction was calculated from the red cell mass

as follows. A blood volume of 64 ml per kg^{*} was assumed for the Durban subjects (Groups II and III) residing at sea level (Finch, 1959). For the subjects from Johannesburg (Group III), which is at an altitude of 1760 metres, the figure of 68.2 ml per kg was used to calculate the total blood volume (Metz, *et al.*, 1962). This figure was then used in conjunction with the mean of all the haematocrit determinations carried out on each subject over the period of study to estimate the red cell iron as follows:

$$\text{Red cell iron (mg)} = \text{body weight (kg)} \times \text{haematocrit} \times \text{blood volume} \\ \text{per kg (ml)} \times 1.1 \\ \text{..... (1)}$$

The factor 1.1 was applied in order to convert red cell volume (ml) to iron content (mg), (Finch, 1959).

Figures for the non-erythrocytic fraction of the miscible body iron pool were calculated from a formula containing the sum of two terms (Finch, 1959). The formula was derived as follows:

First, it was assumed that 10 per cent of the injected radioiron was localised in tissues other than the circulating red cell mass at the time the first (90-day) blood sample was drawn (Chappelle, *et al.*, 1955; Finch, 1959). This first term representing early tissue localization could therefore be

* A figure for the blood volume calculated from the red cell volume and venous haematocrit was used. Although this gives an underestimate for the true blood volume (Gregersen and Chien, 1968) by a factor of 0.91 (the normal body/venous haematocrit ratio) this figure was used because in the ensuing calculation, red cell volume is derived from blood volume using measured venous haematocrit values, and not the body haematocrit. If a figure approximating the true blood volume was used, then it would have been necessary to correct it by the body/venous haematocrit ratio in order to derive the red cell volume for each subject based upon venous haematocrit determinations.

estimated as:

$$\text{Red cell iron (mg)} \times \frac{10}{90} \dots\dots\dots (2).$$

The second term of the formula for non-erythrocytic miscible iron was obtained by analysis of the form of the curve depicting the fall in red cell radioactivity (Figure 4.2). As noted above, the slope of this curve was steeper during the first year than subsequently, and this more rapid disappearance of radio-iron from the circulation was taken to represent continued mixing during recycling between red cell iron and iron located in other miscible sites. The magnitude of this other miscible iron pool could be assessed by considering the two components of the early steeper part of the curve. It could be assumed that external loss of radioiron was continuing at the same rate, even during the initial period. The exponential line representing external loss, when extrapolated backwards therefore indicated one component of the early portion of the curve. The other component, caused by mixing, was given by the difference between the observed early curve and the extrapolated straight line. From this component, the size of the miscible iron pool with which red cell radio-iron was equilibrating, could be estimated. The way in which this was done is illustrated in the hypothetical curves shown in Figure 4.2.

The point at which the extrapolated line intercepts the ordinate was used in the following manner to quantify the miscible pool. If there were no additional miscible iron pools in the body with which red cell radioiron had to equilibrate, then the graph of falling red cell radioiron would follow single order kinetics and yield a straight line over the entire period of observation, as depicted by the upper line in figure 4.2, which cuts the ordinate at 100 per cent. In the case of the lower line which was extrapolated backwards, the ordinate intercept was 50 per cent.

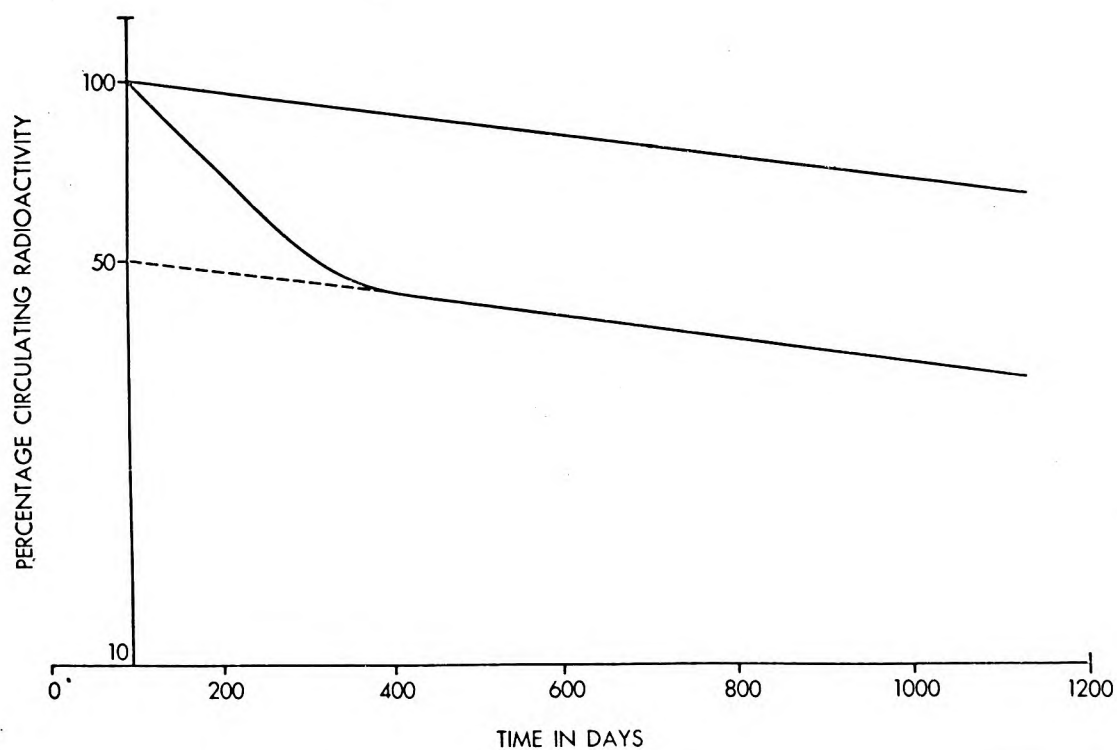


FIGURE 4.2. Two hypothetical curves of blood ^{55}Fe specific activity showing the putative effect of miscible body iron pool size on the form of the curve and the method used for calculating the size of the miscible pool from the ordinate intercept of the extrapolated linear portion of the curve (for description see text, p. 83).

In this example, the specific activity of circulating radioiron fell by 50 per cent or one-half as a result of mixing with other iron pools. In other words, the amount of iron in these pools must have been equal to the amount of iron in the red cell mass for this degree of radioisotopic dilution to have taken place.

The choice of other similar pool analogues shows that the magnitude of the miscible tissue iron pools may in general be obtained from the expression:

Miscible tissue iron =

$$\text{Red cell iron (mg)} \times \frac{100 - \text{ordinate intercept } (y)}{\text{ordinate intercept } (y)} \dots\dots\dots (3).$$

This expression gives the second term of the formula for the calculation of the total non-erythrocytic miscible body iron pool. This formula is now given by:

Total non-erythrocytic miscible body iron pool = Sum of (2) and (3) which is:

$$(\text{Red cell iron (mg)} \times \frac{10}{90}) + (\text{Red cell iron (mg)} \times \frac{100 - y}{y})$$

This may be written as:

$$\text{Red cell iron (mg)} \left[\frac{10}{90} + \frac{100 - y}{y} \right] \dots\dots\dots (4).$$

Finally, since it was assumed that a uniform specific activity was attained during the course of these studies between erythrocytic and miscible non-erythrocytic iron, total miscible iron may be represented by the addition of these two calculated fractions which is given by:

$$\begin{aligned}\text{Total miscible body iron} &= \text{erythrocytic iron} + \text{total non-erythrocytic miscible iron} \\ &= (1) + (4)\end{aligned}$$

The turnover rate for each subject was then applied to the calculated figure for his total miscible body iron in the calculation of daily iron loss as follows:

$$\text{Daily iron loss} = \frac{\text{Turnover rate (per cent per year)} \times \text{total miscible body iron}}{365}$$

The daily iron loss in each subject could be calculated in this manner from the yearly loss. Despite probably day-to-day variations, it is the purpose of this study to arrive at a figure for the average loss per day of iron. If it is assumed that the subjects studied were in a steady state of iron balance, then it is reasonable to calculate iron loss in the above manner, by applying the measured turnover rate of radioiron through the red cell compartment to the total body miscible iron. Iron absorption must then equal iron excretion over the period of study. Although there was no reason to suppose that such an assumption was not valid for the Indian subjects, there were possible difficulties in making the assumption for the Negro subjects.

Iron overload of varying degrees is present in the majority of adult male Negroes. It is in fact for this reason that groups of Negro subjects were included in the investigation, because part of the design of the study was to assess the influence of increased iron stores on the rate of iron loss from the body. Furthermore, the degree of siderosis in Negroes tends to increase with age, which implies that Negro subjects could have been in a state of positive iron balance for the period over which the study extended. During this period, with more iron entering than leaving the body, there would have been a progressive increase in storage iron. This could,

theoretically, have resulted in continual displacement of the radio-iron tracer from the circulating red cell mass into non-miscible stores. The decline in red cell radioactivity might therefore, in part, have reflected this internal redistribution of iron. Even if this postulate is correct, however, evidence from other studies suggests that it is unlikely that the degree of positive iron balance is great enough to represent a major source of error. Chemical analyses of necropsy material (Bothwell and Bradlow, 1960; Charlton, *et al.*, 1970) have indicated that the average quantity of storage iron in middle-aged Negro men is around three grams, which is approximately three times greater than normal. This excess of two grams of iron accumulates over a period of twenty to thirty years, so the annual increment is in the region of 100 mg. Although it is therefore possible that calculated figures for iron excretion based on the drop in red cell specific activity might be too high, the magnitude of positive balance in these subjects was probably too small to have affected the results significantly.

(f) Validation of the experimental procedure

Although it was impossible to validate fully the assumptions on which the study was based, an experiment was carried out to obtain more information on the relative labelling of different body iron compartments. If complete mixing had occurred, and radioiron was uniformly distributed in all miscible body iron compartments, then the specific activities of ^{55}Fe in these compartments should have been the same. To test whether this was so, a sample had to be obtained from a body iron compartment other than blood. A conveniently accessible source of body iron having a sufficiently high iron concentration for the amount of ^{55}Fe present to be readily detectable was chelated storage iron voided in the urine. Desferrioxamine methanesulphonate (Desferal B., Ciba, Basle, Switzerland) was

used, and the ^{55}Fe specific activity of urinary iron obtained after the administration of this chelate was compared with that of a blood sample. Since the ratio of red cell to plasma iron in the blood is of the order of 500 to 1, the contribution of plasma iron to the overall specific activity may be ignored. Although the source of iron chelated and excreted by desferrioxamine is uncertain (Brown, *et al.*, 1967) it neither removes iron from haemoglobin nor from transferrin under *in vivo* conditions (Wöhler, 1964). Harker and co-workers (1968) have provided evidence that chelated iron comes predominantly from the parenchymal stores of the liver rather than reticuloendothelial stores, but there is other evidence that the reticuloendothelial stores do provide a source of this iron (Cummings, *et al.*, 1969).

Desferrioxamine (1 500 mg) was given by intramuscular injection to two Negro subjects in the Johannesburg group 60 months after the study was begun. The two particular subjects selected (J10 and J18) had calculated figures for total miscible iron which were among the highest of the group. Urine was collected over a 12-hour period in specially washed, iron-free, glass containers. Simultaneously, a blood sample was taken. Ferrioxamine (desferrioxamine-iron complex) was extracted from the urine with benzyl alcohol by countercurrent distribution and salting out of the ferrioxamine from the aqueous phase using supersaturated sodium chloride. The ferrioxamine was then returned into an aqueous phase by the addition of diethyl ether (Keberle, personal communication, 1967). Duplicate specimens of both blood and extracted urine ferrioxamine were then wet-ashed and divided into two parts. On one part, the chemical iron content was measured by the thioglycolic acid method after complete wet-ashing of the sample (Bothwell and Finch, 1962, p. 26). The other part was processed and counted in a liquid scintillation counter as previously described.

After due correction for quenching, the specific activities of whole blood and urinary ferrioxamine were expressed in terms of absolute counts per minute per milligram iron. The specific activities in blood and urine for each subject were as follows:

Subject J10

Blood : 1180 cpm per mg

Urine : 1136 cpm per mg

Subject J18

Blood : 1856 cpm per mg

Urine : 2723 cpm per mg.

These results suggested, even in the subjects with very high iron stores, that labelling of the storage iron compartment had become equal to or even greater than that of the circulating red cells during the course of the study.

(g) Exclusion of pathological blood loss

Intestinal amoebiasis is endemic in Durban, and for this reason, it was necessary to carefully exclude the presence of any amoebic infestation or abnormal gastrointestinal blood loss in subjects in whom normal iron loss was being studied. For this reason, preliminary chemical testing for abnormal faecal blood loss using orthotoluidine, as well as examination of stool samples for parasites was carried out on all subjects as part of the routine screening before they were judged suitable for the study. Although the results of these tests were negative, further checking was carried out on a number of Durban subjects during the course of the study using ⁵¹Cr-tagged red cells. The investigation was performed on eight subjects from group II (Durban Negroes) and 10 subjects from group III (Durban Indians).

Donor erythrocytes (group O, Rh-negative) were labelled with approximately 50 μCi ^{51}Cr according to standard techniques (Dacie and Lewis, 1968) and injected intravenously. All faeces passed by the subjects over the following two weeks were collected in waxed 400 ml cardboard cartons, and their radioactive content was measured in a counter consisting of two diametrically opposed five centimetre end-on sodium iodide crystals. Each stool sample was weighed, and counts were then appropriately corrected for weight from a table calibrated for the counter. Three 3 ml blood samples, taken on the 1st, 8th and 14th days were made up to a volume of 400 ml with water and also counted. These served as reference standards for the quantitation of faecal blood loss. The counts in one millilitre of blood on each particular day of the study could be calculated from the straight line graph drawn by interpolation between the points obtained for the counts in the three blood samples. The amount of blood present in stool samples could then be read directly off this graph from the radioactivity present in each sample.

III. RESULTS

(a) Long-term iron excretion

All relevant information and results on each subject are given in tables 4.2, 4.3 and 4.4, together with the means and standard errors of the means for observations within each group. Figures depicting the fall in red cell radioactivity with time, plotted on semilogarithmic paper for each subject are included in the appendix to this thesis.

The calculated mean daily iron losses ($\pm$ standard error) in each group were as follows:-

Group I	(Johannesburg Negroes)	2.43 mg	($\pm$ 0.34)
Group II	(Durban Negroes)	2.03 mg	($\pm$ 0.31)
Group III	(Durban Indians)	1.02 mg	($\pm$ 0.05) ^x

Individual results for the subjects in each group are shown in figure 4.3. The Student's 't' test was applied to these data to test the significance of the observed differences. There was no significant difference ($p > 0.3$) between the daily iron losses in groups I and II, the groups of Negro subjects from Johannesburg and Durban. However, losses in both these groups were significantly higher than in group III, the Durban Indian group (for both, $p < 0.01$).^{xx}

Daily iron losses were calculated from the total body miscible iron and the ^{55}Fe turnover rate. Further statistical analysis was therefore carried out in order to determine which of these variables was responsible when a significant difference in the daily iron losses was demonstrated between groups. It was shown that the higher excretion rates found in the Negro groups were probably due to a combination of both factors, increased total miscible iron and a faster turnover rate.

^x Corresponding results for the two other groups in the collaborative study mentioned previously (Green, *et al.*, 1968) were: Seattle Whites 0.95 mg ($\pm$ 0.09), Venezuelan Mestizos 0.90 mg ($\pm$ 0.09).

