

AN IN VIVO AND IN VITRO STUDY OF SOME LUMINAL AND CELLULAR
FACTORS INFLUENCING IRON
ABSORPTION

by

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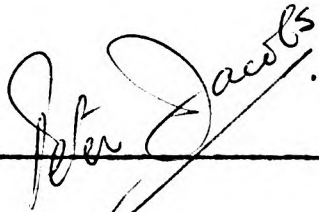
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**This is to certify that this thesis is my own work and has not been
presented for any degree at any other University**



PETER JACOBS

TO MY WIFE

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CHAPTER I

OBJECTIVES OF THIS THESIS

INTRODUCTION

Although the various factors which influence the absorption of iron from the gastrointestinal tract have been extensively investigated, there still remains uncertainty concerning several basic aspects of this process.

In terms of the various luminal factors, controversy exists as to the possible effects of gastric and exocrine pancreatic secretions on the absorption of both simple iron salts and food complexes. Similarly, the role of the liver and intestinal juice remain obscure while information on the absorption of actual dietary iron is still fragmentary.

At the cellular level, it is not clear whether the absorption of this metal is an active process which is dependent upon metabolic energy, or if it takes place merely by passive diffusion. Likewise, while evidence is accumulating that an intrinsic regulating mechanism resides within the cells lining the gastrointestinal tract, it has been suggested that the control of absorption may be achieved by dynamic alterations of humoral agents, such as transferrin.

In an attempt to obtain additional information on some of these luminal and cellular aspects of iron uptake and absorption, a

series of studies have been carried out using a combination of in vivo and in vitro techniques. The results of these investigations are reported in this thesis.

In view of the conflict which exists in the literature over the influence of certain of the factors upon the absorption of iron from the gastrointestinal tract, the first part of this work has been devoted to a general outline of iron metabolism. This is followed by a critical review of what is at present known of iron absorption and special emphasis has been directed to those areas where existing data appeared inadequate. It is hoped that in this way the experimental observations which form the basis of this treatise will be placed in perspective.

Since the experimental data in the various published studies have often been obtained using completely different methods of measuring a similar phenomenon, the currently available techniques of assessing iron absorption have been summarized. This particular section, which is not strictly germane to the body of the thesis, is presented in the form of an appendix.

IN VIVO STUDIES

The investigations outlined in this section were carried out in man using a twin-isotope method. The technical details and the

studies performed to define the overall reproducibility and reliability of this approach are presented in a second appendix.

The first of the luminal factors to be investigated was the role of the gastric secretions upon iron absorption, and for this purpose subjects with histamine-fast achlorhydria were used. Initially, because in vitro studies had indicated a relationship between iron absorption and intraluminal pH, information was sought on the effect of hydrochloric acid per se; data were collected by comparing the absorption of a standard dose of iron from a fixed volume of either water or acid. In an extension of these investigations the possible influence of substances in the gastric juice, other than hydrochloric acid, was examined. In this regard, a comparison was made between the absorption of iron from either normal gastric juice, or an equal volume of hydrochloric acid having the same acid concentration.

Arising out of these observations, a second luminal factor to be scrutinised was the effect of alcohol upon the absorption of iron. These studies were undertaken in an attempt to add further understanding to the well documented relationship existing between excessive consumption of alcohol and the development of iron overload. In this respect there are several reasons why many heavy drinkers should have increased iron stores. Among these is the increased iron intake

due directly to the alcoholic beverages themselves. A second possible way in which increased absorption may be correlated with expanded stores is through the development of pathological sequelae, such as chronic pancreatitis and alcoholic liver disease. However, no evidence was available to support the theoretical possibility that alcohol itself might enhance iron absorption. Such an effect could perhaps be mediated by the alcohol stimulating the gastric mucosa to secrete increased quantities of hydrochloric acid. In an attempt to answer this point different forms of labelled iron were administered with, and without, alcohol to both normal and achlorhydric subjects; absorption was determined using the twin-isotope method.

Once an interest in the relationship between alcohol and iron absorption had been established, an additional study was undertaken in vivo to define the availability of iron in Bantu beer. This particular problem is of considerable local relevance since varying degrees of iron overload occur regularly in the majority of adult Bantu living in South Africa. Although it is probable that this siderosis represents increased absorption from the gut, there is still controversy as to exactly why this should occur. Thus, while it appears likely that Bantu beer provides a major source of iron in the diet of this population, it remains possible that iron absorption is also enhanced by the alcohol which it contains. It was therefore decided to determine

both the amount of beer currently being consumed by the Bantu subjects in Johannesburg and to assess how much of this iron was actually absorbed by the gut. Accordingly, the absorption of labelled iron from Bantu beer was compared to the iron available from aqueous solutions of various iron salts. In view of the siderosis found in the Bantu subjects, similar observations were made in European volunteers. In this way additional information was obtained on the modifying effect of expanded body iron stores on the absorption of this form of iron.

IN VITRO STUDIES

These investigations were carried out using isolated segments of perfused rat intestine. Pilot experiments were directed at developing the perfusion method and characterising its behaviour. Once the performance of this system had been standardised it was applied to an examination of both luminal and cellular factors.

Of the luminal factors, information was obtained on the effect of increasing iron dosage and the role of ascorbic acid. Since the action of vitamin C possibly reflected altered intraluminal pH, the influence of nitric and hydrochloric acids were also studied. The last study provided data which was complementary to investigations carried out with hydrochloric acid in man.

In a second phase of the investigation, attention was directed to the role of metabolic energy in the transfer of iron from the lumen to the venous effluent. This process was examined in the presence of anoxia and metabolic inhibitors. It was also assessed when certain of the metabolic stimulators and methylene blue were present in the perfusate.

A third area to be studied, again using this system, was the effect of previous manipulation of the animal in vivo upon the subsequent performance of the isolated and perfused loop. On the one hand the effect of iron overload was documented. On the other hand the influence of varying diets, iron depletion and acute haemolysis was studied.

In summary, the work in this thesis is presented in three sections. The first is concerned with the general aspects of iron metabolism and includes a critical review of the literature pertaining to the absorption of this metal from the gastrointestinal tract. In the second, the results of in vivo investigations of some luminal factors, using a twin isotope technique, are presented. In a third section the results of studies on both luminal and cellular factors, using an in vitro perfusion technique, are set out. In the last two sections the experimental results are discussed and an attempt made to assess their significance in the light of current knowledge of iron

metabolism. The last part of this thesis summarizes the methods of determining iron absorption and details the twin isotope technique used in these studies. Finally, the various references consulted are listed in a bibliography.

CHAPTER II

THE METABOLISM OF IRON

HISTORICAL INTRODUCTION

The discovery and subsequent translation of the Ebers Papyrus, an ancient compendium of prescriptions, provided evidence that the therapeutic effects of iron had been recognised in Egypt and Mesopotamia as early as 1500 B.C. (Beutler, Fairbanks and Fahey, 1963). It is not surprising that these early Egyptian and Sumerian physicians advocated the empirical use of iron since, being very superstitious folk, they believed that this element, particularly in its meteoric form, was of celestial origin and therefore likely to have been imbued with special healing powers by their gods.

Subsequently, Greek and Roman medical practitioners wrote widely on the efficacy of iron compounds. They advocated, for example, a topical application to battle wounds in the belief that the latter would be healed most quickly by the touch of the metal that had caused them, the so-called Telephian wounds. Similarly, water in which a sword had been allowed to rust was administered as a tonic to those who had lost blood on the battle field. The latter therapy was based on the primitive belief that, since both blood and rust were red, it could be assumed by analogy that blood contained significant quantities of iron. In the light of present day knowledge it is not

altogether surprising that such forms of therapy enjoyed a degree of success, and possibly even prompted the Hippocratic descriptions of the styptic effects of iron salts and their irritative effects upon the gastrointestinal tract.

The writings of Dioscorides and Galen, during the classical period, contained reference to the use of iron in patients with splenic enlargement or menorrhagia, again suggesting an early recognition of the beneficial effects of iron in the treatment of the secondary anaemias. Nevertheless, it remained for Avicenna to draw attention to the fact that this metal was without therapeutic value unless first converted into the salt (Wallerstein and Mettler, 1958).

This link between iron deficiency and its therapy was strengthened in 1554 when Johannes Lange is purported to have first described chlorosis as a clinical entity (Major, 1945). However, it was not until the 17th Century that Sydenham reported the clinical benefit which accrued when he treated this condition by the administration of simple iron salts.

Such empirical observations, all suggesting a possible role of iron in blood formation, were placed on a more rational basis in 1713 when Lemery and Geoffroy established the presence of iron in the ash of blood. Further support for this relationship derived from

Menghini's demonstration that in experimental animals the concentration of this metal could be increased by the feeding of iron-rich foods. A complementary study (Fodish, 1832) demonstrated that the iron content of blood in chlorotics was lower than that found in healthy individuals (Beutler et al., 1963; Underwood, 1962).

Thus, the place of iron in the treatment of chlorosis seemed established and in 1832 Pierre Blaud outlined the principle, valid even today, that treatment should comprise large doses of iron over an extended period of time (Andersson, 1950).

It is not altogether surprising that during this period attention should have centred on the role of iron in the pathogenesis of anaemia. However, while such workers as Bunge and Abderhalden accumulated considerable data on the nutritional physiology of this metal, serious misconceptions of its role in clinical medicine and particularly iron deficiency remained. The latter included the erroneous belief that only "food-iron" or organically bound iron was available to the body (Underwood, 1962).

At least some of this confusion was clarified when Stockman (1893 a ; 1893 b) clearly demonstrated that in chlorotic women an adequate haemoglobin response could be obtained to both parenterally and orally administered iron salts; iron-free diets were without effect.

That a nutritional factor was of significance in the development of this severe iron deficiency state was suggested when this worker showed that diets eaten by chlorotic subjects contained less iron than did his own (Stockman, 1895).

Despite such apparently unequivocal evidence there remained a hard core of medical opinion which held that in treating chlorosis there is little, if any, place for irontherapy. It was not until the first quarter of the present century that this poorly supported dogma was dispelled. Thus, in a series of critical experiments, using phlebotomised dogs, it was established that both organic and inorganic forms of iron promoted significant regeneration of haemoglobin (Whipple and Rabscheit-Robbins, 1925).

Of importance was the demonstration by Heath, Strauss and Castle (1932) that the amount of inorganic iron injected subcutaneously into iron deficient subjects correlated closely with the increase in haemoglobin. It was noteworthy that a far greater quantity of iron was required orally to bring about a comparable response, suggesting that only a small fraction of the oral dose was utilized for haemoglobin synthesis. These findings were confirmed by Reimann, Fritsch and Schick (1936). The Czechoslovakian workers also indicated that the normal daily iron requirement was satisfied

by small quantities of this mineral. This latter observation contrasts with the much larger retention found in patients with so-called "iron-responsive" anaemia. It may be concluded that these studies finally settled the age-old controversy surrounding the role of iron in the pathogenesis of iron deficiency anaemia and irrevocably established its value in subsequent replacement therapy.

In 1937 the investigation of iron metabolism was catapulted into a totally new era by the astute observations of McCance and Widdowson (1937 a). These workers, using a series of balance experiments, established the cardinal point that the body has a very limited capacity to excrete iron. The publication of their monumental studies completely reorientated the approach to iron metabolism and focused attention on the central role of the absorptive mechanism in the homeostatic regulation of body iron stores.

About approximately the same time the investigation of this whole problem was given tremendous impetus by the introduction of suitable radioactive isotopes into medicine. For the first time it was now possible to obtain information about the dynamic structure of this system: quantitative chemical methods had provided only static measurements of intake and excretion or of concentrations in accessible pools (Sharney, Wasserman, Gevirtz, Schwartz and Tendler, 1965).

It is unfortunate that at such a critical time work in the field should have been interrupted by the second world war. However, significant progress has been made in our understanding of iron metabolism during the last two decades. While much of the older work has stood the test of time, critical re-evaluation of some studies has inevitably resulted in revision of out-dated concepts. In the light of modern research it has been possible to formulate a pathway for the metabolism of iron in man and this is outlined in the following section.

IRON METABOLISM - A CURRENT CONCEPT

The biological importance of iron may be gauged from the fact that, while only minute quantities are normally found in the organism, its presence is indispensable to life. Iron enjoys this unique position by virtue of its ability to undergo reversible oxidation and has, as such, a central role in the essential physiological processes of cellular respiration and oxygen transport. By way of contrast, the body has relatively small reserves of iron present in two storage compounds, namely ferritin and haemosiderin.

A. BODY IRON CONTENT

The total amount of iron in the animal or human body is difficult to measure exactly, and will vary not only with the species but also with age, sex and nutritional status. However, it is generally accepted that 0.006 to 0.007 percent of the whole body is present as iron, a value approximately 4 to 5 grams (Moore and Dubach, 1962) in the 70 kilogram individual, or about 45 milligram per kilogram (Finch, 1965).

There is little direct evidence available for this figure which has been derived from estimations of the haemoglobin mass in man, coupled with an analysis of tissues from various animals (Drabkin, 1951). In one study the iron content of 4 cadavers was measured (Widdowson, McCance and Spray, 1951). The latter workers obtained values of 700 milligrams in a $4\frac{1}{2}$ -year old male who had died from tuberculous meningitis, 2.07 grams in a 42-year old female who had drowned, 3.82 grams in a 48-year old male who had suffered from endocarditis and 5.36 grams in a 25-year old male who had died as a result of uraemia.

B. DISTRIBUTION OF BODY IRON

The complete analysis of the various iron compounds found in man has not yet been successfully performed. The distribution of

this metal between the various compartments has therefore been estimated (Moore and Dubach, 1962) on the basis of data obtained from various different sources (Hahn, 1937; Drabkin, 1951).

Accurate assessment of the amount of iron in each of these pools is further hindered by variations occurring both in health and in the disease states. Thus, while marked fluctuations are likely to occur in the red cell iron and in storage compartments, changes taking place in the various iron-containing enzymes do so to a lesser extent. However, the concept that iron in this form was "inviolable" is no longer acceptable.

On the basis of their chemical structure these complexes fall into two groups - either haem or non-haem containing compounds (Table I). Nevertheless, it is more practical to discuss some of the more important physico-chemical and physiological properties of these naturally occurring substances on the basis of their biological activity as either functional or storage iron containing compounds (Table II).

I. The Functional Iron-containing Compounds

a) Haemoglobin

This red pigment has a molecular weight of 67,000 and is a complex of a basic protein, globin, and four haem moieties. The

TABLE I

THE IRON-CONTAINING COMPOUNDS IN MAN

A Chemical Classification

Percentage of Total
IronGrams Percentage Iron
 in Compound

Compound

THE IRON-PORPHYRIN (HAEM) COMPOUNDS

HAEMOGLOBIN

MYOGLOBIN

HAEM/ENZYMES

Cytochromes

Catalase

Peroxidase

800.0

0.340

2.72

70.5

35.5

0.345

0.122

3.2

0.8

0.43

0.0034

0.10

5.0

0.09

0.0045

0.10

-

-

-

-

THE NON-HAEM COMPOUNDS

TRANSFERRIN

STORAGE-IRON

Ferritin)

Haemosiderin)

10.0

0.05

0.003

0.10

-

-

0.8 - 1.5

26.0

ENZYMES

Succinic

Dehydrogenase

Xanthine Oxidase

DPNH -Cytochrome

C. Reductase

-

-

-

-

-

-

-

-

-

-

-

-

TOTAL BODY IRON

3 - 4

100

Modified from Drabkin (1951), Granick (1958) and Moore and Dubach (1962).

TABLE II

THE IRON-CONTAINING COMPOUNDS IN MAN
A Functional Classification

I. FUNCTIONAL IRON-CONTAINING COMPOUNDS (Essential Fraction = 33 ± 5 milligram/kilogram)

- | | | | |
|----|-------------------------|---|--|
| a) | Haemoglobin | - | Found largely within red blood cells |
| b) | Myoglobin | - | Located within muscle tissues |
| c) | Transferrin | - | Mainly as an intravascular plasma protein |
| d) | Labile Iron Pool | - | ? Bone marrow |
| e) | Iron-containing Enzymes | - | Widely distributed throughout all body tissues |

II. STORAGE IRON-CONTAINING COMPOUNDS (Non-essential Fraction = 12 ± 8 milligram/kilogram)

- | | | |
|-----------------------------------|---|---|
| Ferritin and Haemosiderin Complex | - | Located within the reticulo-endothelial cells of the liver, spleen and bone marrow and also in liver parenchyma and muscle. |
|-----------------------------------|---|---|

former is composed of two alpha and two beta polypeptide chains, the synthesis of which are under separate genetic control and may vary not only between species but also between individuals. The haem fraction, or prosthetic group, is a ferrous-iron containing tetrapyrrole and is covalently bound to the protein in such a manner that each of the polypeptide chains is associated with one prosthetic group.

Haemoglobin, which is normally found within the erythrocytes, functions as a buffer system, a mechanism for carbon-dioxide transport and plays its major physiological role in conveying oxygen from the lungs to the tissues. It is ideally suited to the latter process as a result of its dissociation curve and the character of the ferrous-iron: oxygen bond. The small quantities of extracellular haemoglobin found in the plasma are bound to a specific alpha-two globulin, haptoglobin, in which form this pigment has little, if any, physiological significance for oxygen transport.

The amount of iron contained in haemoglobin (0.34 percent by weight), when considered in relation to the total mass of haemoglobin, represents some 75 percent of total body iron. Consequently, with the availability of radioactive isotopes this iron may conveniently act as a marker to study both haemoglobin synthesis and erythropoiesis.

b) Myoglobin

Myoglobin has a molecular weight between 17,450 - 17,920 (Perkoff, 1965) and contains one ferrous-iron porphyrin group per molecule (Moore and Dubach, 1962). This respiratory pigment which is found in both skeletal and heart muscle, was originally believed to be identical with haemoglobin. However, myoglobin is now recognised as a separate entity and it is possible to distinguish it from haemoglobin on the basis of a difference in electrophoretic mobility. Furthermore, myoglobin, unlike haemoglobin, is rapidly excreted by the kidneys when released into the blood stream (Javid, Fischer and Spaet, 1959).

On the other hand this compound, like haemoglobin, functions as an oxygen store, although in this case for the muscles; a property related to its affinity for oxygen (Moore and Dubach, 1962). A further point of similarity exists in that myoglobin may be depressed in iron-deficiency states (Gubler, Cartwright and Wintrobe, 1957).

c) The Plasma Iron

Although it had long been appreciated that a small quantity of non-haemoglobin iron was constantly present in the plasma, it was not until 1937 (Moore, Doan and Arrowsmith) that the true significance of this transport fraction was recognised. In view of

the extreme toxicity of ionic iron to the organism and its relative insolubility at the pH of body fluids, it is not surprising that the plasma iron should be protein bound.

The specific iron transporting beta-one globulin, which is colourless until iron is added when it becomes a salmon pink colour (Schade and Caroline, 1946), is called transferrin or siderophilin. This protein, having a molecular weight of $93,000 \pm 3,000$ (Bezkorovainy, Rafelson and Likhite, 1963), is normally present in the plasma to a concentration of about 250 milligrams percent (Jager, 1949; Moore and Dubach, 1962).

At least eight genetically different transferrins have been identified (Turnbull and Giblett, 1961) and it has been suggested that their synthesis is controlled at a single locus by three alleles (Smithies and Hiller, 1959). Each transferrin, having a half-life of 6.7 to 8.4 days (Katz, 1961), appears to bind and transport iron in the same way. Thus, two atoms of ferric iron are bound to each molecule of siderophilin (Beutler et al., 1963), probably linked via a hydroxamic acid group (Fiala and Burk, 1949).

The capacity of transferrin to bind iron is normally only one third saturated, representing a serum iron of approximately 100 micrograms percent; the remaining two-thirds being the unsaturated

or latent iron binding capacity. These values will be influenced to some extent by day to day (Bowie, Tauxe, Sjoberg and Yamaguchi, 1963), and also by diurnal variation in the serum iron levels - those in the morning being some 10 to 30 percent higher than evening levels (Bothwell and Finch, 1962; Gambino, 1965).

This relatively small plasma iron pool, normally only 3 or 4 milligrams (Moore and Dubach, 1962), is of fundamental biological importance, since all iron exchange within the body takes place through the medium of this compartment. The intense metabolic activity of this fraction is indicated by its very high turnover rate - up to 30 or even 40 milligrams in 24 hours. This measurement may be more conveniently expressed in terms of turnover per 100 millilitres of whole blood, in this way facilitating the comparison between subjects who have different blood volumes (Bothwell and Finch, 1962). The average normal turnover expressed in this way is 0.6 milligrams per 100 millilitres whole blood per 24 hours.

d) Labile Iron Pool

The studies of Greenberg and Wintrobe (1946) and Pollycove and Mortimer (1961), using radioactive iron, have provided evidence for the concept of a functional pool of iron. This fraction, which

exists in equilibrium with the plasma-iron, can be freely incorporated into erythroblasts. Such a pool, while theoretically acceptable, has not been anatomically defined (Beutler et al., 1963).

e) Enzyme Iron

Hahn (1937) recognised the presence in tissues of a small quantity of iron which could not be removed during mobilization and, on the basis of his studies, suggested that this "tissue" or "parenchymal" iron was fixed. More recently, evidence has accumulated that this fraction is present in the various enzyme systems. These, although constituting only a minute percentage of total body iron, are of vital importance to the organism since many essential processes depend upon their integrity.