^{xx} No significant difference was found when losses in Durban Indians were compared with either the Seattle or Venezuelan subjects (for both, $p > 0.05$). Losses found in the Negro groups were, however, significantly higher than those in any other group in the collaborative study (for all, $p < 0.01$), (Green, *et al.*, 1968).

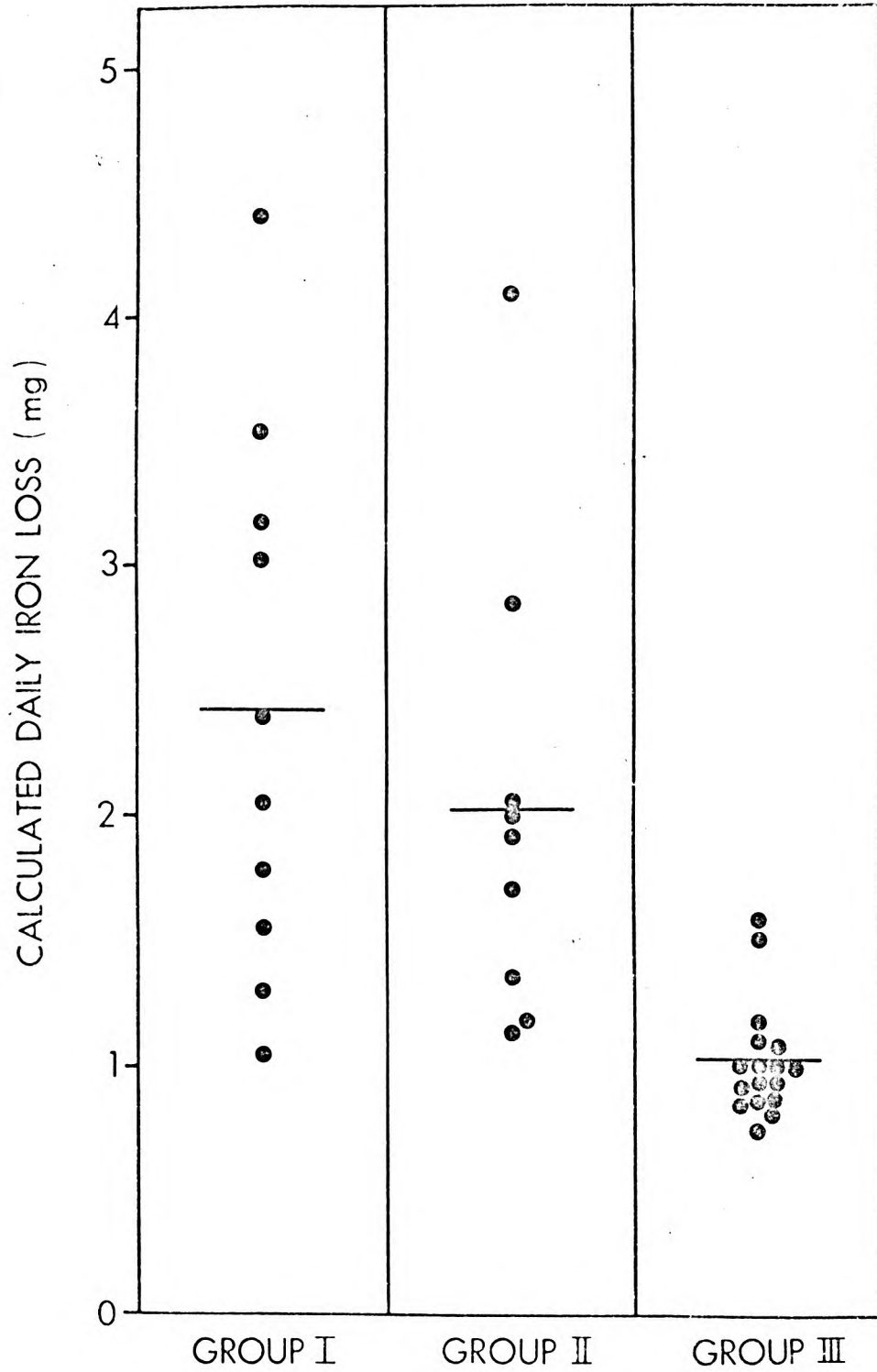


FIGURE 4.3. Calculated mean daily iron losses in subjects in the long-term total body iron excretion study. Group I, Johannesburg Negroes; Group II, Durban Negroes; Group III, Durban Indians.

The mean figures ($\pm$ standard error) for these variables in the three groups were as follows: Total miscible iron:

Johannesburg Negroes (group I),	4242 mg ($\pm$ 364)
Durban Negroes (group II),	4354 mg ($\pm$ 415)
Durban Indians (group III),	2666 mg ($\pm$ 131).

Significance estimates using Student's 't' determinations were: Comparing groups I and III, $p < 0.001$; comparing II and III, $p < 0.005$. These differences were therefore highly significant.

Corresponding figures (mean $\pm$ standard error) for yearly turnover rates were:

Johannesburg Negroes (group I),	20.4 per cent ($\pm$ 1.9)
Durban Negroes (group II),	16.5 per cent ($\pm$ 1.0)
Durban Indians (group III),	14.1 per cent ($\pm$ 0.6).

Significance estimates were: Comparing groups I and III, $p < 0.005$; comparing groups II and III, $0.1 > p > 0.05$.^{*} In the former test, the difference is highly significant; in the latter

^{*} Corresponding figures for the two other groups in the collaborative study (Green, *et al.*, 1968) were:
Total miscible iron: Seattle Whites, 3264 mg ($\pm$ 80); Venezuelan Mestizos, 2738 mg ($\pm$ 111).
Yearly turnover rate: Seattle Whites, 10.8 per cent ($\pm$ 1.2); Venezuelan Mestizos, 12.4 per cent ($\pm$ 1.4).
In both groups, total miscible iron and turnover rates were significantly lower than in the Negro groups (for all, $p < 0.05$). Compared with the Indian group, both groups had significantly lower turnover rates ($p < 0.05$), but total miscible iron did not differ significantly ($p > 0.05$).

test, the difference is not strictly significant, but in view of the close proximity of the 't' value to the level for probable significance at $p = 0.05$, it cannot entirely be disregarded.*

(b) Exclusion of pathological blood loss

The results of the investigation of possible abnormal faecal blood loss in the Durban subjects is given in table 4.5. The mean daily faecal blood loss for each subject was less than the 1.00 ml cited by Hughes Jones (1958) as the upper limit of normal using ^{51}Cr -tagged red cells. The iron contained in the mean quantity of blood lost in the stool per day was calculated for each subject on the assumption that one gram of haemoglobin contains 3.4 mg of iron (Bernhart and Skeggs, 1943). There was a poor correlation between the calculated figures for iron excretion caused by blood loss in the stool and the figures for total daily iron losses obtained from the ^{55}Fe long-term excretion study. Subjects with the highest total daily iron excretion did not have correspondingly high faecal blood losses. Furthermore, the subjects having the highest measured faecal blood losses did not have particularly high ^{55}Fe turnover rates.

* The 't' value was 2.004, just outside the significance limit for $p = 0.05$ which is 2.064 for 24 degrees of freedom. The difference may therefore be significant.

Table 4.5. Faecal blood loss in Durban subjects (Groups II and III) in the long-term iron excretion study estimated by ^{51}Cr -labelled red cell measurement in stools

SUBJECT	MEAN DAILY WEIGHT OF FAECAL STOOL (g)	MEAN DAILY FAECAL BLOOD LOSS (ml)	HAEMOGLOBIN (g per 100 ml)	CALCULATED MEAN DAILY FAECAL RED CELL IRON LOSS (mg)	TOTAL DAILY IRON LOSS FROM ^{51}Cr DATA (mg)	^{51}Fe TURNOVER RATE (per cent per year)
<u>Indians</u>						
D 3	150	0.99	16.3	0.55	1.09	16.9
D 6	40	0.50	18.4	0.31	0.85	11.7
D 7	65	0.51	16.6	0.29	0.95	15.2
D 9	80	0.41	18.0	0.25	1.00	13.1
D12	112	0.29	18.0	0.18	0.73	10.3
D13	104	0.34	14.2	0.16	0.82	11.0
D14	66	0.26	18.3	0.16	1.56	12.4
D15	198	0.33	16.4	0.18	0.95	13.6
D16	158	0.38	15.8	0.20	1.01	17.5
D17	79	0.62	15.8	0.33	1.06	14.8
<u>Negroes</u>						
D22	48	0.36	15.5	0.19	1.75	17.3
D23	95	0.24	16.0	0.13	2.85	18.9
D24	93	0.40	16.3	0.22	1.13	13.6
D25	37	0.78	15.5	0.41	1.17	14.7
D28	145	0.58	17.0	0.33	2.01	19.7
D31	129	0.41	16.8	0.23	4.08	22.0
D36	138	0.23	14.8	0.12	1.70	15.2
D37	87	0.19	13.4	0.09	1.36	12.6
Mean		0.43		0.24		
S.E.M.		0.05		0.03		

IV. DISCUSSION

Previous estimates of total body iron losses have varied considerably, even when similar techniques have been used for their measurement. Most attempts to measure total daily iron excretion have been made using radioisotopic methods, the validity of which are open to question. As detailed in the literature review (Chapter 2), when such methods are applied, it is necessary to make certain assumptions concerning the size of the body iron pools, and the degree of mixing that takes place between the administered radioiron and these pools. These difficulties are exemplified by the calculated figures for total daily losses of iron which have been obtained using whole-body counting techniques. Reported figures for the rate of radioiron loss (per cent per day) in whole-body counting studies, when applied to estimates of the total body iron pool, have put the figure for daily iron loss between 0.4 and 9.0 mg. (See Table 2.3).

In most of these studies, the periods of observation were probably too short for complete equilibration between the injected radioisotope and the total miscible body iron to have taken place. This renders the interpretation of such findings difficult. Finch (1959), overcame this difficulty by following the decline in blood specific activity of injected ^{55}Fe over prolonged periods, and found only modest daily iron losses.

The results obtained in this thesis using the same approach, are in agreement with published figures for daily iron losses not exceeding one milligram in normal male subjects (McCance and Widdowson, 1937a; Dubach, *et al.*, 1955; Finch, 1959; Saito, *et al.*, 1964). Results of the long-term iron excretion studies presented in this thesis on three groups of South African subjects are best considered in conjunction with those obtained in a parallel collaborative study carried out on two further groups of subjects, one from Seattle, U.S.A., and the other from Araya, Venezuela (Green, *et al.*, 1968).

A comparison between these five groups, chosen as they were in order to examine the effects of a number of variables on body iron excretion, highlights a number of significant observations which were made on the groups presented in this thesis. Relevant data and results on the additional two groups have been given in footnotes in preceding parts of this chapter.

The mean calculated daily iron losses in all five groups were as follows: Johannesburg Negroes 2.43 mg, Durban Negroes 2.03 mg, Durban Indians 1.02 mg, Seattle Whites 0.95 mg and Venezuelan Mestizos 0.90 mg. In assessing the significance of these findings, it is pertinent to consider the effects on iron losses of climatic environment, occupation, age, body weight, ethnic group and the size of the body iron pools, either singly or in combination.

(a) Climatic environment and occupation

There was no significant difference in the mean iron loss for Indian subjects who were engaged in manual labour in a working environment with an extremely high temperature and humidity and the Seattle subjects with sedentary occupations living in a temperate climate. This suggests that environmental extremes and the degree of physical activity, factors which both profoundly influence the rate of sweating, play insignificant roles in determining body iron loss. The conclusion is further supported by the similarity of daily iron losses in the Venezuelan compared with the Seattle groups. Venezuelan subjects were doing strenuous manual work in a hot, relatively dry climate.

With regard to the Indian subjects, it should, however, be pointed out that although their calculated mean daily iron losses were the same as in the subjects from Seattle, there were significant differences in both the total miscible iron pools and the radioiron turnover rates between the two groups. The Indian group had smaller miscible iron pools but faster

turnover rates. Since the calculated daily iron loss is based on these two variables, it is the mutually opposite effects of these differences in body pool size and turnover rate which give rise to the similarity in iron loss of the Durban Indian and Seattle groups. The Indians were therefore losing a greater amount of iron relative to the size of their body iron pools, although the absolute figures for iron loss were the same as in the Seattle subjects. Excessive gastrointestinal blood loss in Indian subjects was excluded by means of ^{51}Cr -tagged red cell measurement in stools, and it therefore seems likely that their faster radioiron turnover rates resulted from a relatively greater iron loss through the skin. However, the difference in turnover rates between the Indian and Seattle subjects was not great (14.1 and 10.8 per cent per year respectively), which suggests that even extremes of environmental temperature and humidity exert only a modest influence on iron excretion via this route.

(b) Age

It is possible that age may influence iron losses. The mean ages of the five groups of subjects were similar, but the effect of this variable was examined by correlating iron excretion with age within each group. Significantly less iron was lost at older ages in the Venezuelan subjects ($r = 0.601$; $p < 0.05$). However, no corresponding decrease in iron excretion with age was found in Johannesburg Negroes ($r = -0.280$; $0.2 > p > 0.1$), Durban Negroes ($r = +0.551$; $0.1 > p > 0.05$), or Indians ($r = +0.141$; $0.2 > p > 0.1$). The lack of correlation in Negro subjects may be explained, in part at least, by the fact that body iron stores tend to increase with age in this population group, and iron loss is greater with increased body iron stores, as will be discussed later.

There was also no correlation between iron excretion and age in the Seattle subjects ($r = +0.094$; $0.2 > p > 0.1$). However, the mean figure for this group (mean age 43 years) was 0.95 mg daily, which is somewhat greater than the figure of 0.61 mg daily found in an older (mean age 69 years) but otherwise comparable group of Seattle subjects studied previously by the same technique (Finch, 1959). Body size is positively correlated with iron loss (see below), and the mean weight of the group originally studied in Seattle was less than the later one (66 kg compared with 78 kg). However, this difference was not large enough to explain the difference in iron excretion, so that age could also have had an influence on the difference in iron excretion. It is therefore possible that increasing age, by resulting in decreased physical activity and a consequent reduction in dermal iron losses, may have a small effect on iron excretion.

(c) Body weight

There is evidence to indicate that both the body iron content and the turnover of iron within the body are related to body size. It might therefore be anticipated that iron loss, which depends on these two variables, would be influenced by the body size. There was a significant positive correlation between daily iron loss and body weight in each of the three groups of South African subjects presented in this thesis. The correlation coefficients for the groups were: Johannesburg Negroes, $r = +0.697$ ($p < 0.001$); Durban Negroes, $r = +0.603$ ($p < 0.01$); Durban Indians, $r = +0.635$ ($p < 0.001$). The correlations in Seattle Whites ($r = -0.332$; $p > 0.1$) and Venezuelan Mestizos ($r = +0.226$; $p > 0.4$) were not significant. The reason for a lack of correlation in these two groups is not clear.

(d) Ethnic group and size of the body iron pools

The ethnic group of the subjects studied does not appear to have any influence on iron loss. Mean daily iron losses were similar in Indian Caucasoids, White Caucasoids and Mulattos from Venezuela. Although the mean calculated figures for daily iron excretion were approximately twice as great in Negro subjects as in any of the other groups, this almost certainly relates to their increased iron stores, rather than their ethnic group. A high incidence of varying degrees of iron overload has been reported in the adult Negro males of Southern Africa (Strachan, 1929; Bothwell and Finch, 1962, p. 394). Autopsy studies have found mean hepatic iron concentrations which were three-fold greater in South African Negro subjects than in Whites (Mayet and Bothwell, 1964; Charlton, *et al.*, 1970). In the present study, the calculated mean total miscible iron pools of Johannesburg and Durban Negroes were 4242 and 4354 mg respectively, compared with 2666 mg in Durban Indians, 3264 mg in Seattle Whites and 2738 mg in Venezuelan Mestizos. As the red cell iron pools were similar in all groups, the higher figures for total miscible iron in Negro subjects reflect an increase in the storage iron component.

Assuming uniform radioisotope labelling of miscible iron pools in Negro subjects, then with the same turnover rate, Negro subjects would have a proportionately greater iron loss from their increased body iron pools, resulting in greater daily iron losses. However, not only the miscible iron pools but also the rates of decline in blood specific activity (and hence the turnover rates) were significantly faster in Negro subjects than in any other groups. The reason for this difference in turnover rates is not clear, but there are several possible explanations.

In the long-term iron excretion studies it was assumed that equal labelling of all miscible body iron compartments was complete after the end of the first year of observation. This was supported by the fact that the drop in red cell specific activity was much steeper during the first year than thereafter. Also, if uniform labelling of the body iron pools had not been achieved, and if there were continuing redistribution of tracer from the erythron to miscible storage iron pools having a lower specific activity, then the fall in red cell specific activity after the first year should not have followed a single order kinetic pattern. There is, however, no assurance that some degree of slow redistribution of radioiron between the red cell compartment and relatively non-miscible storage pools did not occur throughout the period of study in the Negro groups. Included in these groups, were subjects who had increased body stores, and were therefore probably in states of positive iron balance. In clinical studies using tracers, such as the long-term iron excretion method, the initial specific activity will be high in the pool into which the tracer is first introduced and in pools having a rapid uptake and turnover of the tracer (in this case, the erythron). Later, however, the specific activity of pools with a slow turnover and release of tracer (in this case, storage iron pools) will become higher (Reizenstein, 1966). Some evidence of this pattern of redistribution was possibly obtained in one of the two Negro subjects (J18) on whom specific activity determinations were carried out on red cell iron and urinary desferrioxamine-chelated storage iron. In the other subject (J10), the specific activities of these two compartments were very similar.

In the event of uniform specific activity of all the miscible body iron pools not having been attained, and if Negro subjects were in positive iron balance, then their faster measured fall in red cell specific activity might have

reflected, in part, a gradual movement of radioiron from the erythron into stores. In this case, the calculated figures for iron excretion in Negro subjects would represent an overestimate of the actual quantities being lost.

If, however, the figures and the assumptions upon which their calculations were based were valid, this would imply that iron excretion was greater in the Negroes than it was in other subjects, and that this increased loss was a function of their greater miscible iron pools. Possible avenues for increased iron losses would be the gastrointestinal tract and the skin. It has been suggested that in states of iron overload, excretion of iron from the body may be augmented by the shedding of iron-loaded reticuloendothelial cells through the gut wall (Thirayothin and Crosby, 1962; Astaldi, *et al.*, 1966). Another possible mechanism whereby excessive iron might be excreted is through ferritin loss in the bile (Bradford, *et al.*, 1969). Also, as will be presented and discussed in the section dealing with compartmental iron losses, there is evidence to suggest that skin iron losses may be increased when circulating iron levels are raised. The results obtained in the present studies support the view that iron excretion is controlled, in part, by the state of the body iron stores. The mean iron excretion in Negro subjects was greater than two milligrams per day, and individual subjects had calculated losses of up to four milligrams per day. This confirms the suggestion made by Crosby, *et al.*, (1963) that iron loss could be considerably increased in iron overload. These workers studied a patient with transfusional siderosis who had recovered from his anaemia and was venesected to remove the accumulated iron. When iron deficiency finally developed, the total amount of iron which was recovered was less than half of the 32.5 g given in transfusions 13 years previously. It was calculated that during those years, the patient was losing iron at a rate of about four milligrams per day.

The present observations also complement the work of Dubach, *et al.*, (1955) who calculated that the daily obligatory iron loss could be reduced to 0.5 mg per day in iron deficient subjects.

V. SUMMARY AND CONCLUSIONS

The evidence obtained in long-term iron excretion experiments on different groups of subjects confirms that in subjects with normal body iron stores, daily iron excretion does not exceed one milligram. Furthermore, daily iron loss does not appear to be influenced by factors that are known to affect the rate of sweating. Iron losses were not greater in subjects doing strenuous work in hot humid environments than in sedentary subjects living in a temperate climate. There was a suggestion that iron losses might be diminished by increasing age. The only variable which clearly affected iron excretion was the state of the body iron stores. Negroes with increased iron stores lost twice as much iron as did subjects with normal iron stores. This implies that body iron loss is regulated, at least to some extent, by the degree of iron repleteness, and is in keeping with previous suggestions that the excretory mechanism is a variable one, dependent on the body iron content.

CHAPTER 5

COMPARTMENTAL IRON LOSSES

I. INTRODUCTION

In order to study iron losses through the known normal routes of excretion for this element, both chemical and radioisotopic methods were used. As detailed in the general introduction to this thesis, most available techniques for the measurement of iron loss from the body are open to criticism on methodological or interpretive grounds. With these limitations in mind, it was decided to employ a combination of both methods in order to gain an overall assessment of compartmental iron losses. Although neither of the techniques which were used is ideal in itself, it was hoped that together, the results obtained from such a dual approach could yield meaningful information on this subject.

II. GENERAL DESCRIPTION OF METHODS

(a) Radioisotopic Method

(i) Material and equipment

For technical reasons, the iron isotope ^{59}Fe was used in radioactive studies. Because of the high-energy gamma-radiations emitted during its decay, this radioisotope which has a half-life of 45 days can be counted more easily and efficiently than ^{55}Fe . Because of the radiation properties of ^{59}Fe , its presence can be detected without any further processing of the samples of tissue or body fluids. Furthermore, the high attainable counting efficiency enables reliable measurement of radioactivity in large volume samples containing little radioiron.

Radioactivity determinations on small volume samples (four millilitres or less) were carried out in a well-type counter consisting of a thallium-activated sodium iodide crystal linked through a photomultiplier tube to an IDL 1700 scaler.

Larger volume samples were counted in a well-type counter made of plastic phosphor material (Nuclear Enterprises NE 220) which was connected through a perspex light guide to a photomultiplier tube and a Phillips high voltage scaling unit. The design of this counter was similar to that described by Warner and Oliver (1962).

(ii) Principle of method

Ten microcuries of high specific activity ^{59}Fe (12 - 19 μCi per μg) was injected intravenously.* The iron was given as the citrate salt (in 0.1 per cent sodium citrate). It is not necessary to preincubate iron with plasma for clearance studies if it is given in the form of ferric citrate (Loeffler, *et al.*, 1955; Bothwell, *et al.*, 1957). However, in any subjects who exhibited clinical or biochemical evidence of iron overload, the radioiron was incubated before its administration with plasma obtained from a screened normal healthy blood donor who had no history of serum-communicable disease.

The exact amount of radioiron administered was determined gravimetrically by the weight difference between the syringe before and after injection. A small weighed sample of the injection solution was also diluted in a volumetric flask and set aside to serve as a standard. At the time of injection of radioiron, a blood sample was withdrawn for the estimation of the serum iron level and unsaturated iron binding capacity. The modified method of Bothwell and Mallet (1955) was used for

* This is within the safe limit laid down by the ICRP Committee (1960). The maximum permissible burden for ^{59}Fe is 20 μCi for the spleen which is the critical organ.

serum iron determinations, and the method of Bothwell, *et al.*, (1959) for measurement of unsaturated iron binding capacities.

Serial blood samples were then collected at intervals (10, 20, 30, 60, 120 minutes) after the injection of the radioiron, for determination of the plasma iron turnover (Bothwell and Finch, 1962, p. 67). Details of the method, which assumes no significant feedback, are as follows: from the exponential plot of radioiron clearance which is obtained by counting unit volumes of plasma, the plasma-clearance half-time ($T_{1/2}$) may be derived. Furthermore, if this line indicating the clearance of radioiron from the plasma is extrapolated back to zero time, the activity at this point (ordinate intercept) represents the level of maximum circulating activity at the time of injection in the unit volume of plasma counted. The total radioactivity injected, based on the counts in the retained standard aliquot of the dose, may then be used to calculate the plasma volume as:

$$\text{plasma volume (ml)} = \frac{\text{total injected radioiron (cpm)}}{\text{radioiron in plasma at zero time (cpm per ml)}}$$

It is then possible to calculate:

- 1) the total amount of iron in the plasma since:

$$\text{total plasma iron (mg)} = \frac{\text{plasma iron } (\mu\text{g per ml}) \times \text{plasma volume (ml)}}{1000}$$

..... (1)

- and 2) the fraction of iron removed per hour which is given by:

$$\text{fraction of iron removed per hour} = \frac{0.693}{T_{1/2} \text{ (in hours)}} \quad \text{..... (2)}$$

(where 0.693 is the natural logarithm of 2)

Plasma iron turnover is then calculated as:

$$\begin{aligned} \text{Plasma iron turnover (mg per 24 hours)} &= (1) \times (2) \times 24 \\ &= \frac{\text{total plasma iron (mg)} \times 0.693}{T_{1/2} \text{ (hrs)}} \times 24 \end{aligned}$$

Using this approach, it is possible to obtain a measure of the absolute amount of iron which passes through and leaves the plasma each day. Despite considerable diurnal variations in the plasma iron level, there is a complementary and reciprocal change in the rate of plasma iron clearance, so that the calculated plasma iron turnover remains reasonably constant, regardless of the phase during the diurnal fluctuations at which the measurement is carried out (Bothwell and Finch, 1957; Lockner, 1966).

The calculated figure for the plasma iron turnover was used for the estimation of compartmental iron losses when the radioisotopic approach was employed. In general, the percentage of injected radioiron which subsequently appeared through various excretory routes (in the case of faeces and bile) or became sequestered in sites that supposedly would lead to its eventual loss from the body (in the case of epidermis) was measured. This percentage was then related to the plasma iron turnover in order to quantify the actual (in the case of faeces), and maximum possible (in the case of bile and epidermis), amounts of iron derived from the plasma which could be lost through these various avenues. Details of modifications and additions to this general approach will be given in the appropriate section dealing with each particular avenue of loss.

(b) Chemical methods

In complementary studies to document compartmental iron losses, the chemical iron content of urine, bile, sweat and

stripped epidermis was measured directly. One of two methods was employed. In all but one study, the thioglycolic acid method was used after wet-ashing of the samples (Bothwell and Finch, 1962, p. 26). The only exception was sweat iron determination which was carried out using a modification of the bathophenanthroline method for serum iron (Bothwell and Mallet, 1955). For all chemical iron determinations, acid-washed, iron-free glassware was used, and special care was taken to avoid any possible contamination.

III. GASTROINTESTINAL TRACT

(a) Total gastrointestinal iron losses : Experimental

It has been pointed out in the introduction that the chemical estimation of total endogenous iron loss from the bowel is not possible unless a careful balance study is carried out. Results using such an approach have been documented (McCance and Widdowson, 1938; Green, *et al.*, 1968). In the studies described here, a radioisotopic technique was preferred for the estimation of daily iron loss due to blood and other sources through the gastrointestinal tract.

(i) Subjects studied

Relevant information on the subjects studied is given in Table 5.1. Thirteen subjects were studied, comprising one White woman, three White men, two Negro women and seven Negro men. Five of the Negro men had convincing clinical and laboratory evidence of haemosiderosis. All subjects were hospital in-patients during the time of study. They were haematologically normal, and had no evidence of any disease associated with abnormal gastrointestinal blood loss. Subjects were not receiving salicylates or any other medications likely to

Table 5.1. Description of subjects in radioisotopic study for measurement of gastrointestinal iron loss

SUBJECT	SEX	ETHNIC GROUP	AGE (years)	DIAGNOSIS	HAEMOGLOBIN (g per 100 ml)	SERUM IRON (μ g per 100 ml)	SERUM TOTAL IRON BINDING CAPACITY (μ g per 100 ml)	TRANSFERRIN SATURATION (per cent)	Tl ⁵⁹ Fe CLEARANCE (minutes)	CALCULATED PLASMA VOLUME (ml)
1	F	White	66	Bronchopneumonia	12.6	40	298	13	38	2256
2	M	White	54	Lobar pneumonia asthina and emphysema	19.8	68	422	16	28	2103
3	F	Negro	39	Hypertensive heart disease	14.5	64	372	17	74	2518
4	M	Negro	50	Pneumonia and cerebral abscess	11.1	47	259	18	60	3608
5	M	Negro	25	Tapeworm	12.6	55	267	21	40	2554
6	M	White	56	Lobar pneumonia and emphysema	16.5	51	232	22	31	2691
7	M	White	60	Ischaemic heart disease	16.2	87	352	25	91	2800
8	F	Negro	39	Cerebral thrombophlebitis	15.2	124	436	28	90	2248
9	M	Negro	46	Haemosiderosis osteoporosis and scurvy	14.8	173	340	51	65	2438
10	M	Negro	40	Chronic pancreatitis and haemosiderosis	17.2	227	263	86	154	2592
11	M	Negro	48	Cerebral thrombophlebitis and haemosiderosis	15.0	182	209	87	105	2825
12	M	Negro	-	Pulmonary and cerebral tuberculosis and haemosiderosis	15.0	154	162	95	70	2527
13	M	Negro	59	Haemosiderosis osteoporosis and scurvy	15.8	205	212	97	128	2645

produce gastrointestinal erosion or haemorrhage. Benzidine testing for occult blood in the stools were repeatedly negative in all subjects.

(ii) Details of method

Ten microcuries ^{59}Fe was administered intravenously to each subject, and the plasma iron turnover was calculated as described above. A carmine-dye stool marker was given by mouth to each subject at the time of the injection of radioiron. All faeces passed after this time and for the full period of hospitalization were collected in waxed cartons. The ensuing period of follow-up ranged from seven to 49 days (mean, 20 days). Blood samples were also withdrawn every day during the first week and thereafter every two to three days over the entire duration of the study for radioactivity measurements. All stool samples were weighed and counted for radioactivity. One millilitre of whole blood was diluted with water in a waxed carton to the weight of the corresponding day's faeces, and was counted at the same time. The counts recorded in blood and in faeces were then appropriately corrected for decay, and for day-to-day variations in volume from a table of correction factors established for the counter. A diluted aliquot of the retained standard dose was counted and used to calculate the total number of injected counts. The counts in every stool and blood sample were then related to the counts injected, and expressed as a percentage of injected counts. Stool samples were examined for the presence of the carmine marker, and the time taken for this to pass through the gastrointestinal tract was noted.

(iii) Calculation of results

For the calculation of results, the percentage of intravenously injected radioiron tracer that was excreted in the stool on each day was plotted against the time in days after injection. The actual time of appearance of radio-

activity was corrected for delay due to intestinal transit time by subtracting the length of time that it took for the carmine marker to appear in the stools. This was usually one or two days, and was never more than three days. The pattern observed in all subjects was moderately constant, so that certain general conclusions could be drawn, from which assumptions were then made concerning the source of the excreted radioiron. By the considerations detailed below, it was possible to arrive at an estimate of total daily faecal iron loss, as well as the relative contributions made by the loss of red cells through the bowel and by other sources including secretions such as bile and mucosal cells.