This enzyme iron has been investigated in some detail by Beutler and his colleagues (Beutler, 1957; Beutler and Blaisdell, 1958a; Beutler and Blaisdell, 1958 b; Beutler, 1959 a; Beutler, 1959 b; Beutler and Yeh, 1959; Beutler and Blaisdell, 1960), who, together with other workers (Gubler et al., 1957), have indicated that under certain circumstances this pool, with the apparent exception of catalase, may be depleted. However, at least in the case of cytochrome oxidase, it appears to return rapidly to normal following iron therapy (Jacobs, 1961).

Beutler et al. (1963) have suggested that iron may form part of these various enzyme systems in three distinct ways. Thus, the iron may be present as an integral part of the haem group, conferring upon the molecule characteristic reactions which are lost when this metal is replaced by others (Theorell, 1947). Typical examples are the catalases and peroxidases which contain iron in the ferric state and liberate oxygen from peroxides (Moore and Dubach, 1962). Further examples are the cytochromes which, occurring in the mitochondrial membranes of cells, support respiration by providing a system of electron transfer mediated through the capacity of the iron atoms to undergo reversible oxidation.

Secondly, the structure of the molecule may include iron, without its apparently being incorporated into the haem moiety. Enzymes falling into this category would be the metalloflavoproteins (Xanthine Oxidase, Succinic dehydrogenase and DPNH-cytochrome reductase) in which both iron and riboflavin are present in the prosthetic group (Moore and Dubach, 1962).

Finally, iron may participate in the enzyme activity of a protein without forming part of the enzyme at all. This iron may be present in only loose combination, such as aconitase (Dickman and Cloutier, 1950; Dickman and Cloutier, 1951; Beutler, 1959 c). Similarly, recent evidence indicates that the enzymes involved in the

metabolism of one carbon units are also iron dependent (Vitale, Streiff and Hellerstein, 1965).

II. The Storage Iron-containing Compounds

Throughout childhood and adolescence this element accumulates slowly, so that in the adult approximately one gram of relatively inert iron is present in the parenchymal cells of various organs and in the reticuloendothelial system within the liver, spleen and bone marrow.

This storage iron is present in two biochemically distinct forms, namely, ferritin and haemosiderin. While these compounds have clear-cut chemical differences they subserve similar storage functions.

Thus, iron is available from either form for haemoglobin synthesis (Shoden, Gabrio and Finch, 1953). This iron may be rapidly mobilised in the case of haemorrhage - as much as 25 or 50 milligrams daily being delivered to the erythroid marrow (Finch, 1965); the more recent deposits being mobilised first (Hampton, 1954). Furthermore, the stores will support an almost constant level of haemoglobin in the face of negative iron balance, provided that loss occurs slowly; hence, they constitute a "buffer" to the development of iron deficiency anaemia. Consequently it is not until storage iron is exhausted, or near-exhausted, that anaemia will become manifest - any change in their

size being resisted by alterations in absorptive or loss mechanisms.

Similarly, when iron is administered parenterally incorporation takes place rapidly into both the storage compounds. While this phenomenon is complex and poorly understood it is clear that, from a functional standpoint, a basic attribute of these cells must be their ability to maintain a tremendous iron gradient across their membranes. In considering this accumulation further, it is interesting that parenterally administered iron, or iron from effete erythrocytes, is predominantly stored as ferritin and haemosiderin in the reticulo-endothelial system; this is easy to understand in view of the phagocytic activity of these cells. Enhanced intestinal absorption, on the other hand, results in increased deposition initially in the parenchymal cells of the liver, and later in other organs. However, there may occur a variable degree of interchange and redistribution of the iron between these sites (Cappell, 1930; Finch, Hegsted, Kinney, Thomas, Rath, Haskins, Finch and Fluharty, 1950^a).

Also of interest is the observation that the rebuilding of the stores takes place very slowly, even under the most favourable conditions, and aided by enhanced intestinal absorption. This facilitated absorption, which may continue in the absence of anaemia, is thought to reflect the size of the body iron stores and suggests that they can influence this process independently of other factors.

It is then interesting to speculate that such control may be mediated in some way by altered behaviour of the reticuloendothelial system, perhaps by "conditioning" the absorbing cells of the gastrointestinal tract.

Although these two compounds are intimately related on a functional or kinetic basis, they will be discussed separately in the interests of clarity.

a) Ferritin

Chemically, studies on this iron-protein complex have been facilitated by its stability in aqueous solution at 80° Centigrade. At this temperature most of the other proteins are coagulated, and following their removal, the ferritin may be crystallised with cadmium sulphate (Weinfeld, 1964). These crystals contain the homogenous yellow iron-free protein, apoferritin, having a molecular weight of 460,000 (Rothen, 1944) and iron hydroxide - iron phosphate micelles of varying size and concentration. The iron content of ferritin usually ranges from 17 to 23 percent by weight (Granick, 1942; Granick, 1946 c) although Harrison (1964) reports that iron-rich ferritin may have a molecular weight of 800,000 to 860,000 and would then contain approximately 26 percent of iron, the micelles accounting for 44 percent of the molecular weight. It has been suggested by Granick

(1946 c) that these micelles have the approximate composition $(\text{FeOOH})_8 (\text{Fe OPO}_3\text{H}_2)$. However, it is the iron itself that is of particular interest since this possesses a characteristic magnetic susceptibility which is not observed in any other iron compound (Michaelis, Coryell and Granick, 1943; Michaelis, 1947).

Ferritin, which is normally present in significant amounts in the liver, spleen and the bone marrow (Moore and Dubach, 1962), is not visible by conventional light microscopy. However, its characteristic structure may be readily identified in electron microscopic preparations. Thus, Farrant (1954) was able to demonstrate that each molecule was composed of a protein shell of apoferritin around a core of inorganic ferric hydroxide. Support for such a suggestion is to be found in the identical electrophoretic mobility of both ferritin and apoferritin which occurs despite differences in molecular weight and structure (Mazur, Litt and Shorr, 1950). Also, in this regard, it should be appreciated that both compounds have similar immunochemical properties (Granick, 1943; Mazur and Shorr, 1948; Mazur and Shorr, 1950). Later studies (Richter, 1959) have suggested that the characteristic tetrad of ferric hydroxide micelles in the molecules of ferritin is specific, thus facilitating their distinction from such micelles occurring in other iron compounds (Figure 1). There is some evidence (Kerr and

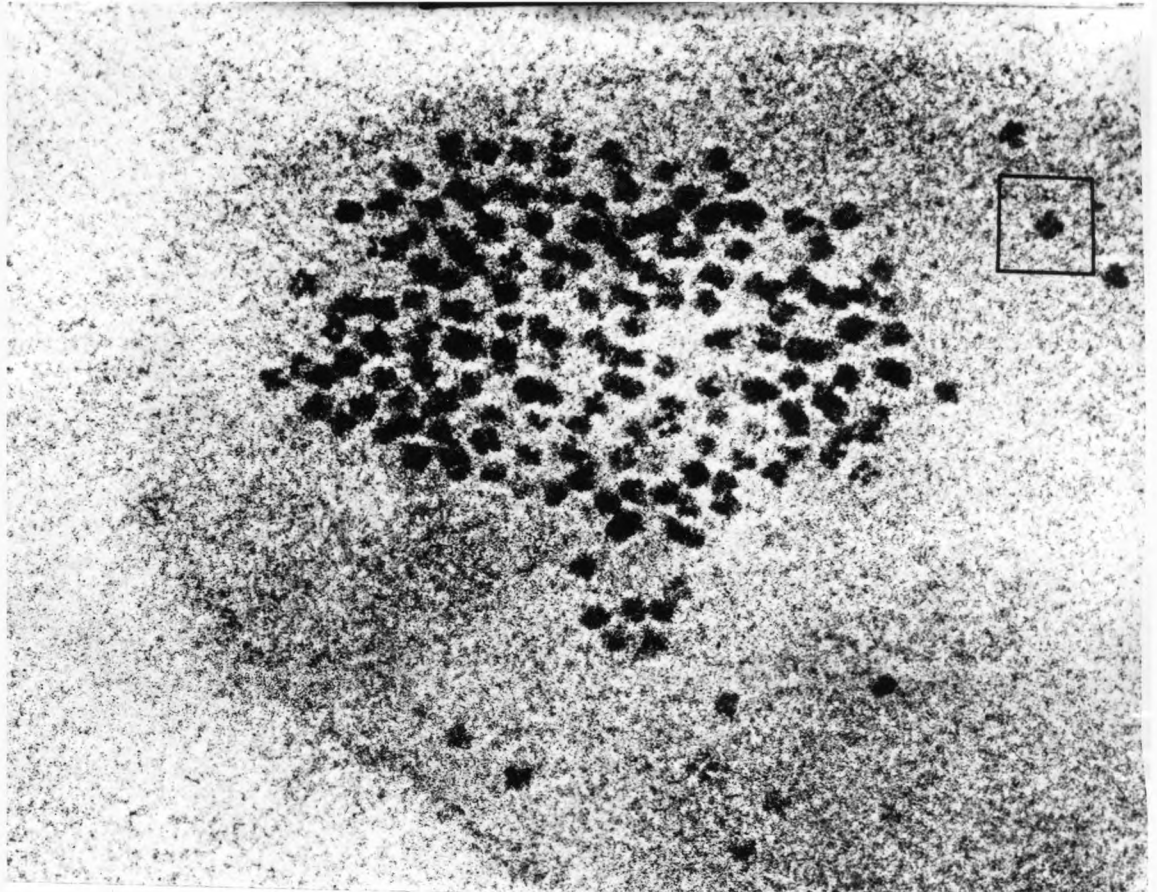


FIGURE I

Ferritin molecules seen on an electron microscope preparation demonstrating the characteristic clusters of four dots representing iron micelles.

Magnification 850,000 X

From Bessis, 1960.

Muir, 1960) that there may actually be six micelles of iron present within the protein shell (Figure 2).

Superficially, there appears to be little difference between ferritin isolated from various species, but definite immunological distinctions exist. Hence, it is possible to produce potent anti-serum against one type of antigen by injecting it into animals of another species. The availability of such anti-sera have made possible studies previously limited by technical methods not sensitive enough to detect the presence of small quantities of antigen.

Considered from a functional standpoint, ferritin appears to be primarily a storage compound. While information is available on both its chemistry and its distribution, the manner in which iron enters and leaves the ferritin molecule remains obscure (Bothwell and Finch, 1962). It has been demonstrated (Gabrio and Salomon, 1950) that although cells normally contain only a little apoferritin, many different types are capable of synthesizing this protein and forming ferritin when iron is presented to them. Typically, the cells of the gastrointestinal tract become ferritin-laden when large amounts of iron are given orally (Granick, 1946 a). It is this process, by which iron is incorporated into ferritin, that has not been defined. However, there is good evidence that transferrin-bound iron enters hepatic ferritin by means of an active biological mechanism in which

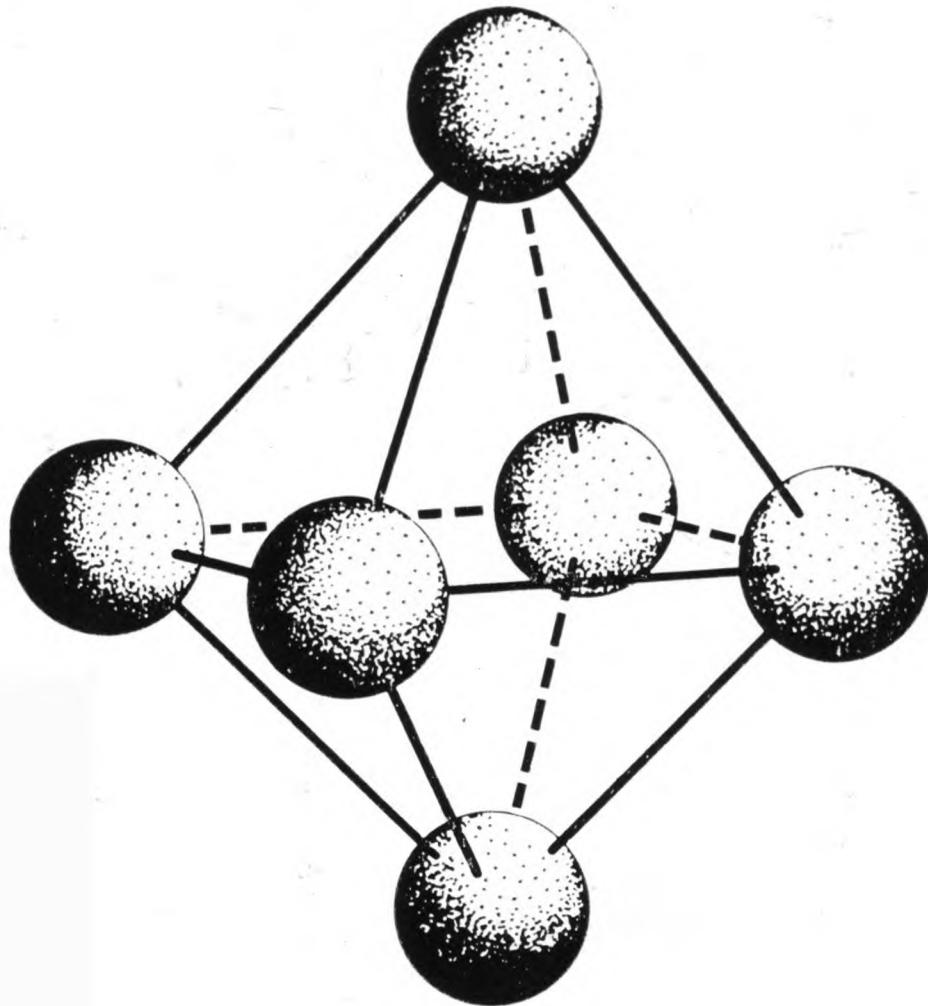


FIGURE 2

Hypothetical model of the spatial distribution of iron in the ferritin molecule

From Bessis, 1960.

both adenosine triphosphate and ascorbic acid are involved. It has been suggested that the ferric iron is reduced to the ferrous state, and in this way released from its bond to transferrin and made available for ferritin synthesis (Mazur, Green and Carleton, 1960; Mazur, Carleton and Carlsen, 1961).

Conversely, the release of iron from ferritin is equally interesting in that reducing agents such as ascorbic acid, glutathione and cysteine appear to play a part (Mazur, Baez and Shorr, 1955). Furthermore, it has been suggested that under anaerobic conditions the enzyme xanthine oxidase participates in this reaction (Green and Mazur, 1957; Mazur, Green, Saha and Carleton, 1958). Of note in this respect is the report that L-histadine can apparently increase plasma iron by releasing this metal from ferritin within the liver (Ohkido, 1964). However, the importance of such mechanisms in the normal mobilization and transport of iron have not been definitely established.

The most obvious role of ferritin is concerned with iron storage. However, the demonstration of significant amounts of this compound in the mucosal, reticuloendothelial and placental cells, all of which are concerned in active transport, suggest that it may also subserve a transfer function. In this context ferritin possibly represents a means of temporary storage and so increases the capacity

of such cells to take up and transmit iron (Bothwell and Finch, 1962). The possible way in which ferritin is involved in iron absorption is discussed in some detail in the following chapter.

Of additional interest, but probably of lesser physiological importance than its role in iron storage and transport, are the other functions that have been assigned to this compound. Thus, ferritin has been demonstrated in the blood of experimental animals in the terminal stages of shock, and it was reported (Mazur and Shorr, 1948) that this substance was identical with a previously described vaso-depressor material (Baez, Zweifach, Mazur and Shorr, 1947). The further investigation of ferritin and its possible role in the production of irreversible shock has been inconclusive. While it was suggested that in haemorrhagic shock some of the storage ferritin iron is converted to the ferrous state, and in this way produces irreversible vascular changes (Mazur et al., 1955), this hypothesis is by no means proved (Bothwell and Finch, 1962). Ferritin is currently thought to play little, if any, physiological role in the regulation of blood pressure, either normally or under these pathological conditions (Beutler et al., 1963).

Similarly, ferritin activity has been demonstrated in the blood of patients in whom oedema is present as a consequence of the

nephrotic syndrome, congestive cardiac failure and decompensated portal cirrhosis. It has been suggested that this substance may be concerned in the oedema formation in such subjects (Shorr, Baez, Zweifach, Payne and Mazur, 1950). This postulate is based upon the described antidiuretic properties of ferritin which are thought to be mediated through the posterior pituitary (Baez, Mazur and Shorr, 1950; Baez, Mazur and Shorr, 1952; Mazur, Baez and Shorr, 1952). There is at present little definite evidence to support this suggestion and it is still possible that the ferritin found in the plasma of oedematous subjects is the result, rather than the cause, of the oedema (Bothwell and Finch, 1962).

b) Haemosiderin

This compound, which apparently only functions for iron storage, has an essentially similar distribution to ferritin throughout the body tissues. From this standpoint, there is little doubt that these two compounds are closely related. Thus, under conditions of need iron is mobilized from both fractions, and conversely, incorporation takes place into both ferritin and haemosiderin following parenteral iron administration (Shoden, Gabrio and Finch, 1953).

On the other hand, there are striking differences when the compounds are considered superficially. Thus, in contrast to

ferritin, the golden-yellow granules of haemosiderin are visible under the light microscope and stain readily with Prussian blue, indicating that they contain some trivalent iron (Weinfeld, 1964). In addition, haemosiderin evidences no electrophoretic migration, and is strikingly insoluble in water.

On closer scrutiny, however, the distinction is not so clear-cut. This is possibly because even purified haemosiderin is not homogenous and its exact nature remains variable and ill-defined. Thus, while haemosiderin contains up to 45 percent by weight as iron (Ludewig, 1957; Shoden and Sturgeon, 1960; Richter, 1960) this is also largely present as a colloidal hydroxide. It is noteworthy that the magnetic moment of haemosiderin is similar to that of ferritin, although its magnitude is rather more variable (Michaelis et al., 1943; Shoden and Sturgeon, 1960). In addition, apoferritin has been isolated from iron-free preparations of haemosiderin (Greenberg, 1956) and ferritin itself has been demonstrated in haemosiderin granules both in situ (Richter, 1958; Bessis and Breton-Gorius, 1962), and following chemical purification (Richter, 1960; Shoden and Richter, 1960).

These observations have been extended by McKay and Fineberg (1964) who demonstrated that haemosiderin granules contain not only apoferritin but also other proteins, fatty acids, cholesterol

and "haem-like" compounds. These authors have concluded that haemosiderin is not simply aggregated ferritin, but rather that it represents an iron-loaded subcellular organelle, perhaps a mitochondrion.

C. BODY IRON EXCHANGE

Physiologically, the metabolism of iron takes place for practical purposes in a closed system, with actual exchange between man and his environment being very limited. The small quantities of this metal lost each day via bleeding, exfoliation of cells from the gastrointestinal tract or from the skin during sweating (Weintraub, Demis, Conrad and Crosby, 1965) are replaced by regulated absorption from the diet. This daily loss varies widely between individuals, but, in order to maintain balance, an average male will retain approximately 4 percent or 0.6 milligrams of iron from a diet providing 15 milligrams (Bothwell and Finch, 1964). On the other hand, the menstruating female assimilates food iron with an efficiency of about 8 percent, largely to compensate for menses. This may have to increase still further during pregnancy (Finch, 1965). In either case additional increments in absorption are required to replace abnormal blood loss.

It is clear that the varying iron requirement of the individual must normally be provided by absorption from the diet. However, the exact way in which this is achieved remains speculative. Thus, while

much of the information relating to the nutritional aspect derives from studies using simple iron salts, the extent to which these observations can be extrapolated to the physiological mechanism is not clear. It is, however, reasonable to assume that any fraction of the dietary iron that is present in this form or is liberated from organic compounds, perhaps with the aid of digestive enzymes, will follow such a pathway. By way of contrast, a significant proportion of food iron is present as stable organic complexes, for example haemoglobin and myoglobin. The manner in which these compounds are absorbed, or even the extent to which their iron is available remains unknown.

Nevertheless, once iron is free within the lumen of the bowel, it is generally thought to be reduced, possibly by substances in the diet such as ascorbic acid and cysteine, and in this form to be readily absorbed by the mucosal cell (Sheehy and Floch, 1964). Again the details of such cellular uptake and subsequent transfer remain matters of conjecture. However, it seems likely that the iron may either rapidly pass across the cell and enter portal blood or be shunted into a storage protein within the cell, probably ferritin. The later fate of this protein-bound fraction is of importance; the iron may be liberated at the capillary pole or alternatively be lost when these cells desquamate.

It is this cellular phase of iron transport that has excited such interest, largely because regulation of body content is apparently controlled at this level by absorption. This mechanism contrasts to other exchangeable substances, most of which are regulated by their excretory pattern. In this regard, the increased absorption associated with iron deficiency and accelerated erythropoiesis probably reflects more rapid movement of iron directly across the cell. Conversely, in conditions of overload or marrow hypofunction a slow plasma iron turnover could saturate the ferritin mechanism of the gut with reduced absorption and greater loss of iron when these cells exfoliate. This mechanism is elaborated in the subsequent discussion.