The typical pattern of radioiron excretion is shown in Figure 5.1. There was a marked early peak in the first two to eight days which was regarded as mainly being due to radioiron lost in secretions and desquamated mucosal cells. The peak gradually tapered off and was then maintained at a fairly constant plateau throughout the remaining period of observation. The sustained low level of faecal radioactivity was taken to represent daily blood loss through the gastrointestinal tract, since red cells with incorporated tracer iron were present in the circulation at this time, and moreover, the degree of labelling of the red cells could be expected to remain unchanged over the survival period of this labelled cohort.

The actual amount of blood lost each day over this later period could be quantified by comparing the radioactivity in the faeces with the measured level of radioactivity in circulating blood taken on the same day. By averaging the daily faecal blood loss, a figure was obtained for the mean daily iron loss from the bowel due to blood. This was given by:

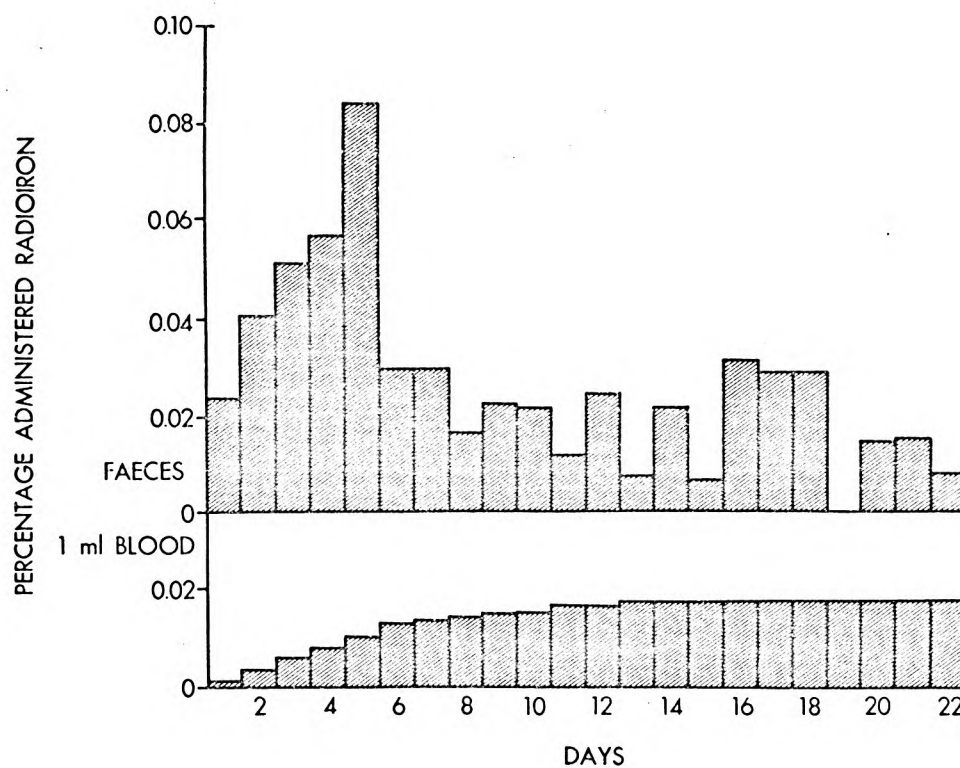


FIGURE 5.1. Representative pattern taken from the results in one subject of daily faecal radioiron losses following intravenous injection of ^{59}Fe tracer. The amounts of radioactivity present in one millilitre whole blood on each day are also shown.

$$\begin{aligned}
 &\text{Mean daily faecal iron loss due to blood (mg)} = \\
 &\text{Mean calculated daily faecal blood loss (ml)} \times \\
 &\text{haemoglobin concentration (g per ml)} \times 3.4^* \dots\dots\dots (1)
 \end{aligned}$$

The amount of radioiron present in the faeces each day over the later period remained fairly constant, and since the level of circulating radioactivity was also constant, this suggested that the daily quantity of blood being lost through the gastrointestinal tract normally did not vary much. It could therefore be assumed that the estimate of mean daily faecal blood loss was equally valid for the earlier period. It was then possible to calculate how much of the peak of radioactivity excreted during the early period was contributed by blood. During the first seven to ten days, the radioactivity in blood would be rising steadily as newly formed red cells which had incorporated radioiron were entering the circulation. The amount of radioactivity due to a constant volume of blood in the faeces would, therefore, also rise steadily. Taking into account the amount of radioactivity present in blood on each day, it was then possible to calculate how much of the faecal radioactivity was due to blood loss on that day. This was done by applying the figure for the mean estimated daily faecal blood loss (ml) in each subject, to the radioactivity (counts per ml) in the circulating blood. Figures for faecal radioactivity on the days spanning the early peak of excretion were then corrected by subtracting the calculated number of counts assumed to be due to blood from the total number of stool counts on each day. The fraction that remained was assumed to be derived from sources other than blood.

* One gram haemoglobin contains 3.4 mg iron (Bernhart and Skeggs, 1943).

Although this non-haemoglobin fraction of radioactivity was lost over several days, it was derived from the injected dose of radioiron, almost all of which was cleared from the circulation within the first day. From what is known of the plasma iron clearance curve, it would appear that feedback re-entry of radioiron into the plasma consists of two components (Morgan, *et al.*, 1967; Hosain, *et al.*, 1967). The first, is rapid, with a $T_{1/2}$ in the region of eight hours, but constitutes only 5 to 10 per cent of the injected dose of radioiron. The second component, although it may represent 25 per cent of the administered radioiron, has a slow reflux ($T_{1/2}$ of around eight days). Neither of these feedback mechanisms would therefore have any significant bearing on the fraction of the tracer that is initially cleared from the plasma to mucosal cells or secretions of the gastrointestinal tract which are then lost in the faeces within the eight days of the early peak of radioiron excretion. The non-haemoglobin fraction of faecal radioiron loss could therefore be regarded as being derived from a fixed proportion of the iron passing through the plasma which is distributed to various parts of the gastrointestinal tract and then lost. The actual amount of iron that this radioactivity represented was calculated by expressing it as a percentage of the injected tracer dose, and then relating this to the daily plasma iron turnover in each subject. Thus:

$$\begin{array}{lcl} \text{Daily gastrointestinal iron} & & \text{Cumulative non-haemoglobin} \\ \text{loss excluding blood} & = & \text{stool counts} \\ \text{(mg per day)} & & \text{Counts injected} \end{array} \times \begin{array}{l} \text{Plasma} \\ \text{iron} \\ \text{turn-} \\ \text{over} \\ \text{(mg per day)} \end{array}$$

..... (2)

A figure for total daily gastrointestinal iron loss was obtained by adding the amount due to blood and the calculated non-haemoglobin iron loss. This was given by the sum of the figures obtained in equations (1) and (2).

(iv) Results

In the studies designed to estimate gastrointestinal iron losses using the ^{59}Fe tracer technique, the percentage of intravenously injected radioiron that was excreted in the stool each day was measured, as well as the circulating level of radioactivity. Because of the uniform pattern obtained in each subject (Figure 5.1), it was possible to quantify two components of daily faecal iron loss by employing the calculations detailed above. These two components, due to blood loss on the one hand and sources such as iron lost in cells and secretions on the other, were added together to give the total daily loss of iron from the gastrointestinal tract. Results on the 13 subjects studied is given in Table 5.2.

In eight of the 13 subjects daily gastrointestinal blood loss could be quantified by comparing the radioactivity present in the stool after the early peak of radioiron excretion with the corresponding level of circulating blood radioactivity. When stool weights were compared with the radioactivity in the specimens passed after the initial peak had subsided, a directly proportional relationship was observed. This confirms the findings of Levin, *et al.*, (1959) who used ^{51}Cr -tagged red cells to measure gastrointestinal blood loss, and lends supportive evidence to the assumption in the present studies that radioiron loss over this time reflects faecal blood loss. The mean calculated daily faecal blood loss in these eight subjects was 0.77 ml (SD 0.16). Taking into account the haemoglobin concentration in each subject, the mean daily faecal iron loss due to blood was 0.38 mg (SD 0.07) in this group. In the remaining five subjects, in whom it was not possible to extend the period of observation for long enough beyond the early peak of radioiron loss to reliably estimate daily faecal blood loss, an assumed maximum of one millilitre blood loss in the stool each day (Hughes Jones, 1958) was used

Table 5.2. Total gastrointestinal iron losses (haem and non-haem) measured by a radioisotopic method using ^{59}Fe

SUBJECT	PLASMA IRON TURNOVER (mg per day)	URATION OF STOOL COLLECTION (days)	MEAN CALCULATED DAILY FAECAL LOSS (ml)	MEAN CALCULATED DAILY FAECAL IRON LOSS DUE TO BLOOD (mg)	COMPUTED DAILY IRON LOSS EXCLUDING BLOOD (mg)	CALCULATED TOTAL DAILY GASTROINTESTINAL IRON LOSS (mg)
1	23.7	18	0.81	0.35	0.04	0.39
2	50.9	33	0.55	0.37	0.22	0.58
3	21.7	33	0.65	0.32	0.05	0.36
4	28.2	49	0.97	0.36	0.20	0.56
5	35.0	18	0.58	0.25	0.13	0.38
6	44.1	23	0.81	0.45	0.13	0.58
7	26.7	7	1.00*	0.55*	0.04*	0.59*
8	30.9	13	0.88	0.45	0.12	0.57
9	64.8	33	0.92	0.46	0.23	0.69
10	38.1	6	1.00*	0.58*	0.12*	0.70*
11	45.8	9	1.00*	0.51*	0.16*	0.67*
12	55.5	10	1.00*	0.51*	0.22*	0.73*
13	42.3	10	1.00*	0.53*	0.20*	0.74*
MEAN	39.05	20	0.771	0.376	0.140	0.514
S.D.	13.10		0.158	0.074	0.072	0.121

* Assumed maximum for daily faecal blood loss. Not included in calculating the mean.

in the calculation of the non-haemoglobin component of gastrointestinal iron loss.

By subtracting the amount of radioactivity present in a volume equal to the mean daily faecal blood loss from the measured daily stool radioactivity during the early peak of radioiron excretion, a measure of the gastrointestinal radioiron losses arising from sources other than blood could be derived. In each of the eight subjects studied over prolonged periods (longer than 12 days), the value for the mean daily faecal blood loss was obtained by direct measurement, as described previously. In the five subjects studied for shorter periods, an assumed daily loss of one millilitre per day was employed for each subject.

The calculated figure for cumulative non-haemoglobin stool radioactivity excreted over the period spanning the early peak of radioiron loss was expressed as a percentage of the injected dose of tracer. It was assumed that a similar percentage of the daily plasma iron turnover would be lost through the gastrointestinal tract, and the daily non-haemoglobin iron loss was calculated for each subject. The mean daily faecal iron loss, excluding blood, for all 13 subjects calculated in this way was 0.14 mg (S.D. 0.07).

The five subjects with clinical and biochemical evidence of iron overload (transferrin saturation greater than 50 per cent) had higher calculated daily non-haemoglobin iron losses (mean 0.19; S.D. 0.05 mg) compared with the other subjects studied (mean 0.12; S.D. 0.07 mg). Although suggestive, this difference was not statistically significant when Student's 't' test was applied ($t_{11} = 1.962$; $0.1 > P > 0.05$).

Also, when the group was considered as a whole, the correlation between the amount of non-haemoglobin iron lost through the gut and the percentage saturation of circulating transferrin was not statistically significant ($r = + 0.436$; $0.2 > P > 0.1$).

The sum of the figures for mean daily iron loss due to blood (eight subjects) and due to other non-haemoglobin sources (13 subjects) gave a figure of 0.52 mg (S.D. 0.12) for total daily gastrointestinal iron loss.

(b) Biliary iron losses : Experimental

(i) Subjects studied

The daily excretion of iron in bile was measured in 20 White subjects, using chemical and radioisotopic techniques either singly, or in combination. Daily bile specimens were obtained from patients who had undergone gall bladder surgery with common bile duct exploration and who were continuously draining bile through an externalised T-tube placed in the common bile duct. The total duration of bile collection varied between two and 10 days.

(ii) Details of methods

Bile was collected in specially washed, iron-free containers, and when chemical estimations were performed, bile collected during the first 24-hour period was discarded to eliminate possible contamination due to instrumentation and handling during surgery. The daily volume of bile collected was recorded. Chemical estimation of the iron content of bile was carried out after wet-ashing a 5 ml aliquot of the daily specimens obtained from 18 subjects. The iron concentration (μg per 100 ml) and total iron content of each 24-hour bile sample were calculated.

In addition, a radioisotopic technique was used to assess biliary iron excretion in a total of eight subjects (including six of the subjects in whom biliary iron was measured chemically). A method similar to that for studying faecal iron losses was used. Ten microcuries ^{59}Fe was injected intravenously on the day after operation. The plasma iron turnover was then measured, as described previously. All

bile secreted subsequently was collected, and its radioactivity was measured. The radioactivity excreted in the bile each day was expressed as a percentage of the injected dose. The cumulative fraction of injected radioactivity excreted in the bile over the period of observation was then related to the plasma iron turnover, as was done in the case of estimating non-haemoglobin faecal radioiron loss. In this way, the fraction of the daily plasma iron turnover which entered the biliary tract was quantified.

(iii) Results

The iron content of bile collected from a T-tube in the common bile duct was measured chemically in 16 subjects. Relevant information and the results obtained in these subjects is given in Table 5.3. The figure for each subject represents the mean value obtained over a two to ten day collection period. The mean biliary iron concentration in the group of subjects studied was 83.3 μg per 100 ml (S.D. 61.3) and the mean total daily iron content of bile was 0.238 mg (S.D. 0.128).

In eight subjects, biliary excretion of radioiron was studied after the intravenous administration of ^{59}Fe . Radioactivity was detectable in the bile within 15 minutes after the injection of radioiron. The amount of radioactivity excreted in the bile each day was expressed as a percentage of the injected dose. Radioiron excretion rose to reach a peak during the 72 hours following the injection, and then fell to lower levels which were maintained over the period of observation. A fairly constant pattern was obtained in each subject (Figure 5.2). The quantity of chemical iron represented by the ^{59}Fe tracer was calculated on the assumption that biliary radioiron excretion represented a proportion of the radioiron cleared from the plasma, and that a similar proportion of the daily plasma iron turnover would enter the bile. In this way, the mean calculated figure for daily iron in bile was found to be 0.035 mg (S.D. 0.026) (Table 5.4).

Table 5.3. Biliary iron excretion measured by chemical determination

SUBJECT	SEX	AGE	SERUM IRON	SERUM TOTAL IRON BINDING CAPACITY	TRANSFERRIN SATURATION	URATION OF BILE COLLECT- ION	MEAN DAILY BILE VOLUME	MEAN BILIARY IRON CONCENTRATION	TOTAL MEAN DAILY CHEMICAL IRON CONTENT OF BILE
(Years)			(μg per 100 ml)	(μg per 100 ml)	(per cent)	(days)	(ml)	(μg per 100 ml)	(μg)
1	F	52				5	205	S.D. 48 244 182	S.D. 0.507 0.402
2	F	39				2	218	145 131 49	0.249 0.083
3	F	68	33	238	14	3	352	150 31 19	0.125 0.093
4	F	57	51	238	21	8	409	119 37 9	0.154 0.051
5	M	66				9	266	118 92 106	0.158 0.084
6	F	60	16	170	9	6	561	101 24 13	0.135 0.073
7	F	57	116	278	42	6	271	78 27 14	0.075 0.049
8	F	51	42	205	21	6	397	68 53 16	0.205 0.054
9	M	69	51	298	17	6	389	38 44 17	0.169 0.060
10	F	74				9	254	108 136 35	0.325 0.109
11*	F	79	51	222	23	8	483	128 94 33	0.456 0.202
12*	F	45	53	286	19	7	231	66 122 37	0.275 0.090
13*	F	61	34	269	13	10	460	38 42 17	0.193 0.076
14*	F	66	100	253	40	7	267	106 123 14	0.328 0.157
15*	F	55	44	202	22	8	728	258 12 4	0.086 0.047
16*	F	24	13	289	5	5	293	127 121 20	0.371 0.202
			OVERALL MEAN		6.6	361.5	83.3		
			S.D.		143.2	61.3	0.238		
							0.128		

* Subjects also studied using ^{59}Fe radioiron (see Table 5.4).

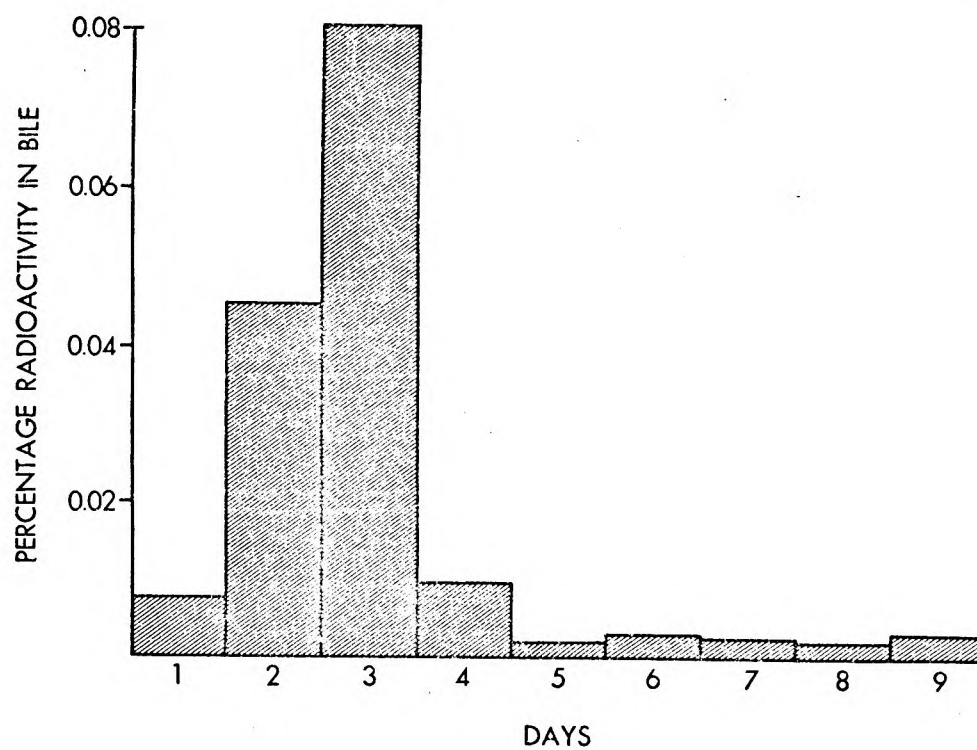


FIGURE 5.2. Biliary radioiron excretion following intravenous injection of ^{59}Fe tracer. Typical pattern taken from the results in one subject.

Table 5.4. Biliary iron excretion calculated using ^{59}Fe radioiron

SUBJECT	SEX	AGE	SERUM IRON	SERUM TOTAL IRON BINDING CAPACITY	TRANSFERRIN SATURATION	$T_{1/2}^{59\text{Fe}}$ CLEARANCE	CALCULATED PLASMA VOLUME	PLASMA IRON TURNOVER	DURATION OF BILE COLLECTION	MEAN DAILY COLLECTED BILE VOLUME	FRACTION OF ^{59}Fe EXCRETED IN BILE	CALCULATED DAILY IRON LOSS IN BILE
		(years)	(μg per 100 ml)	(μg per 100 ml)	(per cent)	(minutes)	(ml)	(mg per day)	(days)	(ml)	(per cent)	(mg)
11*	F	79	51	222	23	35	2324	34.1	8	483	0.058	0.020
12*	F	45	53	286	19	65	2703	22.1	7	231	0.158	0.035
13*	F	61	34	269	13	25	2315	31.2	10	460	0.061	0.019
14*	F	66	100	253	40	89	2928	32.8	7	267	0.104	0.034
15*	F	55	44	202	22	57	2695	20.8	8	728	0.019	0.004
16	F	24	13	289	5	12	2280	25.0	5	293	0.072	0.018
17	F	62	62	283	22	61	2893	29.4	10	368	0.211	0.062
18	M	62	33	250	13	30	2680	29.8	9	286	0.305	0.091
			MEAN						8	389.5	0.124	0.035
			S.D.							164.3	0.095	0.026

* Subjects in whom the chemical iron content of bile was also measured (see Table 5.3).

In six of these subjects, chemical iron estimations were also carried out on the bile samples. These results were several fold higher (mean 0.285; S.D. 0.131 mg) than the calculated figures based on kinetic measurements.

(c) Discussion : Gastrointestinal including biliary iron losses

Assessment of total gastrointestinal iron losses was attempted by measuring the faecal excretion of intravenously injected ^{59}Fe tracer. Quantitative losses, excluding faecal blood, were then calculated by relating the computed figures obtained for non-haemoglobin iron loss to the plasma iron turnover. The calculation was based on the assumption that the mucosal cells of the gastrointestinal tract derive their iron only from the plasma. Using this approach, it was not possible to document any iron losses which might have resulted from non-plasma derived sources such as the shedding of iron-containing reticuloendothelial cells, since such sources would not have become labelled during the relatively short periods of observation. However, no late peaks in stool radioactivity were recorded in previous radioiron excretion studies in which the periods of observation extended over 140 and 300 days respectively (Dubach, *et al.*, 1955; Saito, *et al.*, 1964). Such peaks might have been expected as a result of the tracer mixing with reticuloendothelial iron when successive generations of labelled erythrocytes disintegrated.

After correcting for the estimated amount of radioiron due to blood, it was assumed that the initial wave of radioactivity which appeared in the faeces following the intravenous injection of ^{59}Fe represented loss of mucosal cells, as well as some of the iron excreted through the biliary tract. The calculated mean daily iron loss arising from this excretory mechanism was 0.14 mg, and was not significantly greater in iron-overloaded compared with normal subjects. Data from

the radioisotopic biliary iron excretion studies suggested that about one-quarter of this iron (0.035 mg), could have been derived from the bile, not taking into account the probable reabsorption of a proportion of biliary iron. The temporal appearance of peak radioiron excretion in the bile would correspond with a portion of the peak of faecal radioiron excretion, allowing for transit time delay in the bowel from the level of the common bile duct to the rectum.

The remainder of the peak of faecal radioiron excretion was probably derived from gastrointestinal secretions and in sloughed mucosal epithelial cells. The turnover rate of the majority of cells lining the gastrointestinal tract is between one and four days (Lipkin, *et al.*, 1963). It has been shown in animals, that transferrin-bound radioiron given intravenously enters newly formed epithelial cells in the crypts of the small intestine, and migrates with the cells as they move to the villus tip (Conrad, *et al.*, 1964). These workers also found that iron entered the gastric and colonic epithelial cells. These findings were confirmed by Loehry, *et al.*, (1969a) who also obtained evidence to suggest that there is early loss of a small portion of transferrin-bound iron into the bowel lumen by exudation.

When the calculated early iron losses in bile based on the present radioisotopic studies were compared with the chemically measured iron content of bile, there was an obvious discrepancy. The mean daily iron content of bile (0.24 mg) was greater than the mean figure calculated from the radioisotope excretion data (0.035 mg). Furthermore, the mean daily chemical iron content of bile was greater even than the mean computed total daily non-haem iron loss from the bowel (0.14 mg), which was obtained from faecal radioiron excretion experiments. Since every effort was made to avoid iron contamination in chemical estimations, the differences noted probably indicate

that the bulk of biliary iron is not derived directly from the plasma. Studies with the iron chelate desferrioxamine suggest that at least some of the iron in bile is derived from parenchymal cells (Harker, *et al.*, 1968). In addition, it has been shown that the excretion of iron in bile increases after the induction of haemolysis in dogs (Hahn, *et al.*, 1939; Hawkins and Hahn, 1944). This suggests that reticuloendothelial cells in the liver may contribute to the iron content of bile. Bradford, *et al.*, (1969) also found that ferritin accounts for at least a portion of the biliary iron in rats. If most of the iron in bile is indeed derived from parenchymal and/or reticuloendothelial cells, it would not be possible to quantify such losses using the type of short-term radioisotopic technique employed in the present studies. The labelling of hepatic storage iron, from which biliary iron may be largely derived, would occur only over a longer time. Finally, in attempting to explain the discrepancy between the measured amounts of iron in bile and the computed total daily non-haemoglobin iron lost in faeces, it should be pointed out that a proportion of biliary iron is undoubtedly reabsorbed in the small intestine. It is not known how much of the iron in bile is ultimately lost from the body, and how much is reabsorbed by the gastrointestinal tract. Invoking this as a complete explanation for the discrepancy would imply that reabsorption of biliary iron is highly efficient.

Radioactivity appearing in the stools beyond the early peak of radioiron excretion was taken to represent faecal blood loss. The magnitude of this loss was quantified by relating the radioactivity in stools to that present in the circulating blood on the same day. On this basis, the mean daily blood loss was found to be 0.8 ml, equivalent to approximately 0.4 mg/iron. The mean figure for daily faecal blood loss obtained in Indian and Negro subjects in the long-term iron excretion study (Chapter 4), was approximately 0.4 ml (ca 0.2 mg iron)

when measured using ^{51}Cr -tagged red cells. Faecal blood loss determination using this technique, while suitable as a screening procedure to detect abnormal gastrointestinal bleeding, is not ideally suited to the accurate quantitation of faecal haemoglobin iron losses. A variable fraction of the radiochromium may be eluted from red cells in the gut, and be reabsorbed independently of haemoglobin iron (Hughes Jones, 1958). The fate of haemoglobin iron in red cells which are shed into the gut lumen is not known, but a portion must almost certainly be reabsorbed. Regardless of the extent of this process, however, the technique of direct ^{59}Fe labelling of red cells to measure faecal blood loss should give an index of the net loss of red cell radioiron. It is this figure for external loss which is important for the purpose of quantifying iron excretion, rather than the amount of blood shed into the lumen of the gastrointestinal tract.

The composite loss of iron from the bowel obtained by adding the calculated non-haemoglobin loss represented by the early peak to the figure for blood loss was between 0.5 and 0.6 mg daily. The relative contributions to this figure made by blood and non-haemoglobin sources were approximately two-thirds and one-third respectively. In addition, however, a further unknown fraction of the 0.24 mg iron present in bile, and derived largely from sources other than the plasma, must also be lost.

The difference in mean daily non-haemoglobin iron loss between the normal and the iron-overloaded subjects, approached, but did not quite attain a level of statistical significance (p between 0.1 and 0.05). This could be taken as suggestive evidence that the iron excretory mechanism of the bowel is a variable one, dependent on the state of body iron repletion, and mediated through differences in the amounts of iron delivered to the mucosal cells from the plasma. Dubach, *et al.*, (1955) reported that gastrointestinal iron losses in iron

deficient subjects, were about one-third of the amounts found in normal subjects. Regarding possible differences in the amounts of iron lost through the gastrointestinal tract, which depend on an individual's iron status, another probable mechanism of control should be considered. The absorption of all forms of iron from the intestine is more efficient in iron deficient persons (Bothwell and Finch, 1962, p. 108; Monsen, *et al.*, 1967). This implies that endogenous iron lost into the bowel lumen would also be reabsorbed to a greater extent in states of iron deficiency, hence providing a further mechanism for the conservation of body iron. However, the range of non-haemoglobin iron losses obtained in the present studies was not great (0.04 to 0.23 mg per day), so that this mechanism would appear to play a relatively minor role in the regulation of body iron excretion. Any control of iron excretion vested in the gut is therefore more likely to depend on biliary or reticuloendothelial cell iron losses.

The quantitative estimates of gastrointestinal iron losses obtained in the present investigations are in good agreement with the findings of several previous studies which used either carefully controlled chemical techniques (Ingalls and Johnston, 1954; Green, *et al.*, 1968), or radioisotopic methods (Dubach, *et al.*, 1955; Baird, 1973). In these studies which were reviewed in Chapter 2, gastrointestinal iron losses ranged from 0.2 to 0.75 mg per day, compared with the present mean of 0.51 mg per day.

One important point of difference between the findings presented in this thesis and the findings reported by Singh (1970) and by Baird (1973) concerns the relative contributions to faecal iron losses made by blood and by other, non-haem sources. Both workers, using combined ^{51}Cr and ^{59}Fe labels to discriminate between haem and non-haem iron losses concluded that the greater part of excreted faecal iron was derived from

non-haem sources. For reasons discussed above, criticisms of the ^{51}Cr labelling technique to accurately quantify faecal blood loss casts some doubt on the validity of these findings. However, since the technique used in this thesis suffers from the drawback of being based on several assumptions, it would be fair to conclude that the question remains unanswered. In order to resolve the problem of whether haem or non-haem losses constitute the major source of excreted faecal iron, further investigations are necessary.

(d) Summary and Conclusions

The major source of gastrointestinal iron loss appears to be from red cells shed into the gut lumen. Radioisotopic experiments using a novel approach to determine the quantities of iron lost via the gastrointestinal tract, suggested a daily loss of approximately 0.4 mg in blood, and a further 0.1 mg from sources other than blood, such as desquamated mucosal cells. By the nature of the calculations upon which it was based, the estimate of non-haemoglobin iron losses took into account only that iron which entered the gut from the circulating plasma pool. It is possible that some iron may also be lost through the gut by other mechanisms, not derived directly from the plasma. This possibility is supported by the studies on biliary iron excretion. It was found that the chemical iron content of bile (0.24 mg per day) was considerably greater than the calculated figure for plasma-derived biliary iron (ca 0.04 mg per day) based on radioisotopic experiments. It is not known what proportion of the iron in bile is reabsorbed into the body.

Excluding blood loss, the amounts of iron excreted through the gastrointestinal tract are probably small, but there is a suggestion that they may be influenced by the state of the body iron stores. Although not investigated in the

present studies, it is possible that in iron overload, additional iron may be lost through the gut in bile and reticuloendothelial cells. It is therefore not possible to state with any certainty, the maximum figure for iron excretion through the gastrointestinal tract. However, in subjects with normal iron stores, gut losses are not likely to exceed 0.6 mg per day. This figure is in good agreement with the findings of several other workers. However, the present findings are not in accord with the view that non-haem iron losses exceed the iron loss due to blood. This question, although not central to the problem of quantitative external iron losses, remains unresolved, and requires further study for its elucidation.