A number of luminal and cellular factors are currently recognised as influencing iron absorption. Since these have a special importance for this thesis, their relative contributions are considered in detail in the following chapter.

On leaving the capillary pole of the epithelial cells in the upper small bowel the absorbed iron is oxidised (Granick, 1956) and transported in the ferric state by transferrin to the bone marrow and the reticuloendothelial storage sites: the major movement of iron through the plasma being to the red cell precursors in the marrow. From here the iron, as part of newly formed haemoglobin, appears

in erythrocytes (Figure 3). As these red cells age and become effete they are removed from the circulation by the phagocytic cells of the reticuloendothelial system. The globulin is degraded and the component amino-acids enter a common pool, while the porphyrin is oxidised and excreted by the liver as bilirubin. The iron is released back to the plasma with a turnover rate of 20 to 25 milligrams daily; this fraction being preferentially re-utilized for haemoglobin synthesis.

Finally, the exchange between plasma iron and stores is normally very limited and remains in the order of half a milligram daily.

D. SUMMARY

The interest of physicians in iron and its relationship to chlorosis has been briefly traced to emphasize early preoccupation with purely therapeutic and nutritional aspects.

Attention has then been directed to the major reorientation in research which took place around 1937 when the significance of the absorptive mechanism in maintaining optimal iron nutrition was recognised. At approximately the same time radioactive isotopes of iron first became available for research and the study of iron metabolism entered what may be called 'the modern era'.

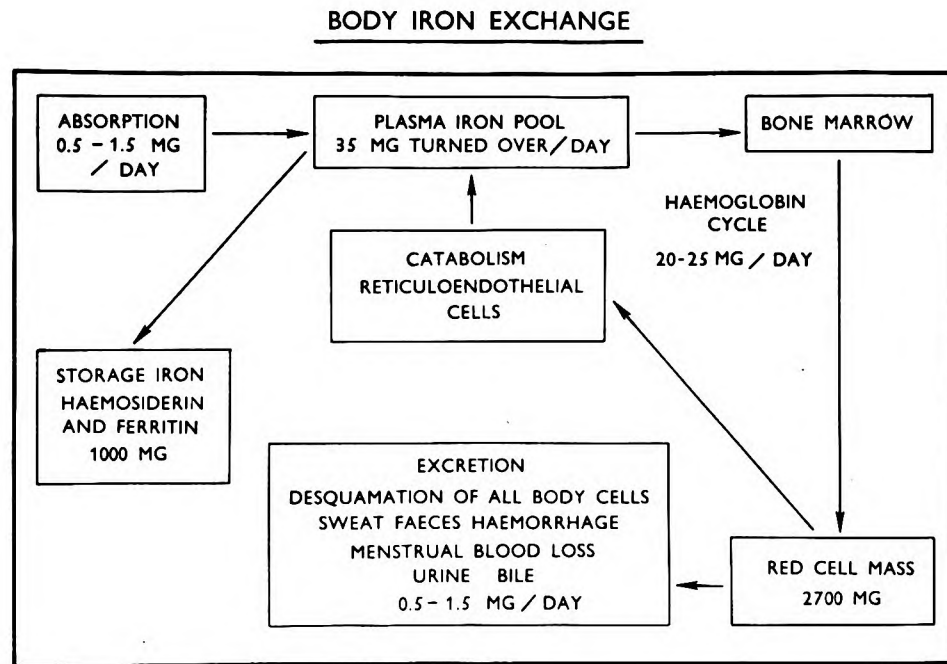


FIGURE 3

An Outline of Body Iron Exchange

Subsequently, technical methods have also improved allowing investigators to design more sophisticated experiments. Arising out of such studies, the various iron-containing compounds have been more accurately defined and certain kinetic aspects of their metabolism documented. In this chapter some of the chemical and functional properties of these substances have been considered in the light of relevant publications and a modern concept of iron metabolism described.

The object of these introductory remarks has been to provide a general background to the metabolism of iron before proceeding to a more detailed review of iron absorption and the factors which influence this process.

CHAPTER III

IRON ABSORPTION - A REVIEW OF THE LITERATURE

The cells of the gastrointestinal tract have the intriguing property of being able to take up and excrete a number of substances, including iron, in a highly selective way. Although the individual stages in the physiological pathway of iron absorption have not been completely defined, a number of factors are currently recognised as influencing the manner in which iron is processed, both within the lumen and by the mucosal cells. It is the purpose of this review to consider these in some detail, and for the sake of clarity it is proposed to do so under several rather artificial subdivisions (Table III).

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TABLE III

- A. ANATOMICAL SITE OF ABSORPTION
- B. THE INFLUENCE OF LUMINAL FACTORS
 - I. Effect of Dose
 - II. Effect of Valency
 - III. Agents which Enhance Iron Absorption
 - a) Ascorbic Acid
 - b) Succinic Acid
 - c) Fructose
 - d) Sorbitol
 - e) Inosine
 - f) Surface-active Agents
 - IV. The Luminal Factors which Depress Absorption
 - V. The Absorption of Food Iron
 - a) Simple iron salts given together with food
 - b) Absorption of food iron
 - VI. The Gastric Secretions
 - VII. The Pancreas and Iron Absorption
 - VIII. The Bile
 - IX. The Liver, Cirrhosis and Iron Absorption
 - X. The Intestinal Secretions
- C. THE INFLUENCE OF CELLULAR OR MUCOSAL FACTORS
 - I. The Role of Metabolic Energy
 - II. The Intracellular Pathway of Iron Transport
 - a) Mucosal Uptake
 - b) Mucosal Transfer
 - i) The Phase of Rapid Transfer
 - ii) The Phase of Slow-release
 - III. Iron Prefeeding
 - IV. Body Iron Stores
 - V. Transferrin and Plasma Iron Levels
 - VI. Erythropoiesis
 - VII. Hypoxia

A. ANATOMICAL SITE OF IRON ABSORPTION

A gradient apparently exists in the gastrointestinal tract for the absorption of iron, having a maximal efficiency in the duodenum and decreasing progressively in the more distal segments. Support for the presence of such a gradient derives from studies in experimental animals where the maximal deposition of radioiron has been demonstrated in the duodenum and upper small bowel following oral iron feeding (Granick, 1946 b; Endicott, Gillman, Brecher, Ness, Clarke and Adamik, 1949; Brown and Justus, 1958; Wack and Wyatt, 1959). Similarly, it has been shown that after large doses of iron ferritin accumulates in the same site (Granick, 1946 b; Gabrio and Salomon, 1950; Heilmeyer, 1958). It should be appreciated that these studies only demonstrate iron in the bowel wall and not the amount absorbed, which may well have passed into the body.

Furthermore, the introduction of radioiron into the terminal segment of a jejunal fistula resulted in lower absorption than when a comparable dose was given into the stomach (Hahn, Bale, Ross, Balfour and Whipple, 1943; Stewart, Yuile, Claiborne, Snowman and Whipple, 1950). Similarly, it has been shown that iron absorption may take place in the distal gastrointestinal tract of rats (Copp and Greenberg, 1946), rabbits (Ohkawara, Bamba, Nakai and Nonomura, 1961) and man (Ohkawara, Bamba, Nakai,

Kinka and Masuda, 1963).

Support for a gradient was also obtained in vitro. Thus, iron transport in everted segments of rat intestine was invariably maximal when the segments were taken from near the pylorus (Dowdle, Schachter and Schenker, 1960).

A possible explanation for these findings is that in the normal animal the main site for absorption of dietary iron is in the duodenum and possibly the upper jejunum. Proceeding distally a physiological gradient may develop, possibly determined by such factors as pH, redox potential and perhaps hepatic and pancreatic secretions, these being optimal in the upper small intestine. On the other hand, it remains possible that these cells may, in fact, have a special intrinsic ability to absorb iron.

The relative importance of the upper small bowel and especially the duodenum in the intestinal absorption of iron is emphasized when some stress is applied, such as a large dose of iron (Duthie, 1964). Conversely, in anaemia there is an extension of the site of maximal absorption to small intestine and possibly even to the colon (Wack and Wyatt, 1959). The greater absorption occurring in the anaemic rat (Wack and Wyatt, 1959; Duthie, 1964) may also be explained by the fact that, under these circumstances, iron takes

longer than normal to pass along the bowel (Austoni and Greenberg, 1940; Mori, Barker, Juras and Wissler, 1957).

Finally, anatomical continuity between the stomach and the small bowel is also important. Hence surgical procedures, such as partial gastrectomy and short-circuiting operations, alter the pattern of iron absorption (Choudhury and Williams, 1959). Thus, while ferrous salts may be normally retained, iron given with food is poorly absorbed and the predicted increase does not occur when iron deficiency develops (Smith, 1956; Baird, Podmore and Wilson, 1957; Stevens, Pirzio-Biroli, Harkins, Nyhus and Finch, 1959; Baird and Wilson, 1959).

It has been suggested, on the basis of histological evidence, that the lymphatics play a critical role in the absorption and transport of iron (Gillman and Ivy, 1957; Gabrio and Salomon, 1950). However, where the lymphatic transport of this metal has been measured directly, only negligible amounts were in fact demonstrated (Endicott et al., 1949; Peterson and Mann, 1952; Everett, Garrett and Simmons, 1954; Reizenstein, Cronkite, Meyer and Usenik, 1960). Similarly, Moore and Dubach (1956) are of the opinion that iron is absorbed directly into the blood stream rather than by way of the lymphatics.

B. THE INFLUENCE OF LUMINAL FACTORS

Although knowledge concerning the absorption of food iron itself remains fragmentary, it is quite clear that this is a highly complex process.

However, it is currently believed that solution and reduction of iron are prerequisites to mucosal cell uptake (Moore and Dubach, 1959). Furthermore, it has also been assumed that dietary iron in general may follow this pathway, perhaps with the assistance of digestive enzymes, but there is evidence that this is not always the case (Turnbull, Cleton and Finch, 1962). For this reason, and because many more studies have been carried out using simple salts, it is logical to consider the behaviour of the latter before that of food iron.

I. The Effect of Dose

A definite inverse relationship exists between the size of the dose administered and the percentage absorbed. On the other hand, however, the total amount of iron absorbed depends directly upon the dose. Thus with small doses, while the efficiency may be greater, the total amount retained is often entirely inadequate to meet requirements, an observation of importance in clinical therapy with iron (Gubler, 1956).

This problem has been investigated in dogs rendered anaemic by repeated removal of blood (Hahn, Ross, Bale and Whipple, 1940); it was found that as the iron intake increased the percentage absorption rapidly falls. In non-anaemic rats (Brading, George and Walsh, 1956) a similar phenomenon was demonstrated in a range which corresponded proportionately to therapeutic dosage in man.

The relationship has recently been analysed in considerable detail by Bothwell and Finch (1962) with doses ranging from 50 micrograms (Bonnet, Hagedorn and Owen, 1960) to therapeutic levels of 400 milligrams of ferrous iron salt (Bothwell, Pirzio-Biroli and Finch, 1958). These workers graphed the dose administered in 12 different published studies against the iron absorption, as measured either on red cell radioactivity or faecal radioiron content. They illustrated well a linear relationship between the two parameters.

Since the biological processes governing the relationship between iron dosage and iron absorption are not clear, an attempt has been made to analyse these kinetics in mice (Gitlin and Cruchaud, 1962). It was demonstrated that in the mouse a similar pattern of absorption exists to that recognised in man. On the basis of their studies these workers suggested that two different mechanisms

might be operating simultaneously: namely, a process with enzymatic or carrier characteristics in which the limiting factor appeared to be the amount of enzyme or carrier available, and secondly, a first-order process in which the limiting factor appeared to be the amount of absorbable iron in the gastrointestinal lumen available to the absorbing cells. It was believed that the first process would play the major role in the absorption of physiological amounts of iron while, with increasing doses, absorption depends to greater degree upon the first-order mechanism. Gitlin and Cruchaud conclude that both mechanisms are influenced by the state of the body iron stores but to a different degree, with the enzyme-limited process affected to a greater extent.

In a critical review (Beutler, Kelly and Beutler, 1962) attention has been directed to the fact that the logarithm of the dose of iron administered to the mouse and to man, bears a linear relationship to the logarithm of the amount absorbed. These workers believe that the study of Gitlin and Cruchaud (1962) can be criticised on the small numbers of animals in each group and suggest that the details of their proposed relationship may not be correct. This opinion is based on studies using larger numbers of animals, in which distinction could not be made between a linear and a log-log relationship at high iron dosage. Beutler et al. (1962) believe that

no simple model can be made to fit iron absorption data on the intact mouse. However, they are inclined to the view that the same absorptive mechanisms are active both when small doses of iron are given and when massive amounts of iron are fed. Hence they believe that acute iron poisoning represents, not destruction of the intestinal mucosa with subsequent "leakage" across the membranes, but rather an extension of the normal absorptive process into the lethal range.

It may therefore be concluded that while such a relationship exists both in man and in certain experimental animals, considerable difficulties in interpretation exist and further critical study is clearly necessary.

II. The Effect of Valency

The evidence in favour of the preferential absorption of ferrous iron, rather than its ferric counterpart, rests upon studies carried out in different species of experimental animals and also in man.

Thus, in mice it is believed that ferric iron must be reduced before absorption can take place (Tompsett, 1940) while in rats ferric is absorbed less well than ferrous iron (Groen, van den Broek and Veldman, 1947). However, animals on a low-iron diet showed no marked difference in their ability to use either form for

haemoglobin formation or storage (Underwood, 1938; Blumberg and Arnold, 1947).

By way of contrast, there is at least one publication (Brading et al., 1956) in which more ferric than ferrous iron was absorbed over a wide dosage range in rats. The interpretation of this study is not clear since it has been reported that the administration of a specific ferrous complexing agent, such as alpha-alpha dipyridyl, blocks the absorption of both ferrous and ferric iron (Venkatachalam, Brading, George and Walsh, 1956). If this complex is not absorbed (Wiseman, 1964) then it is likely that ferric salts are reduced to the ferrous state, possibly in the stomach or high up in the small intestine, before absorption. However, there is evidence that such a complex is absorbed (Torrance, 1965) and it is then possible that rapid urinary excretion of the chelate, together with the label, masked absorption. This would occur particularly if whole body counting was used to determine absorption, since a few days are allowed to elapse for faecal excretion of unabsorbed dose.

Further studies in the rat, using everted pouches of intestine (Brown and Justus, 1958) appeared to support the preferential transfer of ferrous iron since mucosal uptake was significantly greater for the ferrous ascorbate gelatin than for ferric citrate.

More detailed studies (Manis and Schachter, 1962 a), using ferrous sulphate and ferric chloride, extended these observations. Thus, mucosal uptake of ferrous or ferric iron was approximately the same, but serosal transfer was 57 percent greater when the mucosal medium initially contained divalent iron. These findings were confirmed using radioactive isotopes under the same circumstances (Manis and Schachter, 1962 a).

When this problem was studied in dogs, the evidence was also conflicting. Some workers (Whipple and Robscheit-Robbins, 1936; Moore, Dubach, Minnich and Roberts, 1944) have not been able to demonstrate a clear-cut difference, while another group (Hahn, Jones, Lowe, Meneely and Peacock, 1945) concluded from their studies in three anaemic dogs that radioiron was better absorbed when given with ferrous salts.

In evaluating this facet of iron absorption in the human subject, Brock and Hunter (1937) studied the retention of fairly large doses of iron. On the basis of balance studies they concluded that there was not any consistent difference between the absorption of ferrous or ferric iron. These studies are of doubtful value when the technique is considered. On the other hand, Lottrup (1934) had earlier reported a better response in haemoglobin formation when ferrous iron was given to anaemic children. There are now many

published investigations, using a number of different techniques, which support this observation (Moore, Arrowsmith, Welch and Minnich, 1939; Moore et al., 1944; Hahn et al., 1945; Henley, Christenson, Grace and Wolff, 1956; Bothwell et al., 1958). More recently studies using a twin isotope technique have provided additional evidence on this aspect of iron absorption (Brise and Hallberg, 1962 a). There is, however, no convincing evidence that the availability of iron from one ferrous salt is any better than from any other (Gram and Leverton, 1952); the possible exception being in the case of succinate (Brise and Hallberg, 1962 a).

Of some relevance is the observation that reduction of iron occurs in the human stomach (Bergeim and Kirch, 1949). This, coupled with the fact that at very low dosage level (one microgram per kilogram) ferrous and ferric iron was absorbed equally well (Bonnet et al., 1960), suggests that reducing systems may be present in the gastrointestinal tract. It is conceivable that such systems may have certain limits in providing ferrous ions, and in this way determining absorption. However, quantitative studies on this point are not available.

It may be concluded that ferrous iron is better absorbed than the ferric form, possibly due to the greater solubility of

ferrous complexes with a variety of inorganic and organic anions (Thorne and Roberts, 1954; Gubler, 1956). Whether this phenomenon is related to an intrinsic property of the bowel or to reducing mechanisms present in the luminal environment, possibly supplied by various digestive juices or from the diet, remains speculative (Moore and Dubach, 1951; Gubler, 1956).

III. Agents which Enhance Iron Absorption

a) Ascorbic Acid

It is to be anticipated that the absorption of iron salts will be modified by the addition of substances which have a reducing action. The suggestion (Kirch, Bergeim, Kleinberg and James, 1947) that ferric iron was reduced by ascorbic acid when foodstuffs were digested in vitro, directed attention to this vitamin.

In rats (Brading and Cantrill, 1959) it was found that iron absorption from ferrous sulphate was significantly improved when administered together with ascorbic acid. Similarly, in vitro, Dowdle and his co-workers (1960) demonstrated that ascorbate increased transport against a concentration gradient. Manis and Schachter (1962 a) reported that ascorbate had little effect on mucosal uptake but increased serosal transfer significantly - the major effect

being on ferric iron transport. Since this latter step is relatively specific for divalent iron, these workers suggest that ascorbate acts in vitro primarily as a reducing agent.

In human subjects, the administration of ascorbic acid together with ferric salts (Moore, Bierman, Minnich and Arrowsmith, 1940) resulted in a marked enhancement of absorption. Likewise, Gorten and Bradley (1954) concluded that when vitamin C was administered with ferric ammonium citrate to anaemic children, iron absorption and possibly also utilization were increased. The lesser effects of vitamin C observed with ferrous salts (Bothwell et al., 1958) were re-examined by Brise and Hallberg (1962 d). These workers report a study in which 30 milligrams of iron as a ferrous sulphate solution was administered together with quantities of ascorbic acid which varied from 50 to 500 milligrams. It was concluded that when the latter was given in sufficiently high doses it promoted the absorption of iron, and that this effect increased with increasing doses of ascorbic acid. Intravenous infusion of vitamin C did not produce this effect or alter the basal plasma iron turnover. Thus it appeared most likely that the enhanced absorption was due to a reducing action within the gastrointestinal lumen preventing or delaying the formation of insoluble or less dissociated ferric complexes.

b) Succinic Acid

Brise and Hallberg (1962 e) studied the absorption of 30 milligrams of elemental iron as ferrous sulphate in human subjects using a twin isotope technique. They demonstrated that while 30 milligrams of succinic acid had little effect, doses from 60 to 500 milligrams markedly promoted iron absorption. These findings are of some interest since they were produced when this acid was given intravenously. At this time no change could be demonstrated in the plasma iron turnover or in intestinal motility. It was believed that succinic acid did not exert its effect through an increased iron ion concentration in the gastrointestinal tract or through distribution of iron over a greater gastrointestinal surface. It appeared more likely that its action was due to an increase in the intracellular mucosal metabolism.

c) Fructose

In studies using the intact rabbit, rapid absorption of iron has been demonstrated when this was administered as the fructose chelate. In this situation the ions are present as soluble, uncharged complexes of low molecular weight (Charley, Stitt, Shore and Saltman, 1963 a). This study is an extension of earlier work in which these authors had pointed out that the chemical conditions in the upper small

bowel were particularly favourable for chelate formation (Saltman, Charley and Sarkar, 1962). In addition it appeared that the iron-fructose complex was more rapidly absorbed and deposited in tissues than was ferrous sulphate (Stitt, Charley, Butt and Saltman, 1962). On the basis of such observations, this group of workers have suggested a molecular basis for Bantu cytosiderosis and experimentally induced iron storage in rats (Charley, Sarkar, Stitt and Saltman, 1963 b; Stitt et al., 1962), and have proposed an hypothesis for the regulation of iron uptake. Thus, they have suggested that the control of absorption is achieved by chelation and equilibrium binding in which transport of both ferrous and ferric iron into, and across the mucosal cell is facilitated by a suitable ligand. The rate at which iron subsequently enters the circulating blood from the mucosal cells was thought to be a function of the unbound iron-binding capacity of the serum transferrin (Charley et al., 1963 a). However, the significance of these data is difficult to assess since the animal preparation could be criticized on the grounds that changes in radioactivity are monitored between the carotid artery and jugular vein. These data would, therefore, appear to neglect changes produced in portal blood flow through the liver.

d) Sorbitol

Sorbitol is reported to enhance iron uptake in both normal

and iron-deficient rats (Herndon, Rice, Tucker, van Loon and Greenberg, 1958). Since Sorbitol was thought unlikely to reduce iron to the ferrous form, these authors postulated that it either protected substances within the intestinal lumen, or stimulated absorption by an unknown mechanism within the mucosal wall.

e) Inosine

Inosine given intravenously to rats at the time of an oral dose of iron increased absorption of the metal by 50 to 100 percent, but had no effect when given orally (Cheyney and Finch, 1960). It is not clear why this substance should influence iron transport across the intestinal mucosa or in the liver, but it is possible that soluble iron complexes or chelates of low net charge may be formed facilitating movement through the cells.

f) Surface-active Agents

The observations that surface-active agents increased the absorption of ferric iron in hamsters (Wissler, Bethard, Barker and Mori, 1954) has recently been extended to man (Brise, 1962 c). In the latter studies, no significant increase in the absorption of ferrous sulphate could be obtained with polyoxyethylene sorbitan monolaurate (Tween 20), diocetylsodium-sulfosuccinate, sodium

lauryl sulphate and bile acids. It is uncertain whether the difference in these results can be explained by the fact that ferric iron was used in the hamsters while ferrous iron was administered in man.

IV. Luminal Factors which Depress Absorption

There are only a limited number of publications available on the effect that phosphates and phytates have on the absorption of simple iron salts. Nevertheless, studies in which phosphates have been tested against these salts, in the absence of dietary iron, indicate that they readily form insoluble and undissociable complexes with ferric iron (Gubler, 1956) and are thus likely to block absorption. McCance, Edgecomb and Widdowson (1943) have also reported that phosphates decrease the absorption of ferric salts. Similarly, sodium phytate has been found to lower the absorption of small quantities of ferrous salts by 50 percent (Turnbull and Finch, 1961). In this context phytate is known to form complexes with iron (Vohra, Gray and Kratzer, 1965).

A deficiency of copper lowers iron absorption in animals (Wintrobe, Cartwright and Gubler, 1953), but the way in which this effect is produced remains obscure.

V. The Absorption of Food Iron

a) Simple Salts Given Together with Food

The absorption of dietary iron is acknowledged to be a highly complex process. While little is currently known about the absorptive pathway of the tightly complexed iron (Bothwell and Finch, 1962), limited information is available on that portion of food iron which is present in the ionised pool (Ascham, Speirs and Maddox, 1938; Bergeim and Kirch, 1949). The findings described above for simple salts are of interest, but it is not clear to what extent such observations can be extrapolated to the absorption of a readily ionisable fraction of food iron. A better model is likely therefore to be obtained by feeding ionisable salts simultaneously with food.

The most striking feature of such studies is that absorption is markedly reduced (Brise, 1962 b). Thus, the absorption of ferrous chloride was depressed by the simultaneous administration of non-labelled scrambled eggs (Chodos, Ross, Apt, Pollycove and Halkett, 1957), while a "standard meal" reduced the absorption of a tracer dose of ferric chloride from a theoretical level of between 10 and 15 percent, to 6 percent (Pirzio-Biroli, Bothwell and Finch, 1958). In much the same way, the absorption of radioisotope from iron-enriched bread was significantly depressed (Steinkamp, Dubach and Moore, 1955). In

children iron absorption was moderately impaired by the simultaneous administration of milk (Sharpe, Peacock, Cooke and Harris, 1950; Schulz and Smith, 1958 b) but markedly depressed when given with food (Sharpe et al., 1950).

It has been emphasized (Bothwell and Finch, 1962) that the accuracy of these results depend upon the assumption that the tracer used forms a common pool with the ionisable food iron. It is apparent that any error introduced by this assumption would not invalidate the observation that food causes a considerable reduction in absorption: this method would tend, if anything, to over-estimate the amounts of iron absorbed from food.

Furthermore, the exact interpretation of such experimental data is complicated by observations that there is a progressive reduction in absorption as the bulk of the meal increases (Sharpe et al., 1950). Thus, while food itself may be a factor, alternative theories might relate this effect to the composition of the diet or perhaps even the relative proportions of different constituents. In considering the latter possibilities, the danger of a diet very low in phosphate has been demonstrated (Kinney, Hegsted and Finch, 1949): rats markedly increase their absorption of iron and develop progressive haemosiderosis. In additional studies (Hegsted, Finch and Kinney, 1949) the absorption of iron was shown to be dependent

not only on the quantity of iron fed, but also on the iron-to-phosphate ratio. A linear relationship could be demonstrated between the logarithm of phosphate concentration and the amount of iron absorbed from an iron-supplemented diet. In an extension of this work the same phenomenon was demonstrated in the mouse, the guinea-pig and the chicken (Hegsted, Finch and Kinney, 1952).

The fact that iron absorption could not be diminished when large amounts of simple phosphate salts were added to the diet (Chapman and Campbell, 1957) is relevant. These observations suggested that, while phosphates present in the normal diet could reduce absorption of ferric iron, there existed a critical level above which they had little additional effect.

The effects of phytates are likely to be similar, for although little effect could be demonstrated in rats (Fuhr and Steenbock, 1943), sodium phytate reduced iron absorption in adolescent boys (Sharpe et al., 1950). In addition, Widdowson and McCance (1942) attributed the greater depression produced by brown bread to the presence of phytic acid and perhaps inorganic phosphate. Similarly, it was demonstrated that sodium phytate was more effective than disodium hydrogen phosphate in preventing a rise in serum iron levels following a meal of white bread plus soluble iron salt (McCance et al., 1943).

More recently, attention has been directed to the higher iron

requirements in the Indian population, where the intake of phytate is high (Apte and Venkatachalam, 1962).

Yet another factor which can influence the absorption of food iron is calcium. Thus, while there seems little doubt that the presence of large amounts of calcium salts in the diet may alter absorption (Chapman and Campbell, 1957) their importance in terms of dietary availability is not clear. It is possible that any effects produced may be related to the relative concentration present; small amounts may actually facilitate iron absorption by combining with phosphates and phytates and so rendering them unavailable for combination with iron (Gubler, 1956). On the other hand, there is some evidence (Manis and Schachter, 1962 a) that iron and calcium may compete for one or more rate-limiting cellular constituents, and it is conceivable that under certain circumstances the absolute amounts present are more important. In this regard a recent report from India is of relevance (Apte and Venkatachalam, 1964). These workers indicated that under particular dietary conditions it is the quantity of calcium ingested that may be critical.

Finally, it is of some interest that vitamin C also has an effect upon the absorption of iron when the latter is given with food. Thus, the addition of large amounts of ascorbic acid to a

test meal, given with radioiron, doubled the absorption (Pirzio-Birblli et al., 1958). This substance likewise increased absorption of radioiron from enriched bread (Steinkamp et al., 1955). Furthermore, Williams (1959) reported that ascorbic acid increased absorption when administered with Fe^{59} incorporated into bread, the increase being greatest in subjects with normal gastrointestinal tracts and least in those with achlorhydria.

b) The Absorption of Food Iron

It is against studies carried out with simple iron salts, administered either alone or together with food, that absorption of dietary iron itself must be reviewed. In considering this problem it should be appreciated that, at present, information is still fragmentary. Furthermore, it must be recognised that the iron content per se of individual foods cannot alone form the basis for judging the adequacy of any diet. This is because the actual quantity of available iron varies.

With regard to the first aspect, tables are readily available to indicate the iron content of different foods (Watt and Merrill, 1950/51; Documenta Geigy Scientific Tables, 1962). Bothwell and Finch (1962) have emphasized that the amount of iron present in a particular food is profoundly influenced by such factors as the manner of cultivation and methods used in preparation. These authors cite the example of

Bantu beer made in South Africa, where the iron content may vary from negligible amounts up to quantities as great as 20 milligrams per 100 millilitres. They draw attention to the importance of cooking utensils, the pH of the cooking mixture and the initial iron content of the particular foodstuffs used.

The absorption of Bantu beer and the possible significance of this beverage in the genesis of iron-overload in the Bantu of Southern Africa is further considered in Chapter VI. In this section the experimental data which form part of this thesis are presented and discussed.

It is likely that the question of dietary iron content can be best resolved either by analysing foodstuffs immediately before ingestion, or by determining the faecal iron (Walker and Arvidsson, 1953). In studying this problem, it appears that an average diet contains between 12 and 15 milligrams of iron (Moore and Dubach, 1962; Bothwell and Finch, 1964) while some diets may contain very much more than this (Walker and Arvidsson, 1953).

The second important aspect of dietary iron absorption, namely the actual availability of iron, has long been recognised. Thus

in 1938 Ascham and colleagues investigated the proportion of food iron that could be ionised by dilute hydrochloric acid since it was believed that only this fraction could be assimilated. This method of study has been discarded since it is indirect, and ignores the possibility that enzymatic digestion in the intestine may "free" further portions of food iron (Moore and Dubach, 1962).

More recently, information has been sought on this point using chemical balance studies. It has been possible to establish that absorption, from carefully controlled basal diets, varies between 11 and 14 percent (Widdowson and McCance, 1942; Johnston, Frenchman and Boroughs, 1948; Johnston, Frenchman and Boroughs, 1949; Schlaphoff and Johnston, 1949; McMillan and Johnston, 1951). A second way of determining the availability from food is by measuring the response in haemoglobin levels of animals with iron deficiency anaemia, following the administration of different foodstuffs. On the basis of such experiments, good absorption has been demonstrated with liver iron (Pye and MacLeod, 1946), the results have been somewhat conflicting with wheat flour and soya bean (Free and Bing, 1940; Miller and Louis, 1945; Ruegamer, Michaud, Hart and Elvehjem, 1946; Subrahmanyam, Sathe and De, 1950), while absorption was poor from egg yolk, beef muscle, spinach and rice (Pye and MacLeod, 1946; Ruegamer et al., 1946; Sen, 1952).

There is little doubt, however, that the most reliable information on the availability of iron from foodstuffs has been obtained since the introduction of radioactive isotopes (Moore and Dubach, 1951; Moore and Dubach, 1956). In studies of this type, the radioiron has been biosynthetically incorporated into milk (Schulz and Smith, 1958 a), eggs (Moore and Dubach, 1951; Moore and Dubach, 1956; Chodos et al., 1957; Halkett, Peters and Ross, 1958), haemoglobin (Callender, Mallett and Smith, 1957), animal tissues (Moore and Dubach, 1951; Walsh, Kaldor, Brading and George, 1955; Moore and Dubach, 1956), various vegetables (Moore and Dubach, 1951; Moore and Dubach, 1956; Chodos et al., 1957; Halkett, Chodos and Ross, 1959), wheat and ferritin (Hussain, Walker, Layrisse, Clark and Finch, 1965). The results obtained from these investigations are striking in that, although there is a wide variation in absorption by different subjects, values are not markedly different to those obtained with chemical methods. Thus, normals retain approximately 5 to 10 percent, while in iron deficient subjects this rises to between 10 and 20 percent of the iron in food (Moore and Dubach, 1962) and often exceeds this level.

The importance of availability has recently been commented upon by Finch (1965). He suggested that a diet containing a total of 20 milligrams of iron, corresponding to between 3 and 4 milligrams of "available" iron, may be adequate for man. It was felt that woman,

with her greater requirements, should have perhaps 30 to 40 milligrams of iron a day. In any event, it is clear that the iron content of the diet will determine, to some extent, the availability of iron for absorption.

The explanation for these differences in availability is obscure and it has been suggested (Bothwell and Finch, 1962) that they may be related to the types of chemical binding present. For example, it is probable that the iron in eggs is unavailable because it is strongly complexed with the phosphate of yolk phosphoproteins (Chodos et al., 1957); it has been demonstrated (Larsen, Bidwell and Hawkins, 1960) that iron absorption from synthetic chemical chelates is low. Contrastingly, the similar absorptions noted between biosynthetically labelled milk and iron administered simultaneously with milk (Schulz and Smith, 1958^a) suggests that in this instance absorption depends upon such factors as the iron:phosphorus ratio (Bothwell and Finch, 1962). These alternative mechanisms, coupled with the facilitated absorption that large doses of ascorbic acid produce when they are given with certain tagged foods (Moore and Dubach, 1951), emphasize yet again the nutritional importance of the reduced soluble portion of food iron. On the other hand, a further possible reason for the discrepancies observed between the absorption of iron from labelled items may be the presence of alternative pathways for their absorption. In this context, haemoglobin and other iron-containing complexes have been shown to behave differently

from salts in the presence of food, phytate and iron deficiency (Moore and Dubach, 1951; Turnbull et al., 1962).

VI. The Gastric Secretions

Interest in the relationship of the gastric secretions to iron absorption stems from early clinical studies in which it appeared that both inorganic iron salts (Mettier and Minot, 1931; Minot and Heath, 1932; Heath, 1933; Bethell, Goldhamer, Isaacs and Sturgis, 1934) and food iron (Mettier, Kellogg and Rinehart, 1933; Kellogg and Mettier, 1936) were better absorbed in the presence of this juice. These observations appeared to be supported by reports (Kirch et al., 1947; Bergeim and Kirch, 1949) that gastric secretions could solublize and reduce iron. Contrasting studies, using iron absorption curves, have been reported in which large doses of inorganic iron salts were not significantly affected by achlorhydria (Moore et al., 1939; Smith, 1952; Grace, Doig and Wolff, 1954).

The re-evaluation of this problem, using isotopic methods, has failed to clarify the confusion. There is evidence on the one hand that the absorption of ferrous salts (Dubach, Callender and Moore, 1948), haemoglobin iron (Biggs, Bannerman and Callender, 1961) and food iron (Pirzio-Biroli et al., 1958; Moore and Dubach, 1951) was not influenced by gastric secretions. On the other hand, a defective

absorption of radioiron from bread has been reported both in iron deficient patients (Williams, 1959) and non-anaemic subjects (Cook, Brown and Valberg, 1964). In addition, Goldberg, Lochhead and Dagg (1963) demonstrated a significant depression in the absorption of a tracer quantity of iron when this was administered with a standard meal to iron deficient achlorhydric individuals. An increased absorption of radioiron has been reported in gastrectomised patients by the addition of hydrochloric acid (Moeschlin, Schmid and Schnider, 1965).

In considering the above remarks, it should be appreciated that the gastric juice is unique in its acidity and digestive properties (Sparberg and Kirsner, 1964). In addition the possible effects of contamination with salivary enzymes should not be ignored (Johnston and Clark, 1959). It is then conceivable that during the digestion of food, substances such as pepsin could promote absorption by releasing iron bound to protein (Kirch et al., 1947), by enhancing the reducing properties of dietary protein due to sulphhydryl group release (Cook et al., 1964) or by increasing the concentration of amino-acid residues in the upper small bowel (Klavins, Kinney and Kaufman, 1959; Kroe, Kinney, Kaufman and Klavins, 1963). However, since this enzyme requires an acid medium for its action, it is difficult to separate these effects from that of acid per se. Similarly, the presence of reducing substances in the gastric juice may explain its enhancing effect on the

absorption of dietary iron, but these substances likewise perform more efficiently in an acid medium (Grabick, 1949).

The role of the gastric secretions in iron absorption is further considered in Chapter IV. In this section experimental results which form part of this thesis are presented and discussed.

Experimental evidence has recently been presented (Koepeke and Stewart, 1964) suggesting the existence of a substance in gastric juice obtained from anaemic dogs, which facilitates iron absorption when administered orally to normal dogs. These authors believe that iron may be absorbed in a manner similar to vitamin B₁₂, an intrinsic substance being secreted by the stomach which is necessary for absorption of iron. Supportive evidence for this contention has been obtained by Murray (1965) who demonstrated that neutralised gastric and intestinal juices increased the uptake of labelled ferrous iron by isolated segments of rat intestine. A similar enhanced uptake was noted when secretions from haemochromatotic subjects was used. However, when the secretions were heated these effects were abolished.

The majority of these investigations have been carried out in subjects with iron-deficiency or with achlorhydria. It may be concluded, that under specific circumstances, the gastric secretions influence the absorption of simple iron salts, and certain forms of food iron. Evidence is at present insufficient to indicate that these juices play a critical role in iron absorption under normal dietary conditions.

VII. The Pancreas and Iron Absorption

Since the exocrine pancreatic secretions are rich in both phosphorus and bicarbonate it might be anticipated that they could produce a luminal environment which is unfavourable to iron absorption. In this regard, there does appear to be a factor in pancreatic secretion which inhibits the absorption of iron under certain specific circumstances, although the exact mechanism remains obscure. Thus, ligation of the pancreatic duct produced haemosiderosis in cats (Taylor, Stiven and Reid, 1931; Taylor, Stiven and Reid, 1935) and dogs (Kinney, Finch, Kaufman, Hegsted and Partington, 1950). Similar findings have been obtained in animals following ethionine-induced pancreatic necrosis (Kinney, Kaufman and Klavins, 1955) and in protein malnutrition (Kaufman, Klavins and Kinney, 1958). More recently Davis and Biggs (1965)

have demonstrated that the addition of a pancreatic extract reduced the uptake of iron from an isolated loop of rat jejunum.

Clinical studies likewise are not in complete agreement. On the one hand, chronic pancreatitis in man does not apparently cause iron overload (Moore and Dubach, 1959). On the other hand, an increased tissue iron level has been reported at autopsy in some children who suffered from mucoviscidosis (Andersen, 1938). More recently ferric iron, but not haemoglobin iron, has been noted to be strikingly increased in children with cystic fibrosis (Tönz, Weiss, Strahm and Rossi, 1965). However, the early development of iron deficiency following pancreatin therapy is worrying. Could this represent occult bleeding or accelerated loss via bile or gut (Pollack, 1966)? A defective absorption of haemoglobin iron has been demonstrated in children with cystic fibrosis who were on long term treatment with pancreatin (Smith, 1964). This author believes that, in addition to having a direct action on iron absorption, the administration of pancreatin to these children may have produced a malabsorptive process, and that this could continue even after withdrawal of the replacement therapy.

Similarly, the absorption of a physiological dose of iron is increased in patients with calcific pancreatitis (Davis, 1961; Davis

and Badenoch, 1962) and iron absorption can be reduced significantly in subjects with haemochromatosis by the addition of pancreatic extract (Biggs and Davis, 1963; Deller, 1965). Recently, haemosiderosis has been reported in some patients with pancreatic exocrine failure (Banwell, Hutt, Marsden and Blackman, 1964) while Ball (1964) described an inappropriately large absorption of iron in association with pancreatic fibrosis. On the basis of his observations Ball (1964) suggested that this phenomenon may be influenced by luminal pH, and that these patients retain their ability to limit the absorption of iron when the body is overloaded.

It is therefore clear that the exocrine pancreatic secretions may influence iron absorption under defined circumstances. They cannot, on the basis of available information, be regarded as of prime importance in the regulation of iron absorption. Their physiological importance for the overall process of absorption and utilization remains to be determined.

VIII. The Bile

Interest was focused on the bile by a report that exclusion of this substance from the intestine of chronically iron-deficient dogs decreased iron absorption (Hawkins, Rabscheit-Robbins and

Whipple, 1938). Since iron stores are clearly important in determining iron absorption and since the bile offered a direct, non-humoral connection between the liver (an important site of iron storage) and the duodenum (the main area of iron absorption), the role of bile in the control of iron absorption was re-investigated using a cross circulation technique (Wheby, Conrad, Hedberg and Crosby, 1962). These workers were unable to demonstrate a regulating effect of bile upon iron absorption but suggested that its presence may have a facilitating effect, since, exclusion from the iron-deficient dog diminished absorption by approximately one half. These findings are similar to those reported by Hawkins and co-workers (1938) in studies using iron-deficient dogs with biliary fistulas. In later studies, also using dogs with bile fistulas, absorption proceeded normally unless neutral fat was given, when the formation of insoluble iron soaps markedly depressed this process (Smith and Crandall, 1941-1942).

The effect of biliary secretion on iron transfer has also been studied in vivo using isolated loops of rat intestine. No difference in iron absorption could be demonstrated whether the common bile duct was ligated close to the duodenum or not (Manis and Schachter, 1962 a).

It may be concluded that, at present, no effect upon iron absorption can be attributed to bile per se either in rats or in dogs, whether these be iron deficient or normal.

IX. The Liver, Cirrhosis and Iron Absorption

Very little is known of any influence that the liver may normally exert upon iron absorption. Information in this regard has been sought using studies carried out in patients or animals with cirrhosis of the liver.

Thus, although it has been recognised for approximately 20 years that iron overload occurs in patients suffering from cirrhosis (Herbut and Tamaki, 1946), the manner in which this haemosiderosis arises is not clear. In view of the close relationship between excessive alcohol consumption and the development of cirrhosis it is possible that alcohol may contribute to increased stores. In this regard it should be appreciated that many alcoholic drinks contain significant amounts of iron. Furthermore, it is not clear whether the alcohol itself influences iron absorption.

This problem is considered further in Chapter V where experimental data which form part of this thesis are presented and discussed.