IV. URINARY TRACT

(a) Subjects studied and method

The total urinary iron content was measured chemically in six Negro subjects with varying states of iron nutrition. Two subjects were normal, one had iron deficiency anaemia, and three had nutritional iron overload. All urine was collected in acid-washed, iron-free glass containers over seven to ten day periods. Females were studied only after at least four days had elapsed since the last day of a menstrual period. A 250 ml volume of the total, measured 24-hour urine output was boiled down and wet-ashed and the chemical iron content was then measured by the thioglycolic acid method (Bothwell and Finch, 1962, p. 26).

(b) Results

Relevant information in subjects in whom the iron content of urine was measured chemically as well as the results of this study are given in Table 5.5. Mean daily losses (with standard

Table 5.5.

Urinary iron losses measured by chemical determination

SUBJECT	SEX	AGE	IRON STATUS	SERUM IRON (µg per 100 ml)	SERUM TOTAL IRON BINDING CAPACITY (µg per 100 ml)	TRANSFERRIN SATURATION (per cent)	DURATION OF URINE COLLECTION (days)	MEAN DAILY URINE VOLUME (ml)	MEAN DAILY CHEMICAL IRON CONTENT OF URINE (mg)
1	F	44	Iron-deficient	32	410	8	8	1275	205 0.088 0.061
2	F	51	Normal	56	293	19	9	1268	96 0.061 0.031
3	F	29	Normal	112	355	32	9	1699	352 0.142 0.062
4	M	46	Iron-overloaded	142	242	59	9	1626	269 0.107 0.053
5	M	49	Iron-overloaded	221	244	91	10	1509	229 0.112 0.047
6	M	58	Iron-overloaded	178	192	93	9	1339	269 0.162 0.063
OVERALL MEAN 0.1120									
S.D. 0.0346									

deviations) for each subject were as follows: iron-deficient subject 0.088 (S.D. 0.061) mg; normal subjects 0.061 (S.D. 0.031) and 0.142 (S.D. 0.062) mg, respectively; iron-overloaded subjects 0.112 (S.D. 0.047), 0.162 (S.D. 0.063) and 0.107 (S.D. 0.053 mg respectively. There was no significant difference ($t_4 = 1.014$; $0.4 > P > 0.3$) when the results in the iron-overloaded subjects were compared with the other subjects. The mean daily iron loss in urine for all subjects was $0.112 (\pm 0.035)$ mg.

(c) Discussion

Previous studies concerning urinary iron excretion have been reviewed in Chapter 2. The present results of chemical iron determinations on the urine of subjects with low, normal and high iron stores confirm the view that urinary iron losses are normally insignificant, regardless of body iron content (Dagg, *et al.*, 1966). The mean daily excretion of approximately 0.1 mg iron in the urine is small in relation to the other major avenues of iron loss, the gastrointestinal tract and the skin.

The mechanism of iron loss from the epithelial surface of the urinary tract is analogous to that of the gastrointestinal tract. Iron loss through the two avenues occurs both as a result of desquamation of iron-containing epithelial cells, and from the shedding of small numbers of erythrocytes. The problem of discriminating between unabsorbed dietary iron and excreted iron in the faeces precludes the use of chemical techniques for measuring gastrointestinal iron losses. Although this problem does not apply in the case of the urinary tract, chemical iron estimation does not give any indication of the relative contributions made by epithelial cells and by erythrocytes to the total urinary iron content. Such an analysis was possible in the case of gastrointestinal iron losses measured by the radioisotopic technique. Nonetheless, it is possible to arrive at some estimate of

the relative contributions made by epithelial cells and by erythrocytes to the urinary iron content, and it is useful to compare this with the gastrointestinal tract.

The maximum normal daily loss of erythrocytes in the urine could account for only about 0.005 mg iron (Gadeholt, 1964). This suggests that almost all of the 0.11 mg iron present in urine arises from cellular desquamation. By comparison calculated mean daily non-haemoglobin iron excretion from the gut, comprising both cellular and biliary losses, was found to be 0.14 mg. The turnover rate of epithelial cells lining the urinary tract is considerably slower than that of gastrointestinal tract. (Leblond and Walker, 1956). Also, the total surface area of the urinary tract must be less than the gastrointestinal tract. This implies that the mass of cells lost from the urinary tract is small compared with the gastrointestinal tract. Despite this, comparable amounts of iron are lost from both routes through cellular desquamation. This suggests that a considerable proportion of the iron contained in gastrointestinal mucosal cells is conserved by the body, either by reabsorption, or by retention of cellular contents prior to desquamation (Cutright and Bauer, 1967). Alternatively, the radioisotopic technique used for measuring gastrointestinal iron losses may have underestimated the total cellular iron loss, since it took into account only the plasma-derived iron.

(d) Summary and Conclusions

Urinary iron excretion is small (ca 0.1 mg per day) and does not seem to be influenced by the size of the body iron stores. Of the three known routes of iron excretion, the urinary tract is the least significant, and is surrounded by the least controversy.

V. SKIN LOSSES

In an attempt to assess iron loss through the skin and its appendages, both a radioisotopic and a chemical technique were used. In each case, the approach was designed to obtain a measure of the maximum amounts of iron that could be lost from the skin. In these studies, it was assumed that iron is not returned to the body once it has been taken up by the epidermal cells of the skin and its appendages.

(a) Subjects studied and methods

(i) Radioisotopic technique

To obtain an estimate of skin iron losses, a radioisotopic approach similar to that used for the measurement of gastrointestinal and biliary losses was employed. Twenty-four subjects who were to undergo major surgery were studied. Patients were selected who were to have operations at which appreciable amounts of redundant skin were to be removed, such as amputations or mastectomies. Subjects were given 10 μCi ^{59}Fe by intravenous injection approximately 20 hours before operation. The iron was administered as the citrate salt to subjects with normal or low serum iron concentrations and percentage transferrin saturations. In those with a percentage transferrin saturation greater than 50 per cent, the ^{59}Fe was pre-incubated with plasma taken from a normal donor before injection. The plasma iron turnover was then measured as previously described. In four subjects, the percentage saturation of circulating transferrin was artificially elevated, half to one hour before the injection of radioiron. This was achieved by the intramuscular injection of iron-sorbitol-citrate (Jectofer, Astra, Södertälje, Sweden) in doses which ranged from 20 to 100 mg. It has been shown that such injections produce a rise in the level of transferrin-bound plasma iron for up to 12 hours after injection (Wahlqvist,

personal communication, 1966). The height and weight of each subject was recorded and from this the total body surface area was obtained using nomogram tables (Documenta Geigy, 1962).

At operation a template of the area of skin to be removed was made *in situ* by applying '3M steridrape' (Minnesota Mining and Manufacturing Co., U.S.A.) directly to the skin over the site of incision. When this was not possible (e.g. amputations) a template was made by the application of the non-stretch adhesive dressing 'Sleek' (Smith and Nephew, England) to the surface of the amputated limb. The surface area of the template was measured by manual planimetry after tracing the outline of its contours on square-lined graph paper.

A blood sample was taken at the time of operation and the level of circulating radioactivity was determined. At this time (between 16 and 24 hours after the injection) circulating radioactivity should have been at a minimum, since the radioiron would have been almost completely cleared from the plasma, and would have not yet appeared in newly formed erythrocytes. There was therefore little chance of contamination of the specimen of skin by any radioactivity present in the blood. However, all specimens were meticulously cleaned free of any subcutaneous fat and visible traces of blood by gentle scraping using a scalpel blade. As a check on the efficacy of this procedure, haem iron estimations were carried out using a pyridine-haemochromogen method (Bothwell, *et al.*, 1964) on specimens of nonpigmented skin treated in the same fashion as that used in the radioisotopic study. There was no detectable haem pigment in these specimens. Furthermore, the several amputations included in the series were carried out using bloodless-field surgery.

After cleaning, the amount of radioactivity present in the skin sample was measured. Assuming an even distribution of radioiron in the entire integument, the amount of radioiron

contained in the total body skin area was calculated by simple proportion. This was done by multiplying the radioactivity present in the specimen by the factor : total body surface area/area of skin counted. The calculated figure for radioactivity in the total skin area was then expressed as a percentage of the injected dose of radioiron. Further, it was possible to obtain an estimate of the amount of iron taken up from the plasma by the skin each day by applying the percentage of injected radioiron in the skin to the measured daily plasma iron turnover.

In the 21 subjects studied, skin was obtained from a number of different sites. In 17, skin was taken from an eccrine sweat gland-bearing area. In three of these subjects, as well as a further four subjects, skin was also taken from an apocrine gland-bearing area (axilla, groin, vulva).

(ii) Chemical technique

In an attempt to estimate dermal iron losses by another technique, the 'epidermal iron pool' was measured using a chemical method. Areas of skin were removed at autopsy from 17 cadavers following acute traumatic death. The skin was taken from the lateral anterior abdominal wall, which is an eccrine sweat gland-bearing site. The area of skin was measured with a template as described above, and the skin was cleaned by gentle wiping with 2N hydrochloric acid before removal. Skin specimens were carefully cleaned of all traces of blood and subcutaneous fat, and then placed in a boiling water bath (iron-free water) for one minute. This enabled easy stripping of the epidermis from the underlying dermis. The iron content of the stripped epidermis was measured chemically using the thioglycolic acid method after wet digestion of the sample (Bothwell and Finch, 1962, p. 26).

The total skin surface area of each cadaver was calculated from the measured weight and height using nomogram tables

(Documenta Geigy, 1962). The total 'epidermal iron pool' was obtained by multiplying the iron content of the stripped piece of epidermis by the factor : total surface area/area of skin specimen. The maximum possible daily iron loss from the epidermis was then computed by relating its estimated total iron content to figures for human skin turnover rates (Rothberg, *et al.*, 1961).

The non-haem iron concentration was also measured in specimens of liver removed from the same cadavers using a pyridine-haemochromogen method (Bothwell, *et al.*, 1964).

(iii) Sweat iron

Finally, an attempt was made to measure the quantities of iron lost in sweat in subjects undergoing acclimatization experiments in a climatic chamber at the Chamber of Mines Human Sciences Laboratory in Johannesburg. Plastic arm bags enclosing the arm and shoulder were used for sweat collection while the subjects performed exercise at a high temperature and humidity. However, for technical reasons, the study was discontinued, because it proved impossible to exclude external iron contamination under the conditions of the experiment. Despite careful and repeated gentle washing of the skin, first with soap and water, then water and finally iron-free distilled water, the final distilled water rinse was found to contain significant quantities of iron (3 to 10 µg per ml) using a modification of the bathophenanthroline method used for serum iron determinations (Bothwell and Mallet, 1955). It was not possible to determine whether this iron was due to external contamination or to desquamation of cells caused by the washing process. Centrifugation of the washings did not decrease their iron content.

(b) Results

(i) Radioisotopic technique

Skin was obtained from different parts of the body at operation 16 to 24 hours after intravenous administration of ^{59}Fe radioiron. As detailed above, contamination of the specimens by radioactivity present in the circulating blood at this time was absent or minimal. Moreover, care was taken to remove all traces of blood from the skin specimen, and there was no detectable haem pigment in cleaned skin specimens.

The amount of radioiron localised in skin taken from eccrine sweat gland-bearing areas in 13 subjects was compared with apocrine-bearing areas taken from seven subjects (Table 5.6). Subjects in whom the iron saturation of transferrin was artificially elevated were excluded from this comparison. The percentage of injected radioiron present in the skin was expressed per square meter, and it was found that apocrine-bearing skin showed a considerably greater radioiron uptake. The mean percentage uptakes per square meter were 2.14 (S.D. 1.98) and 0.35 (S.D. 0.27) per cent for apocrine and eccrine skin respectively. The difference was statistically significant ($t_{18} = 3.273$; $p < 0.005$). The mean circulating transferrin saturation was 27.3 (S.D. 11.9) and 21.0 (S.D. 9.7) per cent for the apocrine and eccrine group respectively. These were not significantly different ($t_{18} = 1.283$; $0.3 > p > 0.2$).

Despite the higher radioiron uptake by apocrine skin, further calculations to quantify the total skin iron uptake were based on the data obtained from eccrine skin only. This was deemed valid, since only a very small proportion of the skin surface area normally contains apocrine sweat glands (Montagna, 1962, p. 374). In all subjects from whom eccrine skin was obtained the total amount of radioiron localised in skin was calculated on the assumption that the radioactivity was uniformly dispersed. This was reasonably validated in two

Table 5.6. Radioiron uptake by skin following intravenous ^{59}Fe injection. Comparison between eccrine and apocrine sweat gland-bearing areas.

SUBJECT	ECCRINE SKIN			SUBJECT	APOCRINE SKIN		
	TRANSFERRIN SATURATION (per cent)	SITE	^{59}Fe RADIOIRON IN SKIN (per cent per meter ²)		TRANSFERRIN SATURATION (per cent)	SITE	^{59}Fe RADIOIRON IN SKIN (per cent per meter ²)
E1	6	arm	0	A1	12	groin	0.96
E2	11	thigh	0.07	A2	16	peri-areolar	0.86
E3	12	abdomen	0.93	A3	24	vulva	2.83
E4	13	abdomen	0.13	A4	25	axilla	0.33
E5	15	neck	0	A5	29	vulva	2.96
E6	16	breast	0.45	A6	43	groin and vulva	1.02
E7	20	leg	0.34	A7	42	groin	6.00
E8	25	buttock and neck	0.33*	MEAN	27.3		2.137
E9	27	leg	0.35	S.D.	11.9		1.980
E10	27	arm	0.34				
E11	30	breasts	0.55				
E12	35	back	0.66				
E13	36	leg	0.38				
MEAN	21.0		0.348				
S.D.	9.7		0.267				

* Mean figure for skin removed from two different sites.

subjects in whom skin removed from two different sites showed the same percentage of radioiron uptake per unit area. In one subject (E8) in whom lipomata were removed from the buttock and neck, the radioiron uptakes in the skin from these two sites were 0.30 and 0.36 per cent per square meter respectively. In the other subject (E11) who had bilateral simple mastectomies, the radioiron uptakes in the skin from each breast were 0.54 and 0.56 per cent per square meter.

All relevant data on the 17 subjects from whom eccrine skin was obtained are given in Table 5.7. The figures for total skin radioiron uptake were related to the plasma iron turnover as described above, in order to obtain a measure of the amount of iron taken up by the skin each day. Calculated figures for daily skin iron uptake in individual subjects were found to vary with the percentage saturation of circulating transferrin (Figure 5.3), and there was a statistically significant correlation between the calculated daily skin iron uptake and the percentage saturation of transferrin ($r_{16} = +0.942$; $p < 0.001$). At low transferrin saturations, little or no iron was sequestered in the skin, at normal saturations the average figure was between 0.2 and 0.3 mg, and figures of approximately 0.6 mg per day were reached when the transferrin saturation had been artificially raised to very high levels.