Once cirrhosis has developed, however, factors such as shortened red-cell life span along with changes in marrow function may operate in influencing the absorption of iron (Conrad, Berman and Crosby, 1962; Deller, 1965).

The observation that crude hog pancreas depressed the elevated absorptions found in a group of cirrhotic patients, suggests that associated pancreatic involvement may be a factor in the pathogenesis of this iron overload (Callender and Malpas, 1963). The reduced absorption of both ferrous sulphate and labelled haemoglobin noted in this report implies that this action was not simply a matter of precipitation of an iron salt. These findings accord well with necropsy studies (Woldman, Fishman and Segal, 1959; MacDonald and Mallory, 1960; van Goldsenhoven, Henke, Vacca and Knight, 1963).

A similar enhanced absorption of iron in patients with cirrhosis has been recognised by other workers (Greenberg, Strohmeyer, Hine, Keene, Curtis and Chalmers, 1964). These

observations lend support to the concept that, in certain patients with haemochromatosis, liver disease may be the primary factor preceding the accumulation of iron in the tissues.

Of particular relevance is the recent demonstration that iron absorption is greater than normal in patients with cirrhosis when the bile, gastric, pancreatic and duodenal secretions were washed from the segment under study (Linscheer, Greenberg, Moore and Chalmers, 1964). The addition of the duodenal aspirate, containing secretions from liver and pancreas, decreased the absorption to normal levels in all but 2 of the 11 patients studied. These workers were unable to determine either the nature of the factors which enhanced absorption, or define those present in the bile or pancreatic juice which reduced absorption.

It is concluded that in patients with cirrhosis of the liver the absorption of iron may be significantly altered. Although evidence is at present limited it appears that intraluminal, as well as extra-luminal, factors may be of importance under these circumstances. It is strongly emphasized that these studies, being carried out in abnormal subjects, may have no bearing under physiological conditions.

X. The Intestinal Secretions

Pirzio-Biroli (1956) was unable to demonstrate complex formation using chemical, electrophoretic or chromatographic methods when radioiron was mixed with the duodenal juice from mice, rats, dogs or humans. This worker then extended his studies by feeding iron and duodenal juice obtained from normal, iron-deficient and iron-loaded mice to normals. The duodenal juice from iron-loaded mice reduced absorption significantly. No difference could be demonstrated between normal or iron-deficient donors. On this basis, Pirzio-Biroli (1956) suggested that a substance was present in the juice which blocked the absorption of iron when stores were expanded.

Murray (1965) has reported that the uptake of radioiron in vitro could be influenced by the prior exposure of segments of rat intestine to neutralised human intestinal secretions. Juice from both iron deficient and haemochromatotic patients enhanced uptake - this effect was heat labile.

Too little information is available on this facet of iron absorption for any meaningful conclusions at this stage.

Conclusion - The Luminal Factors

It may be said of the luminal factors that they profoundly influence the absorption of iron from the gastrointestinal tract. When simple salts are considered, the effects are much more clear-cut and such variables as dose, valency and reducing substances are well defined.

In contrast, the patterns of absorption obtained when simple iron salts are administered together with food, or when food iron itself is studied, are much less obvious. In reviewing this literature it has become abundantly clear that a great deal of work remains to be done before any firm conclusions may be drawn about the absorption of dietary iron, or even the extent to which this pathway parallels that of simple iron salts.

Similarly, while information about the influence of gastric, pancreatic and other secretions is being accumulated, the evidence to date does not permit any unequivocal role being assigned to these substances in the physiological regulation of iron absorption.

C. THE INFLUENCE OF CELLULAR OR MUCOSAL FACTORS

The stability of the body iron stores under normal

physiological conditions reflects the efficiency with which the cellular activity of the intestinal mucosa is capable of regulating iron absorption. The exact manner in which this unique control is exerted remains unknown.

Broadly speaking, this process may be influenced by the state of the body iron stores, by the utilization of iron for erythropoiesis, environmental hypoxia and pregnancy. It has been suggested that, at least at physiological dosage levels, iron absorption is dependent upon metabolic energy. However, this very important point remains a contentious one. It should be appreciated that at unphysiological loads, varying from acute iron intoxication to chronic dietary overload with haemosiderosis, the influence of these factors is often much less clear.

In more detailed studies of this process a stage of mucosal uptake of iron from the lumen, apparently distinct from a phase of transfer to the plasma has been recognised. It is possible to analyse mucosal transfer into two separate mechanisms, namely an initial rapid phase and a later, slower component.

In view of the very complex nature of these mechanisms, and accepting that any division must be highly artificial, these factors are reviewed separately to indicate, where possible, their relationship

to the absorptive process.

I. The Role of Metabolic Energy

The initial studies of Brown and Justus (1958) using an everted pouch of rat intestine (Wilson and Wiseman, 1954; Wilson and Vincent, 1955) are of particular interest. These workers appreciated the short-comings of this approach and concluded that iron uptake and transfer was a passive process.

Dowdle and his co-workers (1960), in studies using everted pouches of intestine (Schachter and Rosen, 1959) prepared from the proximal small bowel, obtained some evidence for the active transport of iron. Thus, they reported that concentration could be demonstrated against a gradient, that the transport mechanism for iron was limited in its capacity (Taggart, 1958), and that it was dependent upon oxidative metabolism and the generation of phosphate-bond energy. Evidence was also advanced for the possible existence of ^Atwo-step mechanism.

Support for the existence of such a two-step mechanism, and its dependence upon metabolic energy has been obtained by Manis and Schachter, (1962 a). These workers used isolated loops of rat intestine in vivo, and everted pouches of rat intestine in vitro.

Both mucosal uptake and serosal transfer appeared to be dependent upon oxidative metabolism and the presence in the medium of a metabolisable hexose. Each step was influenced independently, serosal transfer to a relatively greater degree. It is of some interest that both actively transported monosaccharides (Wilson and Vincent, 1955) and calcium transport (Schachter, Kimberg and Schenker, 1961) inhibit serosal transfer but not mucosal uptake. Furthermore, this process was shown to be adaptive in that it varied with the iron content of the diet, with single doses of oral iron and also in late pregnancy. It was, therefore, postulated to be one of the factors regulating iron absorption (Manis and Schachter, 1962 b). However, no evidence is provided on the specific site of action within the mucosal cells, or even on the possibility that there may be more than two stages in this process.

Of particular importance is the observation that various features demonstrated in vitro correspond to those found when absorption takes place from duodenal loops. Nevertheless, certain quantitative differences exist between the two situations. Such differences are perhaps explicable on the basis of better oxygenation, more physiological gradients and a lesser degree of damage to the intestinal mucosa found in vivo, all making conditions more suitable

for optimal transport.

It is concluded, on the basis of such published reports, that iron transport may be influenced by metabolic energy. However, not all such communications are in agreement.

The role of metabolic energy in iron transfer is further considered in Chapter VII. In this section experimental results which form part of this thesis are presented and discussed.

II. The Intracellular Pathway of Iron Transport

a) Mucosal Uptake

Studies in rats have provided evidence for a process of mucosal uptake which is separate from subsequent release to serosal surface in vitro, or to portal blood in vivo (Manis and Schachter, 1962 a; Manis and Schachter, 1962 b). Uptake at the mucosal surface was rapid, increased in proportion to iron in the ambient medium and extended down the length of the bowel. Contrastingly, serosal transfer was much more specific in that it was selective for

divalent iron, was sharply localised to the duodenum and appeared to be the rate limiting step.

In dogs, a similar two-step process is thought to take place (Stewart and Gambino, 1961).

In man, investigation has also indicated the presence of a two-step transfer process. The first movement of iron being from the gastrointestinal lumen to the mucosal cells, and this is followed by transfer from the cells to the plasma (Hallberg and Sölvell, 1960 b).

A consideration of these publications indicate that a distinct stage of mucosal uptake exists. It remains uncertain as to what terminates this phase, but it is possible that the formation of non-absorbable complexes within the intestinal lumen may be a factor (Duthie, Code and Owen, 1962).

b) Mucosal Transfer

Considerable interest is currently centered upon this aspect of iron metabolism in which two distinct patterns of transfer can be recognised. Thus, the iron may rapidly enter the plasma (or appear in the serosal pouch), or be sequestered within the cells lining the gastrointestinal tract. The latter fraction may either enter the

plasma at a later stage or be lost when these cells exfoliate. Each pathway will be considered separately.

i) The Phase of Rapid Transfer

Although it is generally agreed that such a stage can be recognised, there is much less agreement on the detailed mechanism. Based upon studies with everted gut sacs in vitro and duodenal loops in vivo, Manis and Schachter (1964) have proposed one model to describe the molecular events underlying the regulation of iron absorption. These workers believe that normally a small endogenous ferrous iron pool and an insignificant ferric mucosal iron pool are present within the cells. In the course of active transport divalent iron is taken up into the ferrous pool, which turns over rapidly, and this iron is then either transported towards serosal surface or stored in the trivalent pool.

An alternative model has been proposed by Brown and Rother (1961; 1963 a; 1963 b). These workers isolated iron:glycine and iron:serine complexes in relatively high concentration during the early rapid phase of transport. They showed that these persisted for a longer time than normal in iron-deficient rats absorbing increased quantities of mineral. Based on such observations it was postulated that the iron chelates represented the rapid transfer intermediates.

In an attempt to further define the process, we have also studied this mechanism and likewise observed that a high concentration of activity appeared in a non-protein fraction shortly after the administration of labelled iron, and that it declined rapidly over the next two hours. In addition, some of this activity appeared to be attached to transferrin - a finding of doubtful significance since the iron might have attached to contaminating plasma during initial separation procedures. These results indicate that this fraction was probably concerned in the initial phase of absorption, although no evidence was obtained to support the conclusion that the iron is complexed with either glycine or serine (Charlton, Jacobs, Torrance and Bothwell, 1965). Manis and Schachter (1965) have similarly been unable to isolate these aminoacid complexes during iron absorption.

Clearly then, a non-protein fraction is intimately associated with the phase of rapid transport, and although certain of its characteristics have been defined, the exact composition and the valency state of iron in this compartment remains uncertain.

ii) The Phase of Slow-Release

The behaviour of that fraction of iron which is not immediately transported to the serosal surface in vitro or to the portal

radicles in vivo, has been intensively studied. Here again, there is some divarication of opinion as to both its nature and function.

Manis and Schachter (1964) have indicated that their divalent pool exists in equilibrium with a mucosal trivalent pool. The latter is thought to be available for subsequent transfer into the blood stream although at a slower rate than the divalent iron. Significant amounts of iron in this form would be lost with the cells exfoliating at the tips of the villi. Furthermore, during increasing iron loads the trivalent pool expands, mucosal uptake is decreased and transport to serosa is reduced. This latter effect or "self-inhibition" was thought to be a primary response induced by the iron itself. Corresponding changes, which would likewise tend to limit iron absorption in the face of excess mineral, were observed with duodenal loops following iron prefeeding.

Further studies on this "storage" fraction have demonstrated that it is strongly protein bound. However, the small quantities of ferritin isolated under these conditions were not thought to be significantly involved in regulating iron absorption (Manis and Schachter, 1964). On the other hand, experiments carried out in intact rats, using small physiological doses of iron as ferrous sulphate (Brown and Rother, 1963 a; Brown and Rother, 1963 b), have

led these authors to suggest that the iron-binding protein within the cells is distinct from ferritin.

In contrast to the above opinions, a considerable literature is accumulating which has once again focused attention on the role of ferritin in iron absorption. Originally, it was demonstrated that human subjects with iron deficiency absorbed increased quantities of radioactive iron when compared to normals (Hahn et al., 1943). This group of workers suggested that physiological saturation of a mucosal acceptor of iron prevented absorption of "unneeded" mineral. They suggested that this "mucosal block" was mediated by increasing intracellular formation of ferritin. Evidence to support this suggestion appeared to have been produced by Granick (1946 a) when it was demonstrated that, following pharmacological iron loading, large amounts of ferritin could be demonstrated in these mucosal cells.

Subsequent studies have indicated that the absolute block to mucosal absorption originally postulated is unlikely to be correct since it can be overwhelmed by the administration of large amounts of iron (Smith and Pannacciulli, 1958; Bothwell and Isaacson, 1962). Furthermore, even in the presence of iron excess, a portion of any small dose ingested is absorbed (Bothwell, van Doorn-Wittkamp, Du Preez and Alper, 1953; Pirzio-Biroli and Finch, 1960). In

humans ascorbic acid increases the absorption of food iron (Moore and Dubach, 1951; Brown, Dubach and Moore, 1958), a situation not likely to increase the actual requirements of this metal (Crosby, 1963).

Further doubt was cast on the original theory since ferritin, although demonstrable in gut cells after the feeding of very large doses of iron (Granick, 1946 a; Gabrio and Salomon, 1950; Heilmeyer, 1958), could not apparently be isolated after small quantities were ingested (Brown and Rother, 1963 a; Brown and Rother, 1963 b; Beutler et al., 1963). However, we have been able to demonstrate the presence of ferritin at physiological dosage level (Charlton, Jacobs, Torrance and Bothwell, 1963; Charlton et al., 1965). In addition there is electron microscopic evidence for the presence of this substance within the gut cells (Hartman, Conrad, Hartman, Joy and Crosby, 1963). What then is the role of this iron-protein complex in the absorption of iron from the gastrointestinal tract? Some insight into the function of ferritin in this process can be obtained by studying its behaviour under various circumstances. Thus, during the first hour following the administration of radioiron there is a progressive rise in the percentage bound to ferritin, with a corresponding fall in the non-ferritin fraction. Since most of the mucosal activity is attached to ferritin in the later phases, it seems probable that this complex is

not directly concerned with the transfer of iron across the cell. Results in iron-deficient animals support this interpretation, since a very rapid transfer of large percentages of the administered dose may be observed, although little labelled ferritin is present in the mucosa (Charlton et al., 1965). In a previous study (Brown and Rother, 1963 b) a build-up in a labelled fraction, precipitable with perchloric acid, was observed during the first hour in normal animals. Although these workers did not believe that the fraction represented ferritin, our results indicated close correlation between the perchloric acid and ferritin fractions (Charlton et al., 1965).

The ultimate fate of iron sequestered within mucosal cells appears to depend upon several factors. Using small doses of iron Wheby and Crosby (1963) have produced evidence that some of the iron may feed gradually into the body, and similar findings have been obtained by ourselves (Charlton et al., 1965). However, it was not possible to show the late absorption of mucosal activity when larger doses were given, or when small doses were given to animals that had recently received parenteral iron. Thus, two hours after the administration of radioiron, a mean of 35 percent of the administered activity was in the mucosa and 3.3 percent in the remainder of the carcass. By 24 hours mucosal radioactivity had dropped to negligible

levels, whereas the mean activity in the carcass was only 4.7 percent. It is clear therefore that mucosal iron must have been lost into the lumen of the gut. Theoretically this could occur either by extrusion of ferritin from the mucosa or via exfoliation of the intestinal cells. Conrad and Crosby (1963) have strongly supported the second possibility: their studies were carried out using radio-autographs of rat intestinal mucosa at various intervals after oral administration of labelled iron. These workers demonstrated both the sequestration of radioactive iron within the mucosal cells and the progress of the labelled cells towards the tips of the villi.

Based on such observations a mucosal storage mechanism can be envisaged which significantly influences iron absorption. Thus, iron taken up from the intestinal lumen into the absorbing cells in excess of body requirements is trapped within the mucosa, and discarded when these cells exfoliate (Conrad, van Hoek and Crosby, 1961). It has been postulated (Conrad and Crosby, 1963) that the sequestered iron is in the form of ferritin and further evidence is available in support of this suggestion (Charlton et al., 1965). This mechanism appears to function well when small amounts of iron are presented to the mucosa. However, the percentage of mucosal activity precipitable as ferritin is much lower with large doses,

suggesting that this process is only effective when iron intake is within the physiological range.

A number of factors have been recognised which may influence iron absorption and it is of interest to consider how some of these observations can be reconciled with the cellular regulatory mechanism outlined above.

III. Iron Prefeeding

Rats pretreated with oral iron absorbed less of this element than did control animals both in vitro, and when studied with duodenal loops in vivo (Manis and Schachter, 1964). In addition, data obtained using intact rats have demonstrated that mucosal behaviour could be altered by manipulating dietary iron content (Bannerman, O'Brien and Witts, 1962). These findings have been confirmed (Charlton et al., 1965) and evidence has been presented (Pollack, Kaufman and Crosby, 1964 b) that the changes in absorption were not related to alterations in the half-time or serum iron level when compared to a control group. These reports are supported by the gradual increase in absorption which takes place over several days when rats are switched from a stock diet to one low in iron. Such observations are compatible with the steady replacement of the absorbing mucosa by new cells

"conditioned" during their development, in some ways not yet established, by the altered iron content of the diet (Charlton et al., 1965).

The applicability of these results to human iron metabolism (Rush, Figallo and Brown, 1965) has been tested. In these studies no effect could be attributed to the low iron diet per se. It was suggested that the discrepancy between normal human subjects and rats lies in the suppressive effect on iron absorption due to the high iron content of commercial rat diets.

IV. Body Iron Stores

A decrease in stores results in enhanced absorption of iron salts (Hahn et al., 1943; Dubach et al., 1948; Bothwell et al., 1958), of iron administered with food (Pirzio-Biroli et al., 1958) and of labelled food iron (Moore, 1955). Of significance is the fact that subjects who are otherwise normal, but have increased absorptions, often give a history of having been blood donors in the past (Steinkamp et al., 1955).

It is of some interest that a direct reduction in the content of the liver iron store by partial hepatectomy in the rat did not cause an increase in iron absorption (Weintraub, Conrad and Crosby, 1964 a);

a similar observation having been reported previously (Venkatachalam, Brading, Kaldor and George, 1957). Conversely, an increased iron absorption has been reported as a transient feature following partial hepatectomy in the mouse. This effect possibly reflects proliferation of liver tissue - an effect analagous to erythroid marrow hyperfunction (Mendel, 1964). These conflicting results are likely to be due to the different postoperative times at which the absorption studies were performed. However, it remains just possible that the reduction of storage iron in the first of these experiments was of insufficient magnitude to effect iron absorption quantitatively.

When iron stores are increased, on the other hand, there is a corresponding reduction in iron absorption (Bothwell et al., 1958; Pirzio-Biroli and Finch, 1960). In at least one study it has been demonstrated that these variations in absorption are unrelated to haemoglobin, plasma iron and transferrin levels (Pirzio-Biroli and Finch, 1960).

How then can these various observations be explained on the basis of current knowledge, since it seems clear that the cells of the iron-absorbing portion of the gastrointestinal tract normally maintain iron balance with elegant precision (Conrad, Weintraum, and Crosby, 1964).? Let us consider, at a theoretical or speculative

level, a mechanism when stores are normal, expanded or depleted.

In normal animals the newly formed epithelial cells are variably saturated with intrinsic iron, the quantity depending upon such factors as the state of the body repletion. These young cells are constructed to contain a functional ferritin apparatus which is capable of synthesizing apoferritin, and thus incorporating iron into ferritin (Crosby, 1963). As this cell population grows out along the villus each individual cell will accept a portion of the iron present in the lumen of the bowel, the quantity being governed by its own iron content. "Needed" iron is then received from the intestinal cell by the body, while that which is not "needed" remains within the mucosa, probably as ferritin, to be lost when the cells exfoliate at the end of their life-span.

During stages of iron overload the mucosal cells of the intestine are heavily laden with body iron and this restricts entry of dietary iron into the body. The small quantity of food iron absorbed by iron-loaded animals is less than the amount lost by desquamation of iron-laden intestinal cells. In time, this negative iron balance would reduce the body stores to normal levels (Crosby, Conrad and Wheby, 1963; Conrad et al., 1964). This mechanism may, however, not be adequate to intercept all unneeded iron. Thus, following the ingestion of large doses of iron, accumulations may be found where ferritin bodies are not

usually observed (Hartman et al., 1963). This "acervation" or "intracellular accumulation" of the metal occurs in combination with nucleic acid or some other cytoplasmic component (Crosby, 1963) and further aids in the loss of extra iron when cells are lost into the lumen. A second possible defence mechanism is the interception of iron by tissue macrophages. While it is possible that these deciduous cells may participate in absorption (Gillman and Ivy, 1947) it is considered more likely that they work their way into the lumen taking their relatively large store of nonfunctional iron with them (Thirayothin and Crosby, 1962; Crosby et al., 1963).

At the other extreme, when body stores are depleted, little iron is incorporated into these cells during their formation. This state permits relatively uninhibited passage of dietary iron into the body. Conservation is also effected since only minute amounts of this metal, the physiologically active iron, would be lost when the cells slough off the villus.

V. Transferrin and Plasma Iron Levels

In 1947 Laurell suggested that the transferrin level and its relative saturation with iron might influence absorption. This

opinion has received some support from the observations of Sölvell (1960).

On the other hand, there is considerable evidence that iron absorption is not controlled directly by alterations in plasma iron or transferrin concentrations (Dubach et al., 1948; Stewart et al., 1950; Yuile, Hayden, Bush, Tesluk and Stewart, 1950; Bothwell et al., 1958; Greenberg, Wong, Miller, Scarlata and Chalmers, 1960; Moore, 1961; Wheby and Jones, 1963; Pollack, Balcerzak and Crosby, 1963; Conrad et al., 1964; Wheby and Umplierre, 1964; Charlton et al., 1965).

However, there is little doubt that, while no immediate effect on absorption can be detected paralleling the plasma iron, this pool significantly modifies iron absorption. Thus, in rats maximal depression was observed 18 hours after intravenous iron (Charlton et al., 1965) although parallel studies had indicated that the percentage saturation of circulating transferrin was maintained at high levels only for the first 12 hours of the study. It is thus possible, though not proven, that the changes in mucosal behaviour may be related to this environmental factor. Likewise, absorption was demonstrated to be least at the time when a parenteral dose of iron

was maximally concentrated within the intestinal epithelium (Conrad et al., 1964). It seems likely, therefore, that the subsequent behaviour of mucosal cells is conditioned during their developmental period in the crypts of Lieberkühn (Conrad et al., 1964).

On this basis, any manipulation of the cellular environment must be followed by a latent period during which "conditioned" cells move out into their functional position on the villi. Our recent observations (Charlton et al., 1965) are compatible with this thesis, since the observed changes in absorption correlate well with the turnover rate of intestinal cells (Messler and Leblond, 1960).

VI. Erythropoiesis

There is a decrease in iron absorption when erythropoiesis is depressed (Bothwell et al., 1958; Reynafarje, Lozano and Valdivieso, 1959; Krantz, Goldwasser and Jacobson, 1959).

The opposite effect is observed when erythropoiesis is stimulated either by bleeding or cobalt (Bothwell et al., 1958) or by haemolytic agents (Stewart, Vassar and Stone, 1953; Chappelle, Gabrio, Stevens and Finch, 1955; Bothwell et al., 1958). Similarly, iron overload can develop in subjects with long-standing haemolytic anaemias (Bothwell and Finch, 1962). It is interesting that the

absorptive response to increased blood formation may be modified by the size of body iron stores (Bothwell et al., 1958), however, control is not absolute since haemochromatosis may occur in such individuals. In this regard it has been suggested that preferential utilization of iron by the bone marrow reduces sequestration in the gut, resulting in increased absorption until sufficient mobilization from stores establishes a new state of iron equilibrium (Conrad et al., 1964). The exact manner in which this effect is mediated remains uncertain, but of considerable relevance is the observation that the plasma iron turnover, which closely parallels rate of erythropoiesis, influences the intestinal iron content. It is possible that this in turn determines the ability of these epithelial cells to absorb iron from the lumen (Weintraub, Conrad and Crosby, 1964 b; Weintraub, Conrad and Crosby, 1965).

Similarly, it has been demonstrated that the hormone erythropoietin is capable of enhancing iron absorption, but this property appears to be indirect and dependent upon acceleration of red cell production (Mendel, 1962).

VII. Hypoxia

It is much more difficult to understand the observation

(Mendel, 1961) that under conditions of hypoxia, iron absorption may be increased independently of increased utilization by the marrow. It is not clear whether this is related to a direct effect on the mucosa, or is a reflection of the many changes induced by hypoxia elsewhere in the organism. Under these circumstances it would be important to consider alterations in serum iron and transferrin levels (Colehour, Borsook and Graybiel, 1957), alteration in iron-containing enzymes and other metabolites (Criscuolo, Clark and Mefford, 1955; Tappan, Reynafarje, Potter and Hurtado, 1957) and the relationship of oxidative metabolism to internal movement of iron (Sölvell, 1960).

Conclusion - The Mucosal Factors

The absorption of iron from the gastrointestinal tract is complex. There is some evidence available to suggest that it is an energy dependent process, but further study is necessary to clarify this point.

Iron absorption is thought to take place in two distinct stages. It is possible to identify a phase of mucosal uptake from the lumen, which can be separated from subsequent transfer. This latter mechanism appears to have two components. Thus, iron may rapidly cross the cell and appear in the portal blood, or it may be diverted to an intracellular storage pool of ferritin. It is the fate of the iron

within this transfer system that will determine the quantity of mineral that is ultimately absorbed. The behaviour of newly absorbed iron within the gut cells is thought to depend upon their complement of intrinsic or "messenger iron", the latter reflecting the recent state of ferrokinetics. Thus, body iron not in demand for erythropoiesis enters the intestinal cells during their formation in the crypts, the actual amount depending upon such variables as the level of plasma iron turnover, size of stores and diet. In this way a delicate iron balance can be maintained provided that iron-load remains in the physiological range.

D. SUMMARY

A number of different luminal and cellular factors influence the absorption of iron from the gastrointestinal tract. In considering these, attention has first been directed to the arguments which suggest that the upper small bowel is the area most intimately involved in normal iron transfer. Then, since most of the published information relates to the absorption of simple iron salts, these data have been reviewed in terms of dose, valency and the various substances which either enhance or depress their absorption.

In view of the fact that the detailed pathway for the

absorption of dietary iron remains obscure, the literature pertaining to this area has been divided into two sections. In the first, the effects of administering food together with simple salts has been outlined, while in the second, the absorption of dietary iron itself is considered.

In concluding the review of luminal factors, the influence of the various gastrointestinal secretions upon iron absorption has been discussed.

With regard to cellular factors, the possible influence of metabolic energy has been noted. A hypothetical mechanism for the mucosal regulation of iron absorption is described in terms of such variables as body stores, erythropoiesis and plasma iron turnover.

Certain of the luminal and cellular factors which may influence iron absorption have been studied, both in vivo and in vitro, as part of this thesis.

The original data obtained in these investigations are presented and discussed in Chapter IV

(The Role of Gastric Secretions in Iron

Absorption), Chapter V (The Effect of Alcohol

on Iron Absorption), Chapter VI (Iron Overload

in Bantu Subjects - the Availability of Iron
in Bantu Beer) and Chapter VII (In Vitro
Studies - including the influence of metabolic
energy on iron transfer).

CHAPTER IV

THE ROLE OF THE GASTRIC SECRETIONS IN IRON

ABSORPTION

The role of the gastric secretions, and particularly hydrochloric acid, on iron absorption remains controversial although this problem has been studied for many years. The results of early clinical investigations in which iron absorption was measured by haemoglobin and red cell regeneration in iron deficient patients, suggested that these secretions potentiated the response to therapeutic doses of inorganic iron salts (Mettier and Minot, 1931; Minot and Heath, 1932; Bethell et al., 1934), and also that they influenced the absorption of food iron (Mettier et al., 1933; Kellogg and Mettier, 1936). Similarly, Barer and Fowler (1937) demonstrated a defective retention of dietary iron in achlorhydric subjects using a chemical balance technique.

By way of contrast, the application of iron absorption curves to an investigation of this problem showed that large doses of inorganic iron salts were not significantly affected by achlorhydria (Moore et al., 1939; Smith, 1952; Grace et al., 1954).

More recently various isotopic techniques have been applied to the re-evaluation of this problem, but these studies have likewise

yielded conflicting results. Thus, Dubach et al., (1948) using a combination of faecal excretion and red cell utilization methods reported a normal absorption of large doses of ferrous salts in three cases of Addisonian pernicious anaemia in relapse. Similarly, Biggs et al., (1961) reported that the absorption of haemoglobin iron was normal when this was tested in achlorhydric subjects while Callender et al., (1957) concluded that gastric secretion did not influence the absorption of haemoglobin iron when this was examined in normal subjects. In addition (Pirzio-Biroli et al., 1958) found the absorption of a tracer amount of radioiron to be normal when given with a standard meal to four subjects with Addisonian pernicious anaemia in remission. Similarly, Moore and Dubach (1951) were unable to enhance the absorption of radioiron biosynthetically incorporated into eggs, when this was fed to two achlorhydric subjects, even when hydrochloric acid was added; a similar finding being recorded in two patients having untreated hypochromic anaemia. It is possible, however, that the low absorption in these cases may have masked differences which would have been apparent at higher levels.

In contrast, Williams (1959) demonstrated defective absorption of radioiron incorporated into bread when this was administered to

four iron deficient patients with histamine fast achlorhydria. Other workers (Goldberg et al., 1963) found a significant depression in the absorption of a tracer quantity of iron administered with a standard meal to iron deficient, achlorhydric subjects. Similarly, Cook et al. (1964) reported a significant depression in the absorption of a tracer amount of radioiron, when this was administered with white bread to nine non-anaemic subjects with achylia gastrica. In this study the patients were compared to a group of normal matched controls. These workers furthermore demonstrated that in six patients with achylia gastrica, the addition of normal gastric juice significantly increased the absorption; the addition of neutralised gastric juice produced little effect.

The importance of gastric secretion in iron absorption may also be inferred from data obtained in patients following partial gastrectomy where a defect has been demonstrated in the absorption of iron from a standard meal (Pirzio-Biroli et al., 1958; Stevens et al., 1959), from eggs (Chodos et al., 1957) and from bread (Williams, 1959; Choudhury and Williams, 1959). This defect could be normalised under certain circumstances by acid (Moeschlin et al., 1965) and was thought to have both a secretory and mechanical component (Turnbull, 1965).

The present investigation was undertaken to further define the role of hydrochloric acid in iron absorption and also because studies in vitro had indicated that the pH of the luminal contents had a marked effect on the absorption of iron salts. In order to overcome difficulties resulting from individual variations in absorption the double isotope technique was used; the absorption of iron administered with and without acid being compared in the same subject.

MATERIAL AND METHODS

Thirty subjects with Addisonian pernicious anaemia who had received parenteral vitamin B₁₂ and who were in full clinical and haematological remission were studied. The absence of free hydrochloric acid was established in each subject by the augmented histamine test (Kay, 1953). This study was carried out in two sections. In the first the absorption of iron from water was compared to its absorption from acid. Twenty-four subjects were studied. In this phase of the investigation a standard dose of 5 milligrams of iron was administered by nasogastric tube to the fasting subjects over a period of one hour on two consecutive mornings. The iron was made up in 300 millilitres of water on the one day and in the same volume of 0.05 N hydrochloric acid on the next day. This quantity of acid was equivalent to 15 mEq HCl, which is slightly

more than half the mean output of normal subjects following maximal histamine stimulation (Marks, Bank, Louw and van Embden, 1962). In each case the iron was labelled with 5 microcuries Fe^{59} on the one occasion and 125 microcuries Fe^{55} on the other.

In 10 subjects the iron was given as ferric chloride, in seven subjects as ferrous ascorbate (50 milligrams ascorbic acid being added to the ferric chloride immediately prior to administration), and in the remainder as biosynthetically labelled haemoglobin. The radioactive haemoglobin was prepared from rat blood 14 days after the intravenous administration of a tracer quantity of radioiron.

In the second part of this study the effect of gastric juice of iron absorption was compared to that of hydrochloric acid. Six subjects were studied. On one morning the fasting patients received 5 milligrams of ferric iron together with gastric juice obtained from normal subjects following maximal histamine stimulation. On the following morning the same dose of iron was given with an equal volume of hydrochloric acid of corresponding normality.

Samples of blood were collected from all subjects 14 days after the second dose. Aliquots were then digested, electro-

plated onto copper discs, and differentially counted for the two isotopes. The relative percentage absorption of Fe^{55} and Fe^{59} being calculated from a blood volume of 70 millilitres per kilogram in Johannesburg and assuming that 80 percent of the absorbed dose was in circulation at 14 days.

RESULTS

The mean absorption of ferric chloride in water was considerably less than when it was administered in 0.05 N HCl (2.2 percent as compared with 9.2 percent) (Table IV). Results were less striking with ferrous ascorbate, since acid only caused a marked increase in 3 of the 7 subjects (Table V). The mean absorption with water in this group was 11.37 as compared with 20.6 with the acid. The absorption of haemoglobin iron was not enhanced when hydrochloric acid was present, and in fact, there was suggestive evidence that it caused some depression of absorption in 6 of the 7 subjects (Table VI). The mean absorptions with haemoglobin were 13.8 percent with water and 9.5 percent with acid. These results are summarised in Figure 4.

The results from the second part of this study are set out in Table VII. The mean absorption of ferric chloride from

TABLE IV

Haematological Data and the Results of Iron Absorption Tests in Subjects with Histamine Fast Achlorhydria

Sex	Haemo- globin	P.C.V.	Plasma Iron	U.I.B.C.	Transferrin Saturation	Iron Absorption with acid	Iron Absorption with water
	Grams %	%	micrograms %	micro- grams %	%	%	%
M	11.4	36	58	270	17.6	12.7	0.6
M	15.1	47	61	210	22.5	2.8	0.7
F	13.6	-	85	206	29.2	5.0	3.2
M	14.1	40.5	44	154	22.2	1.5	0.7
M	15.3	49	64	256	20.0	21.0	11.0
M	15.5	48	112	290	27.8	4.4	0.2
F	16.6	50.5	67	255	20.8	1.8	0.2
M	16.4	48	73	350	13.9	27.0	2.0
M	14.9	45.5	110	363	23.2	13.9	2.1
F	15.1	47	63	318	16.5	2.4	1.9
Mean	14.8	45.7	73.7	277	21.4	9.2	2.3

The effect of 15 milliequivalents of hydrochloric acid on the absorption of 5 milligrams iron administered as ferric chloride

TABLE V

Haematological Data and the Results of Iron Absorption Tests in Subjects with Histamine Fast Achlorhydria

Sex	Haemo- globin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption with acid %	Iron Absorption with water %
F	11.7	-	66	152	30.2	63.1	28.7
M	16.4	48.5	74	261	22.0	7.3	5.5
M	13.3	43.0	80	284	22.0	19.8	13.8
F	12.8	40.0	100	419	20.0	15.0	15.5
F	10.9	34.0	52	311	14.0	24.4	7.4
F	10.8	34.5	31	200	13.0	5.0	1.4
F	14.9	45.0	70	361	16.2	9.4	7.3
Mean	12.9	40.8	68	284	19.6	20.6	11.3

The effect of 15 milliequivalents of hydrochloric acid on the absorption of 5 milligrams of iron administered as ferrous ascorbate

TABLE VI

Haematological Data and the Results of Iron Absorption Tests in Subjects with Histamine Fast Achlorhydria

Sex	Haemo- globin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption with acid %	Iron Absorption with water %
M	15.9	43.5	99	192	34.0	3.7	9.5
F	15.9	47.0	53	143	27.0	4.9	12.2
F	14.5	46.0	114	184	38.2	9.2	13.2
M	16.4	50.0	182	151	54.6	7.4	11.5
F	15.5	50.0	162	200	44.7	2.2	4.5
F	8.9	27.5	80	289	21.6	9.8	19.9
M	17.1	52.0	85	268	24.1	29.5	25.5
Mean	14.9	45.1	110.7	203.9	34.9	9.5	13.8

The effect of 15 milliequivalents of hydrochloric acid on the absorption of 5 milligrams of iron administered as haemoglobin.

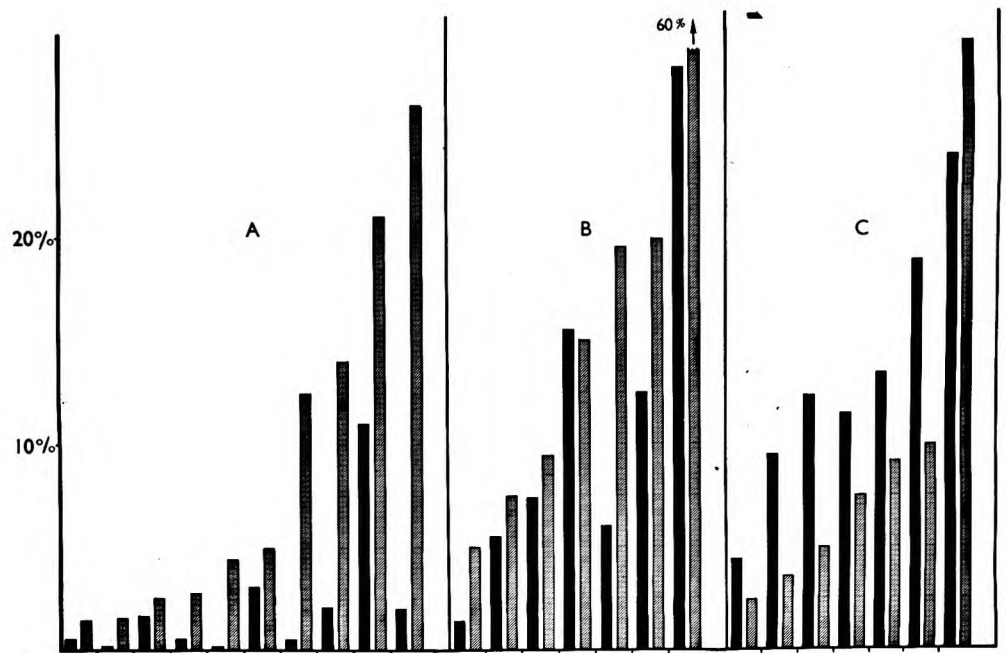


FIGURE 4

A comparison of the percentage absorption of 5 milligrams iron dissolved in 300 millilitres water (Black) with the same dose of iron in 300 millilitres 0.05 N hydrochloric acid (cross-hatched). The iron given as ferric chloride (A), ferrous ascorbate (B) and as haemoglobin (C).

TABLE VII

Haematological Data and the Results of Iron Absorption Tests in Subjects with Histamine Fast Achlorhydria

Sex	Haemoglobin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption with gastric juice %	Iron Absorption with acid with acid %
F	16.4	36.5	95	293	24.5	3.1	1.3
M	13.9	40.5	128	275	31.7	7.8	4.1
F	18.7	39	147	185	44.2	0.7	0.9
F	16.5	39	151	237	38.9	27.6	15.2
M	16.4	42	134	202	39.8	10.0	7.7
F	18.7	52	176	192	47.8	13.8	11.8
Mean	16.8	41.5	138.5	231	37.8	10.4	8.2

gastric juice was 10.4 percent as compared to 8.2 percent from the acid.

DISCUSSION

Although previous workers have demonstrated diminished absorption of ferric iron in achlorhydric subjects (Goldberg et al., 1963) they were unable to establish whether this was due to the absence of hydrochloric acid or to the associated mucosal changes (Ikkala and Siurala, 1964). The enhancement of ferric iron absorption caused by the addition of hydrochloric acid alone in the present study indicates that this per se, is the important factor.

Presumably the acid exerts its effect by keeping the ferric iron in solution until it reaches the absorbing area of the duodenum (Heath and Patek, 1937; Moore et al., 1939; Granick, 1949; Moore and Dubach, 1951; Gubler, 1956). The fact that the absorption of ferrous salts was influenced to a lesser extent by the presence of acid is quite compatible with this thesis, since it is recognised that ferrous ions remain in solution at a much higher pH than do ferric ions (Thorne and Roberts, 1954).

The results obtained with haemoglobin iron are of interest from two points of view. First, it was noteworthy how well haemoglobin iron was absorbed in achlorhydric subjects; and second,

it was apparent that hydrochloric acid did not potentiate absorption. Taken in conjunction with previous similar findings (Biggs et al., 1961) these results suggest that the absorption of haemoglobin iron is not dependent upon any gastric factor, and lend further support to the thesis that haemoglobin iron may be absorbed by a mechanism different from that which operates for inorganic salts. This proposition has resulted from several independent observations. These include the fact that the peak of plasma radioactivity occurs later after the giving of haemoglobin than it does after salts (Callender et al., 1957) and the finding that its absorption is not increased by ascorbic acid or depressed by food or phytate (Turnbull et al., 1962). It has been suggested that haemoglobin iron may be absorbed as a porphyrin complex (Turnbull et al., 1962) and that the porphyrin-iron bond is split within the mucosal cells, with the limiting steps in the absorption of haemoglobin iron being different in normal and iron deficient subjects (Hallberg and Sölvell, 1964). There is at present no evidence to support or refute this speculation.

The importance of gastric secretions in the absorption of dietary iron is rather more difficult to assess and it is doubtful whether the results obtained with inorganic salts can be directly extrapolated to the more complex mechanisms likely to be operative

in the absorption of food iron. Thus, while gastric secretions influence the absorption of some iron salts and certain forms of food iron it is difficult to separate with certainty the enhancing effect of the hydrogen ion from that due to other substances contained within the secretions such as pepsin and reducing agents.

In an attempt to obtain further information on the latter point an additional study was carried out. A standard dose of iron was given either with acid or gastric juice: the normality and volume on the two mornings being the same. In these experiments no significant difference could be demonstrated on the two occasions. These observations lend further weight to the suggestion that the hydrochloric acid in gastric juice is, per se, the important factor.

SUMMARY

A double isotope technique has been used to study the absorption of a 5 milligram dose of iron in subjects with histamine fast achlorhydria. The addition of hydrochloric acid to ferric iron markedly facilitated absorption, while a modest increase only was observed with ferrous salts. It is thought that this is a physical phenomenon dependent upon the different solubilities of these two ions at a given pH. In contrast, the absorption of iron from haemoglobin

is not increased by the simultaneous administration of acid, and here the mechanism is possibly related to an entirely different pathway of absorption. No evidence was obtained to suggest that substances in gastric secretions, other than hydrochloric acid, significantly enhanced this process. It is emphasized that these data cannot be directly related to the physiological role of hydrochloric acid in the absorption of dietary iron.