(ii) Chemical technique

In further studies, the chemical iron content of samples of epidermis removed at autopsy was measured in 17 subjects who died following acute trauma. By extrapolation of these measurements to the entire skin surface area, an estimate of the total 'epidermal iron pool' was obtained. Results, together with the liver non-haem iron concentration in each subject is given in Table 5.8. The mean total epidermal iron content, calculated from material obtained from an eccrine site was 22.5 (S.D. 17.8) mg. There

Table 5.7. Estimate of maximum daily skin iron losses based on skin uptake of intravenously injected ^{59}Fe radioliron

SUBJECT	SEX	AGE	HAEMOGLOBIN	SERUM IRON	SERUM TOTAL IRON BINDING CAPACITY	TRANSFERRIN SATURATION	PLASMA $T_{1/2}^{59\text{Fe}}$ CLEARANCE	CALCULATED PLASMA VOLUME	PLASMA IRON TURNOVER	INJECTED DOSE CLEARED TO TOTAL SKIN	CALCULATED DAILY IRON UPTAKE BY SKIN
			(g per 100 ml)	(μg per 100 ml)	(μg per 100 ml)	(per cent)	(minutes)	(ml)	(mg per day)	(per cent)	(mg)
E1	F	35	14.7	22	367	6	26	2340	19.8	0	0
E2	F	42	11.5	26	244	11	17	3229	48.3	0.13	0.063
E3	F	12	15.7	34	284	12	32	1662	11.3	1.03	0.116
E4	F	50	11.5	40	304	13	39	2605	27.0	0.27	0.134
E5	M	42	8.7	42	285	15	45	3197	29.8	0	0
E6	F	46	12.1	62	388	16	50	2560	31.7	0.82	0.258
E7	M	57	16.3	51	252	20	44	2913	33.7	0.56	0.187
E8	M	46	16.9	87	342	25	80	2619	28.4	0.58	0.166
E9	M	17	17.3	80	301	27	77	1794	18.6	0.56	0.105
E10	M	39	16.3	51	187	27	45	3338	37.7	0.70	0.263
E11	F	33	13.3	96	323	30	88	2119	23.1	0.87	0.202
E12	F	48	17.0	142	409	35	141	1973	19.8	1.04	0.206
E13	M	20	16.1	125	349	35	86	2387	34.6	0.64	0.222
E14*	M	52		167	252	66*	102	2983	48.7*	0.95	0.163
E15*	M	11	13.7	192	219	88*	132	1154	16.8*	2.87	0.180
E16*	M	46	9.0	178	191	93*	74	3063	73.5*	0.88	0.644
E17*	F	42	13.9	327	340	96*	129	1796	45.4*	1.20	0.555
			MEAN			36.2			32.25	0.771	0.2337
			S.D.			30.1			15.30	0.651	0.1942

* Serum iron artificially elevated with intramuscular dextrofer (iron-sorbitol-citrate). In these subjects, skin was obtained from the following sites: E14 (leg), E15 (leg), E16 (thigh), E17 (breast). Sites in other subjects shown in Table 5.6.

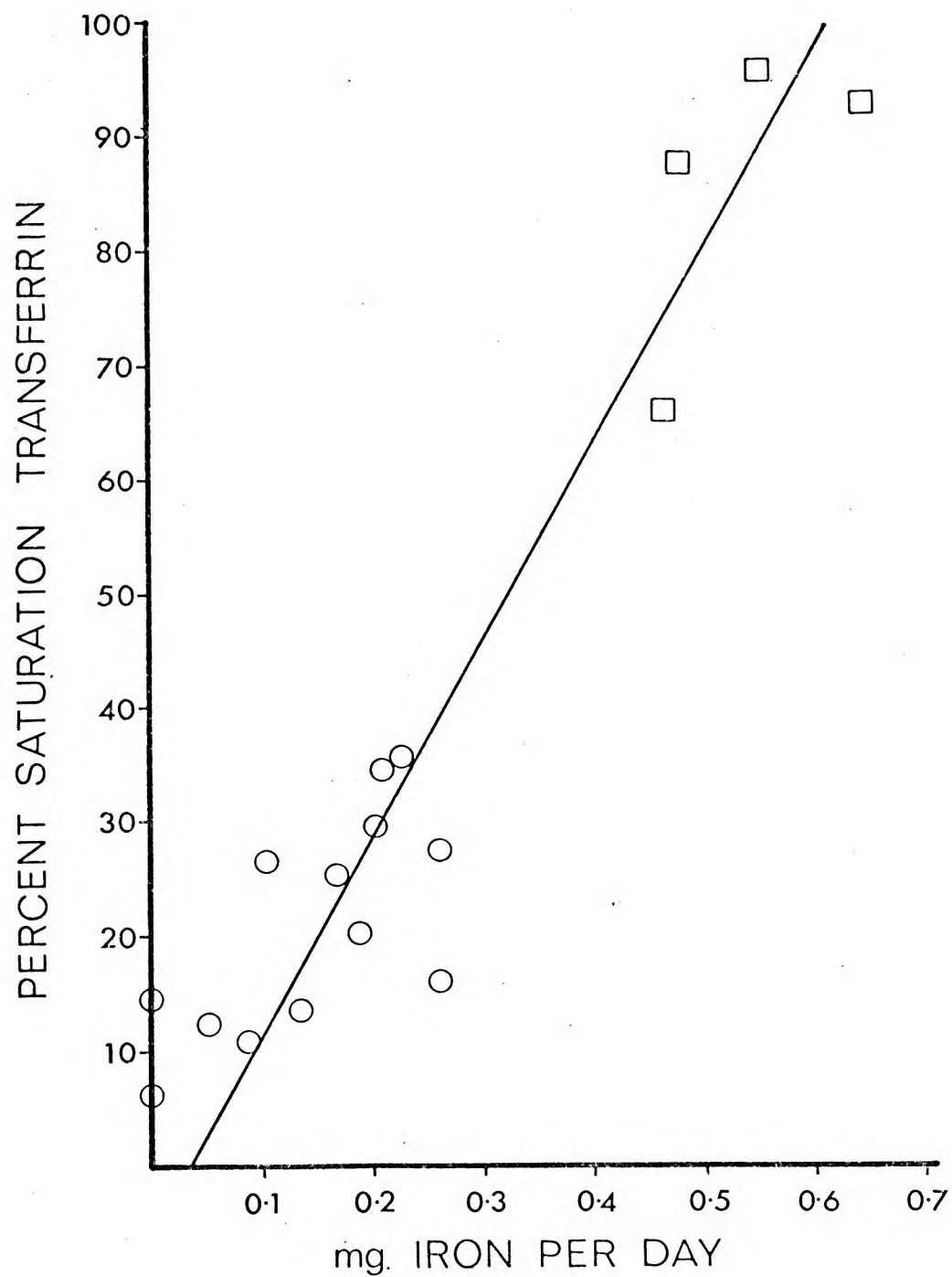


FIGURE 5.3. Correlation between calculated figures for daily uptake of iron by skin and the percentage saturation of transferrin ($r = + 0.942$). The transferrin saturation was artificially raised in four subjects (squares).

Table 5.8. Estimate of maximum daily skin iron losses based on
chemical determination of epidermal iron content

SUBJECT	ETHNIC GROUP	SEX	LIVER NON-HAEM IRON CONCENTRATION (mg per g wet weight)	TOTAL EPIDERMAL IRON CONTENT (mg)	CALCULATED MAXIMUM DAILY EPIDERMAL IRON LOSS (mg)
1	Negro	M	1.477	11.98	0.460
2	Negro	M	2.998	23.90	0.919
3	Negro	M	0.284	19.72	0.758
4	Negro	M	0.389	26.43	1.017
5	Negro	M	0.093	13.77	0.530
6	Negro	M	0.211	14.69	0.565
7	Negro	M	1.861	62.69	2.411
8	Negro	M	0.669	11.09	0.427
9	Negro	M	7.260	11.53	0.443
10	Negro	M	1.665	9.00	0.346
11	Negro	M	0.157	16.70	0.642
12	Negro	M	1.581	12.45	0.479
13	Negro	M	0.558	14.48	0.557
14	Negro	M	0.910	70.89	2.727
15	Indian	M	0.075	17.98	0.692
16	Negro	F	2.829	32.72	1.258
17	Indian	M	0.140	12.37	0.476
	MEAN		1.3621	22.494	0.8790
	S.D.		1.7852	17.834	0.7059

was a wide variation in the hepatic non-haem iron concentration (0.075 to 7.260 mg per g wet weight), but there was no significant correlation between these values and the total epidermal iron content ($r_{32} = + 0.0040$; $p > 0.9$). Figures for the iron content of skin were related to the known average epidermal turnover rate of 26 days (Rothberg, *et al.*, 1961). On the assumption that all the iron present in epidermis is lost from the body, this would represent the maximum daily skin iron loss. The mean daily figure calculated in this way was 0.879 (S.D. 0.706) mg.

(c) Discussion

Much of the controversy concerning iron excretion has centered on the magnitude of its dermal loss. In the studies reported in this thesis, skin iron losses were investigated using both radioisotopic and chemical techniques. Both methods were designed to measure the maximum amounts of iron that could be lost from the body via the dermal route.

In the radioisotopic study, a tracer dose of ^{59}Fe was injected intravenously, and the amount taken up by an area of skin was used to calculate how much iron enters the entire skin surface from the plasma each day. The experimental design was based on the concept of pool replacement, in which it is assumed that whatever leaves the pool in unit time must be replaced, if the pool maintains a constant size. It has been demonstrated, both histochemically and by autoradiography, that iron injected directly into the skin is sequestered in the basal layers of the epidermis and in the glandular and ductal epithelium of the skin appendages (Weintraub, *et al.*, 1965). It has, however, been suggested that this iron is not irrevocably lost to the body, but is available for re-utilization once it has entered the skin (Beamish and Jacobs, 1968; Cavill, *et al.*, 1969). If the iron is labile in the sense that

movement can occur in both directions, then the results obtained with the present radioisotopic technique would overestimate daily losses, since it is based on the assumption that whatever iron enters the integument is eventually lost from the body.

The figures obtained indicate that little or no iron is taken up by the skin when the percentage saturation of circulating transferrin is low. This is in accord with previous observations that the iron content of cell-rich sweat falls in states of iron deficiency (Hussain and Patwardhan, 1959; Prasad, *et al.*, 1963). The finding that the amount of iron entering the skin is decreased in iron deficiency anaemia was also confirmed by Cavill and Jacobs (1971). At normal transferrin saturations, approximately 0.2 mg iron passes from the plasma to the skin daily, and this figure reaches a maximum of 0.7 mg per day when the transferrin saturation exceeds 90 per cent. The figures for uptake of iron by the total skin surface were based on calculations extrapolated from areas of eccrine skin. Since it was shown that apocrine gland-bearing areas derive more radioiron from the plasma than do eccrine gland-bearing areas it is possible that slightly more iron may be localized in the skin than the calculated figures. It is not possible to accurately quantify this fraction, since precise figures for the proportion of the skin surface that contains apocrine glands are not available. However, even taking an extreme estimate that one-tenth of the skin surface contains apocrine glands, not more than a further 0.1 mg could be added to the figure for daily iron clearance to the skin. This is based on the assumed average figures of 1.73 square metres for skin surface area, 2.1 per cent uptake of injected radioiron per square metre of apocrine skin (Table 5.6) and a plasma iron turnover of 30 mg per day.

In the other approach used in the present thesis to estimate maximum possible skin iron losses, the iron content of

epidermis was measured in samples of skin obtained at autopsy. When these figures were related to the total skin area, the mean calculated 'epidermal iron pool' was found to be 22.5 mg (S.D. 17.8). These figures are somewhat higher than those reported by Moore (1964) who used a similar technique. By relating the figures for chemical iron content of epidermis to the known turnover rate of epidermal cells (Rothberg, *et al.*, 1961) and based on the same assumption concerning pool replacement as that made in the radioisotopic study, maximum possible losses through the skin could be roughly estimated. The calculated mean figure of 0.87 mg per day was somewhat higher than was found in the radioisotopic study (mean 0.23 mg). There are several possible reasons for this difference. Firstly, despite scrupulous care, some contamination might have occurred using the chemical method. Secondly, using the chemical method, the total epidermal iron content regardless of its source was measured, whereas with the radioisotopic technique, only the iron which was derived from the plasma was taken into account.

In combination, the chemical and radioisotopic data obtained in the present studies suggest that dermal iron losses are small, and are not likely to exceed one milligram per day. A significant correlation was found in the radioisotopic study between skin iron uptake and the degree of saturation of circulating transferrin, suggesting that dermal iron losses may be regulated, in part at least, by an individual's iron status. There was, however, no such correlation between the 'epidermal iron pool' and liver non-haem iron stores in the chemically determined autopsy study. The reason for this lack of correlation is not clear.

The figures obtained in these studies which are, if anything, overestimates of daily skin iron losses, are at variance with the results of most other investigations (see literature review, Chapter 2). The claim that several

milligrams of iron may be lost via this route each day are based largely on the chemical finding of high iron concentrations in sweat produced under artificial conditions. Results so obtained have then been extrapolated to situations of extreme rates of sweating, and sometimes on the highly unlikely assumption that these rates could be continually maintained over a 24-hour period (Mitchell and Hamilton, 1949; Adams, *et al.*, 1950; Foy and Kondi, 1957; Apte, 1963; Prasad, *et al.*, 1963). Such extrapolations, however, are fraught with difficulty, and have not been attempted in other studies (Hussain, *et al.*, 1959; Moore, 1961; Apte and Venkatachalam, 1962; Coltman and Rowe, 1966; Vellar, 1968). Not only are the conditions of induced sweating highly artificial, but there is also the ubiquitous problem of iron contamination.

The drawbacks of chemical determinations on collected sweat are highlighted by studies in which sweat iron estimations were carried out simultaneously by both chemical and radioisotopic methods (Moore, 1961; Green, *et al.*, 1968). In these studies, the chemically measured sweat iron concentrations were several-fold greater than figures calculated from the radioiron excretion data. Whilst it is possible that the iron in sweat may be derived from a source other than the miscible labelled iron pool, the magnitude of the discrepancy is so great, that this would imply that only a very small fraction of the iron in sweat is derived from the miscible labelled pool. This seems unlikely, and contamination in the chemical method seems to be a more plausible explanation for the discrepancy.

The present findings do not support the suggestion put forward by Weintraub and co-workers (1965), on the basis of a whole-body radioiron retention study, that the skin is a major route of iron excretion. The validity of their findings may be open to question, in view of their short period of follow-up. In an earlier radioisotopic excretion study, in which measurements were made over a much longer period (Dubach, *et al.*, 1955),

figures for sweat iron losses were obtained which were of the same order of magnitude as those found in the investigations reported in this thesis.

It should be pointed out that what is perhaps the most trenchant argument against the skin being a major source of iron loss was obtained from results on the Indian subjects in the long-term total body iron excretion study (Chapter 4). These subjects were working in a laundry with an extremely high temperature and humidity, and might therefore have been expected to exhibit maximal sweat losses. However, their mean calculated total excretion of iron from all sources was only one milligram daily. It appears most unlikely, therefore, that excessive sweating represents a potential source of major iron excretion, even in hot, humid, tropical climates.

(d) Summary and Conclusions

The magnitude of skin iron losses has been the most vexed question concerning body iron excretion. Estimates of the maximum possible amounts of iron that could be lost through the skin were obtained by radioisotopic methods. The results of both approaches indicated that skin iron losses are never likely to exceed one milligram per day, and are usually considerably less than this figure. The figures obtained are probably even overestimates of daily skin iron losses, since there is evidence that not all the iron in skin is lost from the body. That the skin is not likely to be a major route of iron loss, even in tropical climates, is supported by the findings in the long-term iron excretion study. Indian subjects working in a hot, humid environment did not lose more iron than subjects living in a temperate environment. The present findings refute previous suggestions that large amounts of iron are lost in sweat, particularly in the tropics.

CHAPTER 6

FINAL SUMMARY AND CONCLUSIONS

I. SUMMARY OF PRESENT FINDINGS

Accurate quantitative information concerning the daily obligatory losses of iron from the body is essential for the assessment of daily iron requirements. Much controversy has centered on the magnitude of daily iron excretion through various routes and its possible variations, and particularly on the question of dermal losses.