CHAPTER V

THE EFFECT OF ALCOHOL ON IRON ABSORPTION

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

There are several possible reasons for these various associations. In the first place, an association between iron and alcoholic intake has recently been pointed out by MacDonald (1963), who has shown that most European and United States wines contain significant amounts of iron. (For example, the average iron concentration in French wines is 8.8 milligrams per litre, and in United States wines 4.9 milligrams per litre). A second possible

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

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

MATERIAL AND METHODS

The absorption of 5 milligrams of iron was assessed in 31 haematologically normal subjects and in four patients with histamine-fast achlorhydria. Because of the well-known variability in iron absorption from subject to subject, the double isotope technique was used in which each individual served as his own control. The iron was administered in a fasting state. On one day it was dissolved in 250 millilitres of water, and on the following day in 60 millilitres of

whisky or brandy, made up to a final volume of 250 millilitres with water. In half the experiments the order of administration was reversed. Isotope labelling was with 70 microcuries of Fe^{55} on the one occasion and with 5 microcuries of Fe^{59} on the other. In different experiments the iron was administered as ferric chloride, as ferrous ascorbate (with 50 milligrams of ascorbic acid), and as rat haemoglobin. Duplicate blood samples collected two weeks later were digested, electroplated onto copper discs, and differentially counted for the two isotopes. The relative percentage absorptions of the two doses were then calculated on the assumption that 80 percent of the iron absorbed from the gut was present in the red cell mass at two weeks, and that the blood volume was 70 millilitres per kilogram. Results were therefore corrected by a factor of 10/8.

RESULTS

The absorption of iron given as ferrous ascorbate was assessed in six normal subjects. The mean absorption was 18.8 percent in water and 17.5 percent in alcohol (Table VIII). There was also no enhancement of the absorption of haemoglobin iron in the presence of alcohol, the mean figures in eight subjects being 14.9 percent in water and 13.4 percent in alcohol. In contrast,

TABLE VIII

The Effect of Alcohol on the Absorption of 5 Milligrams of Iron Given
as (A) Ferrous Ascorbate and (B) Haemoglobin.

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

alcohol caused a marked increase in the absorption of iron given as ferric chloride. In 17 patients the mean absorption in alcohol was 22.6 percent, as compared with 4.1 percent in water (Figure 5). This effect could not, however, be demonstrated in four subjects with histamine-fast achlorhydria (a mean of 3.5 percent in water and 5.2 percent in alcohol).

DISCUSSION

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

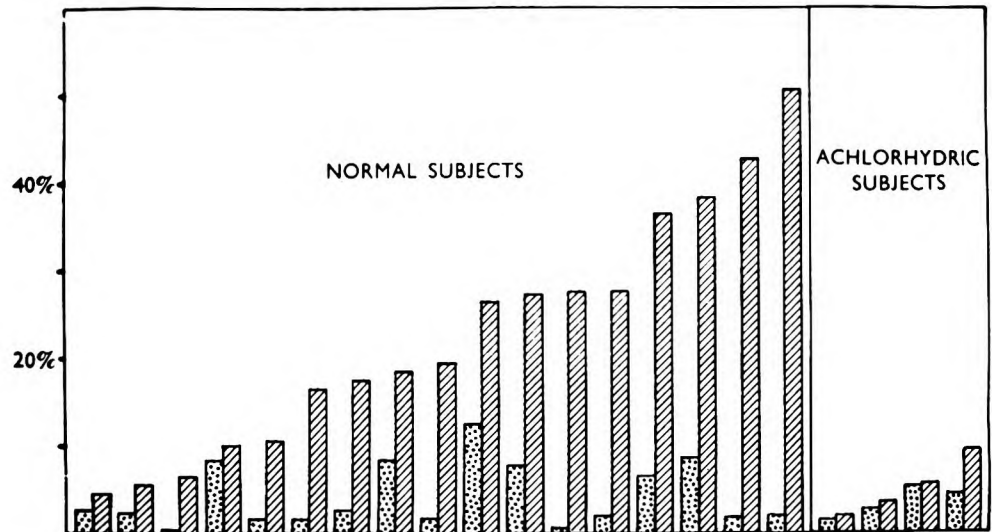


FIGURE 5

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

iron complex is absorbed in a manner different from simple iron salts (Turnbull et al., 1962).

In the present study alcohol was found to potentiate the absorption of ferric iron considerably, but to have no effect on the absorption of ferrous or haemoglobin iron. The similarity between these results and those obtained previously with hydrochloric acid suggested that alcohol might be exerting its effect by stimulating the production of gastric acid. The fact that the administration of alcohol did not increase the absorption of iron in four subjects with histamine-fast achlorhydria would be compatible with this hypothesis.

Viewed in this light the present findings may have some relevance for the pathogenesis of iron overload. Thus, it has been pointed out that many wines from different parts of the world contain significant amounts of iron, and it has been suggested that the high incidence of iron overload in alcoholic subjects is a result of the excessive iron intake (MacDonald, 1963). On the other hand, it should be appreciated that wines contain 10 to 20 percent alcohol, and it remains possible that the absorption of iron taken in this way is enhanced by gastric acid secretion.

These considerations do not exclude the possibility that other factors may contribute to the association between iron overload

and the excessive consumption of alcohol. Pancreatitis is common in alcoholics, and there is now good evidence that deficiency of the exocrine secretions of the pancreas is associated with excessive absorption of iron. This has been shown in animals after pancreatectomy (Taylor et al., 1931), ligation of the pancreatic duct (Taylor et al., 1935; Gillman, Gillman, Mandelstam and Gilbert, 1947; Kinney et al., 1950), ethionine-induced pancreatic damage (Kinney et al., 1955), and in patients with chronic pancreatitis (Davis and Badenoch, 1962). Furthermore, there is evidence that the increased absorption can be reduced by the giving of pancreatic extract (Kinney et al., 1950; Davis and Badenoch, 1962; Saunders, Bank, Airth and Williams, 1963). The fact that cirrhosis is common in alcoholics may also be of relevance, since it has been shown that some cirrhotic subjects absorb excessive amounts of iron even when the body stores are normal or increased (Conrad et al., 1962). It is, however, still not clear whether these effects are secondary to the cirrhosis itself or to associated pancreatic damage (Callender and Malpas, 1963). Lastly, alcoholics as a group eat suboptimal diets, and there are several experimental studies showing increased absorption of iron when the diet is low in certain nutrients but high in iron. These diets have included low phosphate (Hegsted et al., 1949), low protein, high fat (Kaufman et al., 1958) and choline-deficient

(MacDonald, 1960) regimes.

With all these factors contributing to the excessive absorption of iron it is not surprising that doubt has been cast on whether idiopathic haemochromatosis occurs as a distinct entity. The arguments against its existence have been presented by MacDonald (1961, 1963), who believes the condition to be a variant of alcoholic cirrhosis, occurring in subjects exposed to a high iron diet. Some support for this suggestion is provided by the fact that many patients presenting with the disease have a background of alcoholism (Sheldon, 1935; Finch and Finch, 1955). There is, however, a strong body of evidence, accumulated from many sources, which indicates the existence of a specific metabolic disorder in which excessive amounts of iron are absorbed from a normal diet.

Firstly, it should be emphasized that a large number of reported patients with the disease have not been alcoholics, and no other source of excessive dietary iron has been uncovered in these cases. Secondly, there are several family studies in which varying degrees of iron overload have been demonstrated in relatives of patients with haemochromatosis, thus supporting the concept of an inherited metabolic disorder (Bothwell, Cohen, Abrahams and Perold, 1959; Williams, Scheuer and Sherlock, 1962). The fact that

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

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

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

SUMMARY

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

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

CHAPTER VI

IRON OVERLOAD IN BANTU SUBJECTS

Studies on the Availability of Iron in Bantu

Beer

Varying degrees of iron overload occur in the majority of adult Bantu subjects living in South Africa (Gillman and Gillman, 1951; Bothwell and Finch, 1962). Although it is accepted that this overload may be the result of an increased absorption from the gut, there is still controversy as to why this should occur. Originally it was postulated that there was an alteration in the behaviour of the bowel mucosa induced by chronic malnutrition (Gillman and Gillman, 1951). More recently, however, it has been shown that the diet of the Bantu population contains large amounts of iron, and it has been suggested that this is a more likely cause of the tissue siderosis (Walker and Arvidsson, 1953). Although the major foodstuffs consumed by the Bantu population contain only moderate amounts of iron, most of the ingested iron is derived from the utensils which are used in cooking and in preparing fermented alcoholic beverages (Walker and Arvidsson, 1953).

The present study was undertaken to try to assess the amounts of iron currently being consumed by urban Bantu subjects in Johannesburg and to find out how much of this iron is absorbed from the

gut. In addition, it was hoped to gain some further understanding on the role of a high dietary intake on the pathogenesis of Bantu siderosis.

It was decided to use Bantu beer in all these absorption studies for a number of different reasons. In the first instance, although it would have been ideal to label the whole diet, this was not feasible. Secondly, analyses of home-brewed alcoholic beverages consumed by the Bantu have revealed high iron concentrations (Walker and Arvidsson, 1953), and in this regard there is suggestive evidence that the iron overload in these subjects is closely related to their drinking habits (Seftel et al., 1961). Finally, in view of the previous study, it was of particular interest to try to establish whether the alcohol content of the Bantu beer significantly enhanced iron absorption.

MATERIAL AND METHODS

Clinical Material

The Bantu subjects studied included healthy volunteers, patients in a paraplegic unit and convalescent surgical patients. All subjects were men, and none showed any evidence of active infection at the time of the study. In addition, a control group of white male

volunteers were also studied. The mean age of the Bantu subjects was 31 years (range 18 to 60) and of the white subjects 23 years (range 21 to 25).

Chemical Methods

The iron content of the stools was measured by leaving them for seven days in a 1:2 mixture of concentrated nitric and sulphuric acids. Each day the mixture was stirred. Finally, duplicate aliquots were wet ashed, and the iron content was measured using the thioglycollic acid method (McCance, Widdowson, Shackleton, 1936).

The iron content of alcoholic beverages was measured by a modification of the method of Brückmann and Zondek (1939) in which a 4 millilitre aliquot was mixed with 4 millilitre saturated sodium pyrophosphate and 4 millilitres 20 percent trichloroacetic acid. The sample was then maintained at 80 degrees Centigrade in a water bath for 10 minutes. After cooling, the mixture was filtered, and estimation of iron content was carried out on an aliquot of the filtrate using the thioglycollic acid method. Since the beer was coloured, each reading was made against a blank containing an aliquot of the sample and all reagents except thioglycollic acid. (Preliminary studies indicated that this method gave results which

were in good agreement with those obtained by wet digestion of beer samples).

Radioactive Measurements

Tracer amounts of ferric chloride labelled with Fe^{59} were added to samples of fermented Bantu beer (iron content 2.0 milligrams per 100 millilitres) prepared by the municipal authorities, and the beer was then allowed to stand overnight before being used. Although the label was not a physiologic one it was thought that valid results could be obtained by this method since preliminary studies showed that more than 80 percent of the iron in the various types of alcoholic beverage consumed by the Bantu population is in the ionic form. In a second series of studies, carrier ferric chloride was added with the tracer to give a final iron concentration in the beer of 6.25 milligrams per 100 millilitres.

Because of the well-known variability of iron absorption from subject to subject the double isotope technique was used in which the availability of iron in Bantu beer was compared in each individual subject with that of a simple iron salt. On the first day 400 millilitres of beer, labelled with 5 microcuries Fe^{59} , was given, and on the second day a similar dose of iron, labelled with 75 microcuries Fe^{55} ,

was administered either as ferric chloride or ferrous ascorbate. Blood samples collected two weeks later were digested, electroplated onto copper discs and differentially counted for the two isotopes. The relative percentage absorptions of the two doses were then calculated on the assumption that 80 percent of the iron absorbed from the gut was present in the red cell mass at two weeks and that the blood volume was 70 millilitres per kilogram. Results obtained were, therefore, corrected by a factor of 10:8. (Although it has been established that approximately 80 percent of iron entering the plasma is used for red cell production, the figure is somewhat lower in subjects with iron overload. It is, however, rarely below 60 percent in Bantu subjects (Bothwell and Kramer, personal communication), so that levels of radioactivity in red cells should still provide a moderately accurate measure of the percentage of radioiron absorbed from the gut).

RESULTS

Iron Concentrations in Alcoholic Beverages

Analyses were carried out on 169 samples of various types of alcoholic drinks, most of which were home brewed. Although there was a considerable variation (0.5 to 16.0 milligrams per 100

millilitres) the mean concentration for the whole group was high (3.8 milligrams per 100 millilitres) (Table IX). The lowest concentrations were found in beer prepared by the municipal authorities and in distilled liquors.

Drinking Habits of Urban Bantu Subjects

Two hundred Bantu adults attending an outpatient clinic were questioned on their drinking habits with special reference to the relative consumption of home-brewed beverages and those prepared by the municipal authorities. With a knowledge of the adult population served by the clinic and the amount of municipally prepared beer being supplied to the area, it was possible to make a rough estimate of the quantities of fermented beverages being consumed by the urban Bantu population. Results obtained in this way indicated a mean consumption in men of about 13 litres per week, with a ratio of municipally prepared beer to home-brewed beer of 2 to 1. This would suggest an average iron intake from beer of at least 50 milligrams per day.

Iron Content of Stools from Bantu Subjects

Iron concentrations were measured in the stools of a random group of male Bantu adults attending an outpatient clinic. The

TABLE IX

Iron Concentration in Alcoholic Beverages

Name	Ingredients	No. of Samples Analysed	Iron Concentration (mg./100 ml)		Mean pH
			Average	Range	
Bantu beer (municipal)	malt, degermed maize meal	50	1.9	1.1 - 3.8	3.7
Bantu beer (home-brewed)	malt, maize meal	14	8.2	0.8 - 15.0	3.8
Barborton Hops	sugar, malt, bread yeast, sugar, malt, hops	29	4.5	0.5 - 15.8	3.9
Pineapple	yeast, sugar, oats, bread, pineapple	27	5.1	0.6 - 16.0	3.9
Shimeyana Chillies	sugar, malt, potato yeast, sugar, malt, bread, ginger	16 12	2.9 7.5	1.0 - 15.0 0.8 - 15.3	3.8 3.5
Gin, Tot-Tot, etc.	distillates made from other drinks	8 13	5.3 1.0	1.0 - 10.5 0.0 - 5.0	4.1 5.9

mean concentration in stools collected on Monday mornings was 141 milligrams per 100 grams (range 28 to 330) as compared with 34 milligrams per 100 grams on Thursday mornings.

Availability of Iron in Bantu Beer

Fasting subjects were given 400 millilitres of Bantu beer, containing either 8 or 25 milligrams iron, and the percentage absorbed was compared in each subject with a similar dose of iron given as a simple ferric or ferrous salt. In both Bantu and white subjects the percentages absorbed were in the same range as those obtained with ferric chloride (Table X) and were about a third of those obtained with ferrous ascorbate (Table XI). On the whole, Bantu subjects tended to absorb less than did the white volunteer subjects, and this was particularly so at the 25 milligram dosage level.

DISCUSSION

Although siderosis of varying degrees is common in Bantu adults only about 20 percent show severe involvement, and most of these are middle-aged men (Bothwell and Isaacson, 1962). Previous calculations based on chemical analyses of organs obtained at autopsy suggest that the total quantity of iron in such subjects rarely

TABLE X

Comparison of Absorption of Iron in Bantu Beer and a Similar Dose of Iron Given as Ferric Chloride

Subject	Dose of Iron (mg.)	% Absorption	
		Beer	Ferric Chloride
Paraplegic male Bantu patients	8	2.6	0.4
		2.8	0.9
		2.3	1.0
		2.5	3.2
		6.8	1.4
		8.0	0.9
	Mean	4.2	1.3
Healthy white male volunteer subjects	8	2.9	8.3
		17.8	16.6
		1.0	3.6
		1.6	1.6
	Mean	5.8	7.5
Convalescent Bantu male surgical patients	25	2.0	0.4
		1.1	0.1
		1.6	0.4
		1.9	2.6
		0.8	0.8
		1.2	4.3
		2.1	1.4
		0.5	0.3
		10.8	4.8
		1.6	1.0
		1.4	0.3
		3.1	1.0
	Mean	2.3	1.5
Healthy white male volunteer subjects	25	2.6	5.6
		17.5	31.5
		2.4	2.5
	Mean	13.0	16.5
		8.9	14.0

TABLE XI

Comparison of the Absorption of Iron in Bantu Beer and a Similar
Dose of Iron Given as Ferrous Ascorbate

Subject	Dose of Iron (mg.)	% Absorption	
		Beer	Ferrous Ascorbate
Healthy Bantu male volunteer subjects	8	2.8	7.4
		1.6	0.8
		2.3	6.3
		2.3	5.5
		10.3	27.5
	Mean	3.9	9.5
Healthy white male volunteer subjects	8	11.3	19.6
		4.3	18.8
		4.0	17.8
		1.3	9.9
	Mean	5.2	16.5
Male Bantu paraplegic patients	25	0.9	2.0
		1.2	3.6
		0.9	2.8
		0.9	1.9
		1.0	10.1
		2.7	4.0
		2.0	7.7
	Mean	1.2	4.5
Healthy white male volunteer subjects	25	20.1	58.0
		6.5	15.3
		4.5	18.3
	Mean	10.4	30.5

exceeds 15 grams (Bothwell and Isaacson, 1962). The disorder in iron balance necessary to produce this degree of iron overload is not a large one. A mean absorption of 2 milligrams daily between the ages of 20 and 50 years would lead to a positive balance of approximately 1.4 milligrams daily in the male subject, which is enough to account for deposits of this magnitude.

In the present study an attempt was made to assess the amounts of iron present in the diet of urban Bantu subjects and to measure how much of this iron is available for absorption. The importance of Bantu beer as the major source of iron in the diet was evident from both questioning Bantu subjects on their drinking habits and from direct chemical analyses on samples of various drinks. The fact that almost all the iron in these drinks is ionizable indicates that it is introduced from the containers used for brewing.

In order to obtain more direct evidence of the high iron intakes in urban Bantu subjects, stools obtained from patients attending an outpatient clinic were analysed for their iron content. Results were severalfold greater than would be expected in a comparable white population. In addition, it was of interest that the mean concentrations of iron were much higher in stools collected from men on Mondays than in those collected on Thursdays (141

milligrams per 100 grams as compared with 34 milligrams per 100 grams). This difference probably reflects the weekend drinking habits of the subjects.

From these various observations it was concluded that the mean iron intake of urban Bantu men is probably in the range of 50 to 100 milligrams daily, although occasionally it may be a good deal higher than this. These estimates are in good agreement with previous calculations made by other workers (Walker and Arvidsson, 1953).

In assessing how much of the iron present in the Bantu diet is available for absorption, Bantu beer was chosen since it not only represents the major source but also contains the iron in a readily assimilable form. The results indicated that this iron is absorbed to roughly the same degree as is a ferric salt. The relatively low pH of the fermented drinks does not seem to potentiate absorption nor do other constituents have any depressing effect.

These data when viewed in the light of previous studies with alcohol suggest that, under these circumstances, the alcohol content of the beer did not enhance absorption. However, it should be noted that the type of beer tested had a very low alcohol content ($\pm 3\%$) and it remains possible that the more potent adulterated drinks consumed by many of these subjects may, in fact, enhance iron

absorption significantly.

The observation that the Bantu subject tended to absorb less than did white men is most probably due to the fact that the majority had some degree of iron overload since it has been shown that even a moderate increase in iron stores depresses absorption (Pirzio-Biroli and Finch, 1960). Another possible reason for the low absorptions in Bantu subjects was considered. At autopsy many Bantu subjects show deposits of iron in the mucosal cells of the upper portion of the gut and in the tunica propria. It therefore seemed feasible that a proportion of iron absorbed by siderotic Bantu subjects might be fixed in this intestinal pool for variable periods. To test this hypothesis a number of moribund Bantu patients were given 15 milligrams iron as Fe^{59} chloride at various times before death, and the radioactivity present in the duodenum and upper part of the jejunum obtained at subsequent autopsy was measured in a counter composed of a ring of Geiger-Müller tubes (Veall and Vetter, 1952). The mean percentage present in the gut in 12 patients who died between 8 hours and 12 days after the administration of radioiron was 0.4 (range 0 to 1.1). It was, therefore, evident that no pool of significant dimensions exists in this site.

Although the absorptions in Bantu subjects were low the

mean amount of iron retained from beer at the 25 milligram dosage level was 1.9 percent. When these figures are related to a daily consumption of between 50 and 100 milligrams it is apparent that the positive iron balance is of an order sufficient to account for the degrees of siderosis encountered in middle age. The fact that none of the Bantu subjects showed evidence of abnormally high absorption rates can be taken as good evidence that the siderosis so commonly found in these subjects is not the result of any intrinsic mucosal defect.

SUMMARY

A series of studies were carried out to assess the amounts of iron being consumed by urban male Bantu adults. It was found that the majority were taking between 50 and 100 milligrams daily, most of which was present in fermented alcoholic beverages.