An attempt was made in this thesis to document iron losses from the body as a whole, as well as from individual excretory routes, in order to gain a better understanding of external body iron exchange, and to resolve some of the existing controversies. Because of the considerable technical and interpretive difficulties which beset methods for measuring iron losses, a combination of radioisotopic and chemical techniques were employed. It was hoped that by using such a combined approach, meaningful information could be obtained on the magnitude of body iron losses.

In a long-term radioisotopic study, iron excretion was measured in different groups of adult male subjects, chosen in order to examine the effects of a number of variables on body iron losses. The calculated mean daily iron losses were as follows: Johannesburg Negroes 2.42 mg (S.D. 1.09); Durban Negroes 2.01 mg (S.D. 0.94); Durban Indians 1.02 mg (S.D. 0.22). In two further groups which were included in a collaborative study undertaken at the same time as the work presented in this thesis, mean losses were: Seattle Whites 0.95 mg (S.D. 0.30); Venezuelan Mestizos 0.90 mg (S.D. 0.31). Higher excretion figures in the Negro subjects were ascribed to their greater than normal iron stores. The finding that Durban Indian subjects, who were working in an extremely hot and humid environment, did not lose greater amounts of iron than did the sedentary group of Seattle Whites who were living

in a temperate climate, suggested that excessive sweating does not represent a major mechanism for iron excretion.

In studies on compartmental iron losses, the amount of iron excreted through the gastrointestinal tract was measured by a radioisotopic technique. The results indicated a mean daily loss of 0.14 mg within desquamated mucosal cells and secretions, and 0.38 mg in blood. Mean biliary iron excretion was found to be 0.24 mg daily when measured chemically, but the calculated figure from radioisotopic data was only 0.04 mg per day. It was concluded that most of the iron in bile is derived from sources other than the plasma.

Direct chemical measurements on urine revealed a mean daily iron content of 0.11 mg, and this quantity did not appear to be influenced by the size of body iron stores.

In a radioisotopic study, the amount of iron taken up from the plasma by eccrine skin was positively correlated with the degree of saturation of transferrin. At low transferrin saturations little or no iron was taken up by the skin; at normal saturations, uptake was between 0.2 and 0.3 mg daily; at high saturations, the figure rose to between 0.6 and 0.7 mg. By estimating the size of the epidermal iron pool chemically, the mean calculated maximum figure for daily skin iron loss was 0.87 mg.

II. COMPARISON BETWEEN THE RESULTS OF TOTAL BODY AND COMPARTMENTAL IRON LOSSES

In a final analysis of the results obtained in this thesis, the mean figures for calculated daily iron losses from individual body compartments were added together and compared with the figures obtained in the long-term total body iron excretion study.

The sum of the mean figures for daily losses from individual compartments was 0.86 mg. This was made up of 0.51 mg for gastrointestinal losses, 0.24 mg for skin (at normal transferrin saturations) and 0.11 mg for urine. The total of 0.86 mg per day is in moderately close agreement with the figure of 1.02 mg obtained in the total body excretion study in Indian subjects, as well as the figures of 0.95 and 0.90 mg per day found in Seattle and Venezuelan subjects. The small discrepancy may be due to the fact that the figure for gastrointestinal losses does not take into account further possible losses of iron via the bile.

In a further analysis, maximum figures for compartmental losses (0.74 mg from bowel, 0.64 mg from skin and 0.16 mg in urine) were added together in an attempt to gauge the upper limits of daily body iron loss through known excretory routes. The total was 1.54 mg per day, which was lower than the mean figures for total body iron loss in the two groups of Negro subjects who exhibited the highest losses in the long-term iron excretion study. The subjects in these groups had the highest calculated miscible iron stores, and the mean daily iron losses for the groups were between 2.0 and 2.5 mg daily. For some individuals the discrepancy between the sum of maximum compartmental iron losses and total body iron excretion data was still greater, since the Negro groups included five subjects in whom the calculated daily losses were greater than 3.0 mg daily.

The reason for these differences is not certain, but several possible explanations merit consideration. As discussed previously (Chapter 4) there is no assurance that mixing of the labelled miscible iron pools with unlabelled body iron compartments did not continue in Negro subjects during the period of observation. If a slow mixing and redistribution of the tracer was continuing to take place, the radioisotopic method used in the long-term excretion study would have tended

to overestimate actual external iron losses. If, on the other hand, the results obtained with this method were valid, then the possibility exists that significant iron excretion occurs from sites not detected or sources not measured in the studies designed to estimate compartmental losses. In this context it should be pointed out that no information was obtained on biliary losses of iron in subjects with increased iron stores. It is possible that the losses from this route may be greater in iron overloaded subjects, as suggested by experimental evidence in rats (Bradford, *et al.*, 1969). In addition, it has been proposed that gastrointestinal iron losses through the shedding of iron-containing reticuloendothelial cells may be greater than normal in states of iron overload (Crosby, *et al.*, 1963). This has been supported by experimental evidence in rats (Thirayothin and Crosby, 1962). It was not possible to document reticuloendothelial iron losses in the radioisotopic studies presented in this thesis.

When minimum figures for compartmental losses are taken, it may be seen that whilst skin and non-haem gastrointestinal losses may be reduced to virtually zero, obligatory blood loss through the bowel, and urinary iron excretion amounting to roughly 0.38 mg and 0.11 mg respectively, continue to occur, regardless of the state of body iron stores. It was not possible to study subjects who were continually iron-deficient in long-term total excretion experiments, so that the validity of the figure for minimum possible daily iron losses could not be confirmed. However, the figure of around 0.5 mg, representing the sum of mean gastrointestinal blood loss and urinary excretion, agrees exactly with the figure for total body iron loss found by Dubach, *et al.*, (1955) in iron deficient subjects.

It may be concluded from the information presented in this thesis that the hypothesis put forward by McCance and Widdowson (1937a), that external iron exchange in man is limited, is valid.

No support was obtained for contrary views, challenging this hypothesis, which have claimed that body iron losses may be considerably greater than the figure of one milligram per day proposed by McCance and Widdowson. In particular, convincing evidence was obtained refuting the view that increased dermal losses of iron caused by excessive sweating may play an important part in the pathogenesis of iron deficiency anaemia in tropical parts of the world.

The magnitude of daily iron losses in man, although apparently fairly limited are, however, not fixed. Evidence was obtained that the state of the body iron stores does, at least in part, regulate the amounts of iron lost from the body. Iron losses may therefore be reduced in iron deficiency, and augmented in iron overload. Excluding menstrual losses, the possible range of iron losses defined in this thesis is between 0.5 and 4.0 mg per day. It may be assumed that for a normal adult male to replace obligatory iron losses and remain in a state of iron balance, approximately one milligram of iron must be absorbed from the diet each day.

III. QUESTIONS RAISED BY THE PRESENT THESIS

In attempting to define the magnitude and limits of daily iron excretion in the absence of any primary pathological condition giving rise to increased iron loss, information was obtained on total and compartmental body iron losses. Also, the flexibility of the iron excretory mechanism and some of the factors which regulate it were investigated. Whereas further light was thrown on various aspects of iron excretion, and some questions were answered, some residual areas of doubt remain, and a number of further questions may be posed arising from the present investigations. These will be mentioned briefly.

From the results of experiments on total body iron losses there was a suggestion that the variables of age and body size might, in addition to the size of the body iron pools, influence iron excretion. Further investigation of these possibilities merits consideration. Regarding age, and excluding the childbearing period, the prevalence of iron deficiency anaemia rises with increasing age amongst adults. This is mainly due to the higher incidence of abnormal gastrointestinal bleeding in the older age group, and partly because of their poorer diets. A diminution in obligatory iron losses would tend to mitigate against the risk of entering into a state of negative iron balance in the geriatric age group. At the other end of the scale it should be pointed out that information on iron losses in children is scanty, yet there is a high incidence of iron deficiency anaemia, particularly in the one- to two-year age group.

The possible influence of body iron size on iron excretion is also pertinent to the paediatric age group. It may also be of importance in relation to male/female differences in iron excretion. The smaller average size of females, by diminishing obligatory iron losses through other routes, may partly offset the extra losses which they incur as a result of menstruation and childbirth. Alternatively, it may be considered that their smaller obligatory daily losses balance their proportionately lower iron intakes, which result from smaller caloric requirements.

Daily iron losses from the gastrointestinal tract cannot be stated with any certainty. The relative contributions made by blood and non-haem sources remain controversial, and require further study. It seems most likely that the capacity of the body to excrete unwanted iron, although limited, resides in the gastrointestinal tract. Studies on biliary and reticuloendothelial cell iron losses in particular deserve further attention in states of iron overload. The findings on biliary and gastrointestinal

iron losses in this thesis also suggest that a considerable proportion of the iron in bile might be reabsorbed. This question merits further investigation.

Concerning iron excretion through the skin, the present studies defined the uptake and iron content of the skin, and hence the maximum possible losses that could occur through this route. However, recent information from Jacobs and associates which were discussed in the review of the literature (Chapter 2) have indicated that most of the iron in skin is probably not lost from the body with cellular desquamation. In view of this, accurate quantitative figures on skin iron loss may yet have to be defined.

Some information has been obtained in this thesis on body iron excretion in man, but certain facets of the problem still remain unanswered.

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A P P E N D I X

Computer programme (FORTRAN) used for calculation of data in the long-term iron excretion study.

Programme calculated:

- (i) parameters of regression line by the method of least squares.
- (ii) significance of linearity for the line from variance ratio.

```

      DIMENSION X(10),Y(10)
      PRINT 101
101  FORMAT(55H  SLOPE HALF LIFE T.G.RATE 10P1. FACTOR S. ERROR)
      DO 11 I=1,43
      READ 151,XX
151  FORMAT(F3.1)
      READ 150,M
150  FORMAT(I2)
      READ 90,I
90   FORMAT (I2)
      READ 9,(X(I),Y(I),I=1,4)
9    FORMAT (14(F3.1,1X))
      DO 1000 I=1,N
1000 Y(I)=LOG(Y(I))
      XSUM=0
      XSSUM=0
      DO 1 I=1,N
      XSUM=XSUM+X(I)
      XSSUM=XSSUM+X(I)*X(I)
1    CONTINUE
      XYSUM=0
      YSUM=0
      YSSUM=0
      DO 2 J=1,N
      YSUM=YSUM+Y(J)
      YSSUM=YSSUM+Y(J)*Y(J)
      XYSUM=XYSUM+X(J)*Y(J)
2    CONTINUE
      AN=N
      SUMYY=YSSUM-YSUM*YSUM/AN
      SUMXX=XSSUM-XSUM*XSUM/AN
      SUMXY=XYSUM-XSUM*YSUM/AN
      B=SUMXY/SUMXX
      A=YSUM/AN+B*(XSUM-XSUM/AN)
      YY=EXP(A)
      HL=-LOG(2.)/B
      TR=831.72/HL
      SSR=SUMXY*B-SUMXX*B*B
      SSD=SUMYY-SSR
      F=SSR*(AN-2.)/SSD
      SB=SQRT(SSD/((AN-2.)*SUMXX))
      IF (II-11) 3,3,4
3    PRINT 50
50   FORMAT(2H 6)
      GO TO 53
4    PRINT 51
53   PRINT 54,M
54   FORMAT (I3/)
51   FORMAT(2H 0)
      PRINT 100,B,-L,TR,YY,F,SB
100  FORMAT(F10.6,4F10.3,E14.3)
11   CONTINUE
      END

```

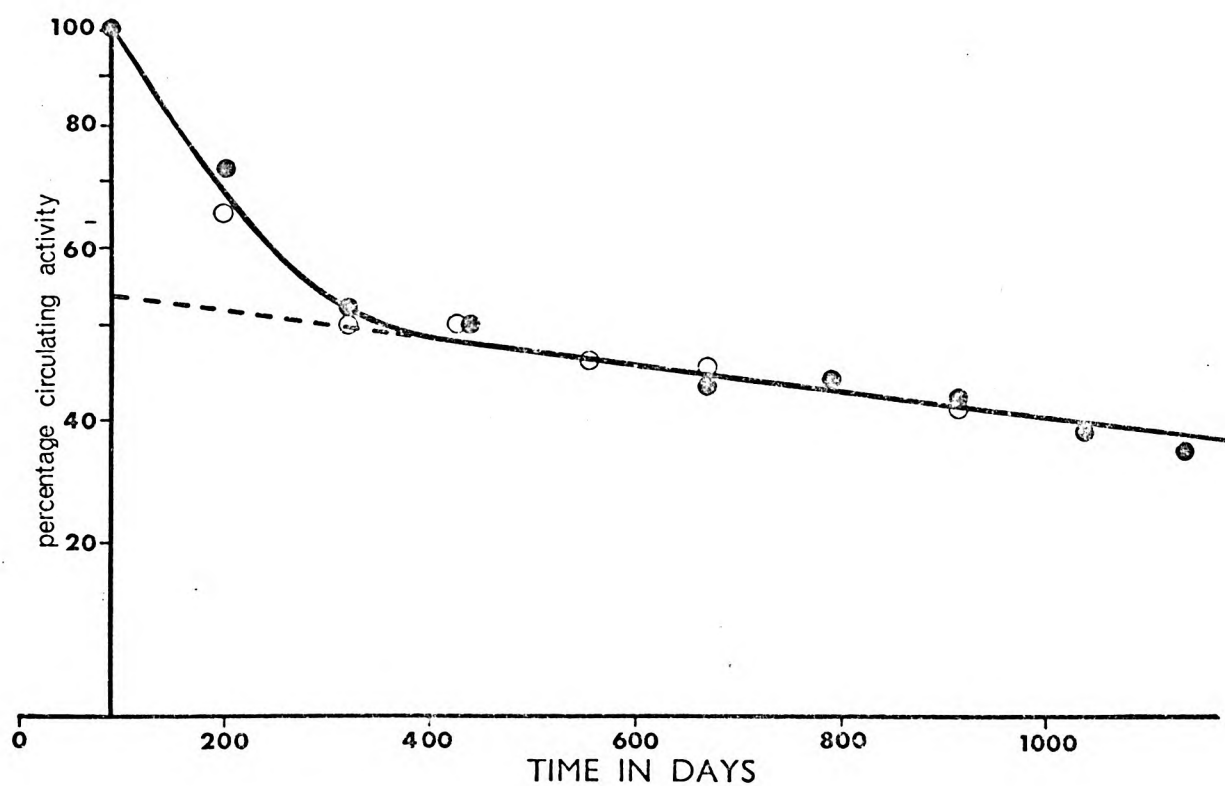


FIGURE A.1. Change in blood specific activity in a Negro subject in the long-term iron excretion study, showing good agreement between the results obtained in this thesis (closed circles) and those obtained in Seattle (laboratory of Dr. C.A. Finch) as part of a collaborative study (Green, *et al.*, 1968).

RESULTS ON INDIVIDUAL SUBJECTS IN THE LONG-TERM IRON EXCRETION STUDY,
SHOWING THE FALL OF RED CELL RADIOACTIVITY WITH TIME (MONTHS).

Results are expressed as a percentage of the blood radioactivity at zero time, taken 90 days after ^{55}Fe injection, when red cell radio-iron utilization would be at a maximum (Chapter 4, p. 72).

Graphs on following pages: 183 - 219