The availability of iron in Bantu beer was measured in Bantu and white subjects. In both groups it was absorbed to the same degree as a ferric salt. On the whole, the percentage absorption was lower in the Bantu subjects, and this was probably due to the fact that body stores are greater than normal in the majority of them.

From the data obtained it would appear that the degrees of

siderosis present in middle-aged Bantu men can be explained on their high iron intake alone. No evidence was obtained in this study to support the possibility that the alcohol content of Bantu beer significantly enhanced iron absorption.

CHAPTER VII

IN VITRO STUDIES

INTRODUCTION

Much of the fundamental research into iron absorption has been carried out in vivo. It is obvious that such techniques approximate most closely to the physiological pathways since the absorptive capacity of the whole bowel is determined together with any modifications produced by its secretions.

On the other hand, a detailed examination of certain phenomena are more advantageously carried out in vitro; for example, the contribution of each segment of bowel to overall absorption can be assessed. Similarly, it should be possible to control simulated environments more precisely than is possible in the intact animal. In this way insight could be gained into the possibility that regulating mechanisms reside within conditioned epithelial cells themselves, rather than being mediated by moment to moment changes brought about by humoral factors. It is therefore understandable that in recent years in vitro techniques have been developed and used more extensively.

In one such system segments of rat duodenum are everted so that the mucous membrane lies ~~on~~ the outside. The transfer of iron from fluid bathing this surface to that inside the sac, lined by

serosal membrane can then be studied (Wilson and Wiseman, 1954). A series of experiments have been reported using this preparation in rats (Dowdle et al., 1960; Manis and Schachter, 1962a; Manis and Schachter, 1962 b; Manis and Schachter, 1964). The observations of these workers have lead them to conclude that absorption is an active metabolic process, and not merely a passive diffusion governed by concentration gradients (Dowdle et al., 1960). In addition, it has been demonstrated that the previous iron content of the animal's diet influences the subsequent performance of the isolated loop (Manis and Schachter, 1962 b).

The advantage of such a technique is its simplicity, enabling a relatively large number of experiments to be carried out each day. However, it is possible to criticise this approach since a separate piece of intestine is needed for each point on a time curve, while the oxygenation of the serosal solution remains unknown and may possibly vary between experiments (Quastel, 1963). In addition, conclusions obtained with this method are open to question, since the movement of iron does not duplicate physiological absorption. Furthermore, some of the findings are different from those obtained in intact animals. For example, the rate of transfer was much slower than occurs in vivo while the amount of iron transferred did not

continue to increase when quantities greater than 14 micrograms were presented to the mucosa (Manis and Schachter, 1962 a). This contrasts with observations obtained in vivo, since it has been shown that absorption is proportional to dosage with no upper limits (Bothwell et al., 1958; Smith and Pannacchiulli, 1958; Beutler et al., 1962). Finally, although Manis, Schachter and their co-workers obtained evidence indicating that iron transfer is an active metabolic process, Brown and Justus (1958) reached the opposite conclusion using a similar experimental preparation.

In view of these discrepancies and because Job and Howell (1962) had already questioned the validity of data obtained using this preparation, an attempt was made to develop an artificial system which more closely approximates physiological conditions.

A preparation has been devised in which a circulation is maintained through the normal vascular channels. In this system, when iron labelled with Fe^{59} is introduced into the duodenal lumen, it rapidly appears in the portal venous effluent. The rate of transfer of the iron across the mucous membrane can then be determined by measuring the amount of isotope in this fluid at regular intervals. It has been found that the rate and characteristics of transfer can be satisfactorily assessed by perfusing for only 30 minutes.

Such a preparation is likely to have several unique features as a research tool. Thus, it might be possible to evaluate the role of the epithelial cells independently of other tissues, while at the same time maintaining normal anatomy and intact cellular membranes. Additional studies would be possible on the role of metabolic stimulators and inhibitors, and the effects of previous in vivo manipulation of the experimental animals could be documented.

PRELIMINARY OBSERVATIONS RELATING TO THE DEVELOPMENT OF THE SYSTEM

Anaesthetic

Various anaesthetic agents have been investigated both in vivo and using this technique. No difference has been demonstrated between diethyl-ether or intraperitoneal pentobarbitone sodium (Sagatal) upon the pattern of iron transfer. This point was of some interest since certain agents are known to influence the excretory pattern of the exocrine pancreas in rats (Lin and Alphin, 1962).

Development of the Anatomical Preparation

One of the major criticisms of the everted gut sacs was the fact that anatomy was disrupted. In developing this perfusion

technique it was anticipated that provided sufficient care was taken during dissection, the normal anatomical relationship between the afferent arteriole, the capillary bed and the small venules could be maintained intact.

Initially, a catheter was introduced directly into the superior mesenteric artery and a second catheter into the portal vein. Following this the bowel was dissected free from the posterior abdominal wall and immersed in warm physiological saline. It was immediately apparent that this approach was unlikely to succeed since even the slightest movement, such as that produced by peristalsis, altered the position of the catheters relative to each other and so obstructed perfusion.

Accordingly, a modification was tried in which the bowel, together with its catheters, was gently ligated onto a plastic template following removal from the animal. Although this approach was clearly an improvement on the previous method it suffered from the same drawback.

Based on these experiments the current dissection technique was established in which catheters were introduced directly into the blood vessels and no attempt was made to remove the viscera from

the peritoneal cavity.

Shock

It is very difficult to assess exactly any effects that the neurogenic shock of these manipulations may have had upon either iron uptake or transfer. However, throughout the dissection care was taken to minimize the handling of viscera, but when this was necessary, to do so very gently with swabs soaked in warm physiological saline. Blood loss was at all times meticulously avoided.

Collateral Circulation

During pilot experiments it was observed that a considerable amount of the perfusate was lost during the course of each study. Observations in which dye was injected into the superior mesenteric artery demonstrated that anastomoses occurred around the rectum and that, depending upon the placement of the arterial catheter, significant loss of dye also took place into both kidneys. In subsequent studies, therefore, care was taken to exclude these vessels from the vascular area to be perfused.

Introduction of Labelled Iron

The effect of perfusing the test substance through the whole

length of bowel from pylorus to ileo-caecal junction (Prasad and Osborne, 1963) was examined. It was found that this method frequently washed off the mucosal cells and also produced kinks in the bowel which led to gross distention of some segments.

For this reason, and because iron absorption is optimal in the upper small bowel (Wheby, Jones and Crosby, 1964) transfer was studied in a standardised length of duodenum and jejunum.

The Perfusing Buffer Medium - Pilot Experiments

The perfusing medium should be physiological from the standpoint of electrolyte concentration, and should provide a ready source of available oxygen. The total osmolarity needs to be of such magnitude that there is no net loss of fluid from the vascular compartment either into interstitial spaces or into the lumen of the bowel, and this will depend to some extent upon the pressure necessary to provide adequate perfusion.

i) Studies Using Erythrocytes

Ideally, all the above requirements would be fulfilled simply and most completely by the use of compatible, and suitably anticoagulated whole rat blood. This approach was the first to be

examined, but the very large quantities of blood required raised prohibitive economic objections. In an attempt to overcome this difficulty, the blood was pooled after each experiment, and following oxygenation was re-used. This approach was discarded because it possibly introduced subtle metabolic alterations which would have provided a variable factor between experiments.

A slightly different technique was next evaluated in which washed rat erythrocytes were suspended in physiological buffer to a final haematocrit of 10 percent. It was anticipated that the rather anaemic level could be compensated for by high flow rate. Using this method de-oxygenation was observed, indicating that the tissue was extracting oxygen and functioning in this one respect as might be anticipated from viable bowel. It was appreciated that the latter observation did not imply that more delicate physiological mechanisms, such as enzyme systems, were necessarily intact. Nevertheless, pilot studies using radioactive iron demonstrated that small, but significant, transfer took place from the lumen into the portal venous effluent. However, in all these experiments very rapid leak of the medium took place into the lumen. To overcome this apparent osmotic deficit, human salt-free albumin was added to a final concentration varying between 3 and 5 grams percent. Despite this

precaution leakage of perfusing fluid still took place into the loop, and finally this approach was likewise abandoned.

Because of their ready availability human blood cells were next examined. Studies carried out in vitro failed completely since no flow could be obtained through the vasculature of the preparation. In addition to this, showers of petechiae were seen on both serosal and mucosal surfaces, associated with free exudation of blood and labelled plasma into the lumen of the bowel. Microscopic examination of blood obtained from the portal vein demonstrated marked agglutination and haemolysis of the human erythrocytes. These observations were readily confirmed by cross-matching the blood in a test tube. It is of interest that the incompatibility could not be influenced by repeated washing of donor human erythrocytes and that the petechial haemorrhages remained present even when anticoagulants were excluded and fresh washed defibrinated blood substituted. These observations clearly precluded the use of human blood for any further study.

Finally, experiments were carried out using washed bovine erythrocytes suspended in physiological buffer. This approach was the most rewarding of studies using red cells. Firstly, it could be demonstrated that the gut utilized oxygen. Thus, at a haemoglobin

concentration of 8.3 grams percent and an initial arterial saturation between 96.5 and 99.0 percent, the perfused system removed 3.98 millilitres of oxygen during the course of study. Values in this range have also been obtained by Kavin (1965). A certain amount of haemolysis took place, and at the completion of the experiment free haemoglobin in the perfusate varied between 54 and 150 milligrams percent. In addition, it was consistently demonstrated that transfer of iron took place from the lumen to the portal venous effluent.

However, the results obtained when erythrocytes were excluded from the perfusate are of particular interest. Thus in 6 experiments using bovine red cells, 5.6 percent (range 2.4 to 8.6 percent) of a 5 microgram dose of ferric iron was transferred. A similar figure of 7.6 percent (range 2.4 to 11.7 percent) being obtained with the cell-free perfusate. Since the presence of red cells had no apparent advantage over the cell-free perfusate in respect of iron transport, they were omitted from subsequent experiments.

ii) Development of Cell-Free Perfusing Medium

The use of cell-free media to perfuse organs is a recognised experimental technique (Carrel and Lindbergh, 1938; Baldwin, 1953; Opie, Shipp, Evans and Leboeuf, 1962), and

accordingly experiments were designed to develop a simple, practical medium for use in this system.

In order to provide a suitable osmotic pressure initial studies were carried out with increasing quantities of large molecular weight dextran in physiological buffer solution (Krebs and Henseleit, 1932; Krebs, 1950; Umbreit, Burris and Stauffer, 1964). In this way a suitable concentration was finally established which, under the conditions of perfusion used in this system, did not produce any significant loss of buffer into the interstitial spaces. The viscosity of this fluid was also low enough to allow a high flow rate, thus reducing anoxic phenomena as much as possible.

It would have been interesting to determine exactly the osmotic pressure of this solution but, in the presence of colloid, this was not possible. In order to establish approximately the osmotic activity of the buffer, it was set up against rat plasma, separated by a semi-permeable membrane. A similar experiment was set up using whole rat blood. These systems were allowed to equilibrate at 37 degrees Centigrade overnight. It was demonstrated that there was no net movement of fluid between the two compartments.

Rheomacrodex, a low molecular weight dextran, was also investigated in this system. A series of experiments failed to demonstrated any advantage resulting from the use of this substance, either in conjunction with, or replacing large molecular weight dextran.

Once a satisfactory medium had been developed (vide infra) a series of experiments were carried out to document metabolic phenomena occurring during the course of perfusion.

Direct measurements on oxygen consumption, using the van Slyke manometric method, were unsuccessful. However, studies with an oxygen electrode indicated that the tissue removed approximately $2\frac{1}{2}$ millilitres of oxygen during each experiment. It is considered of importance that some of this gas remained dissolved in the buffer after it had passed through the tissue, since this suggests that the delivery of oxygen was probably adequate for metabolic processes occurring under these circumstances.

Similarly, observations on the pH of the medium showed that initial levels between 7.25 and 7.40 were unchanged following perfusion through the bowel. These findings indicated that any metabolites produced were adequately buffered by this medium.

Likewise, studies carried out to define the extent of glucose utilization, using a specific glucose oxidase method, demonstrated an average fall of 8 milligrams percent during the course of perfusion. A similar figure has been obtained when gut was perfused with blood (Kavin, 1965). These observations on glucose and oxygen consumption and the stability of the pH applied equally to any period during the 30 minutes of study.

On the other hand, numerous attempts to demonstrate an arterio-venous difference in respect of $p\text{CO}_2$ were unsuccessful. This may have been due to the small quantities of gas produced by the bowel, or perhaps to the high carbon dioxide tension following gassing of the buffer.

Investigations were also carried out to establish whether any of the individual components of the buffer system played a critical role. Thus, although glucose and insulin were present in the perfusing medium in all the subsequent experiments, diminished transfer of iron over 30 minutes could not be demonstrated when they were omitted. These results are presented in detail in the section dealing with the effect of metabolic substrates.

In considering the other components of this buffer solution,

attention was directed to calcium and magnesium by the report (Tidball, 1964) that these ions, being loosely bound in the structure of the membrane, may regulate the permeability of the intestinal cells. Studies in which their concentration in the perfusate were either altered or in which they were left out completely, showed no difference in transfer. Of further interest was the observation by Manis and Schachter (1962 a) that the phosphate buffer used in earlier studies (Dowdle et al., 1960) could decrease iron transport. In view of this report, varying concentrations of potassium dihydrogen phosphate in the buffer were examined, but it was not possible to attribute any effect to this substance.

Thus, specific experiments were unable to demonstrate defects in transfer which could be ascribed to variations in the individual components of the buffer system. It was nevertheless obvious that the ionic environment of these cells should be maintained as close to physiological as possible, and accordingly the buffer system was used as described below.

MATERIAL AND FINAL METHOD

White rats of the Wistar strain weighing between 200 and 300 grams were used, and in different experiments were matched

for weight and sex. These animals were maintained on unlimited supplies of water and mice biscuits, the latter containing 30 milligrams of iron per 100 grams dry weight. The animals were housed in suitable cages with a wire mesh floor to prevent coprophagia. These were kept in a laboratory with a temperature thermostatically regulated between 72 and 80 degrees Fahrenheit.

In a few experiments animals were maintained on a semi-synthetic diet of low iron content for a week prior to use (Valberg, Taylor, Witts and Richards, 1961). Rats were rendered anaemic by removing 4 millilitres of blood 5 and 3 days prior to study or by parenteral administration of an ethanolic solution of phenylhydrazine (30 milligrams per kilogram body weight). Varying degrees of iron overload were produced by parenteral administration of either an iron-dextran complex (Imferon) or an iron-Sorbitol-Citric acid complex (Jectofer).

Preparation of Isolated Loop

Food and water were withdrawn 24 hours before study.

Once surgical anaesthesia had been induced by intra-peritoneal pentobarbitone sodium (20 milligrams per kilogram) 5 units of heparin in 0.9 percent NaCl solution were injected intravenously.

The animal was then attached to an operating board in the supine position, using rubber bands on the limbs. A long skin incision was made commencing on the right fore limb, extending medially onto the manubrium sternum and then caudally in the midline down to the symphysis pubis, at which point it was carried laterally down both legs as far as the knee joints. It was not usual at this stage to encounter significant bleeding despite the fact that the animal had previously been heparinised.

The peritoneal cavity was opened from xiphisternum to symphysis. Particular care was taken to avoid accidentally opening the thoracic cavity or cutting into the bowel. The muscles were retracted laterally and maintained in this position using a self-retaining retractor (Figure 6).

The coils of intestine were gently displaced to the left and after careful dissection of the right renal pedicle the vessels were firmly ligated with black silk. A ligature was placed around the rectum so that flow between superior and inferior mesenteric territories was occluded.

A ligature was also placed around the upper jejunum 10 centimetres from the pylorus, taking care to avoid the accidental

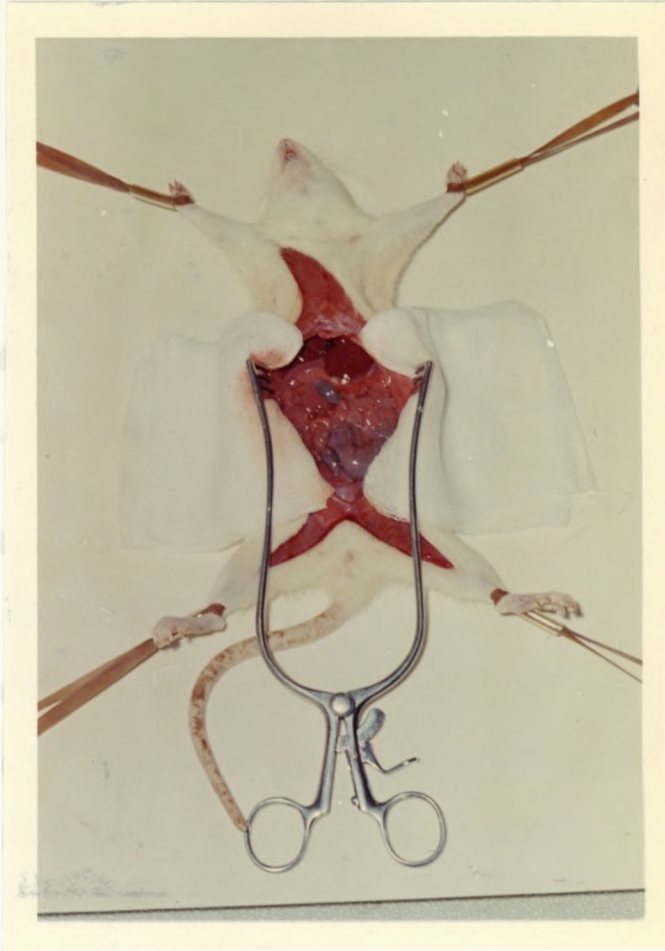


FIGURE 6

ion by liga

Photograph illustrating the extent of the skin incision and the general exposure obtained by the self-retaining retractor.

inclusion of small blood vessels in this ligature. This particular point (Figure 7) was constantly related to a small peritoneal reflection medial and inferior to the porta hepatis, where a rather larger branch of the superior mesenteric artery was usually present. A further ligature which was left untied, was placed around the pylorus itself. The intestines were then displaced to the right and the left renal artery tied.

A thin polythene cannula filled with warm, oxygenated, physiological buffer solution (see below) was introduced through the abdominal wall in the left inguinal region, and the blunt tip apposed to the bifurcation of the aorta. A bulldog clamp was used to occlude the aorta just below the renal arteries, and the cannula was then introduced into the aorta and passed cranially (Figure 8).

The clamp was removed and the cannula advanced to lie with its tip just caudal to the origin of the superior mesenteric artery. It was secured in this position by ligatures just below the tip, at the aortic bifurcation and in the inguinal region.

In order to proceed further with the dissection the parietal peritoneum must be reflected carefully from the superior mesenteric and coeliac arteries. It was appreciated that the failure to expose

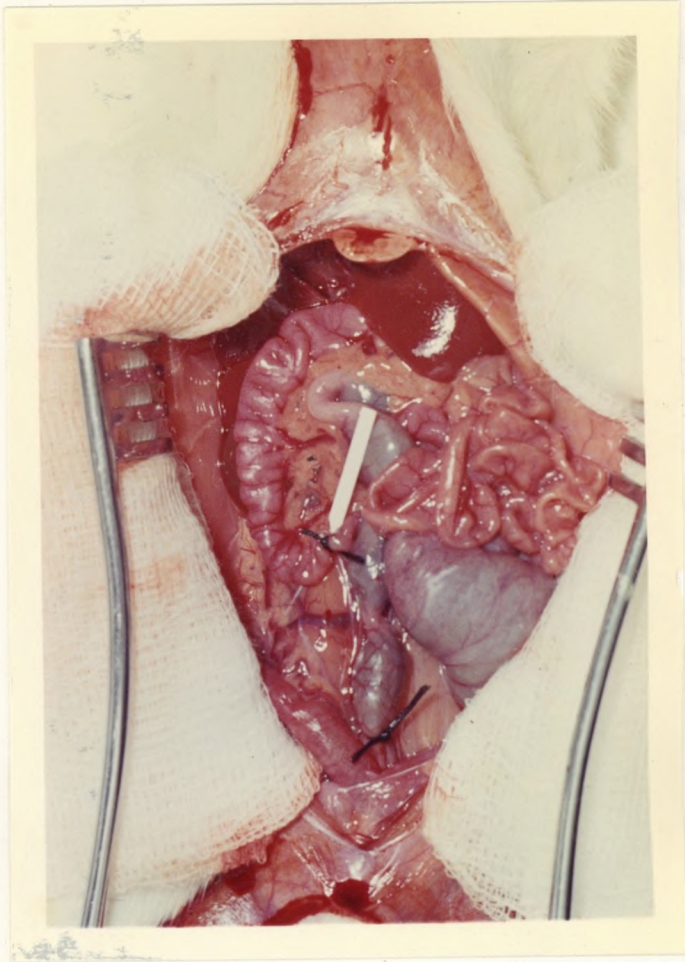


FIGURE 7

Photograph illustrating the distal extent of the isolated loop (White arrow) and ligature around the rectal anastomotic vessels.

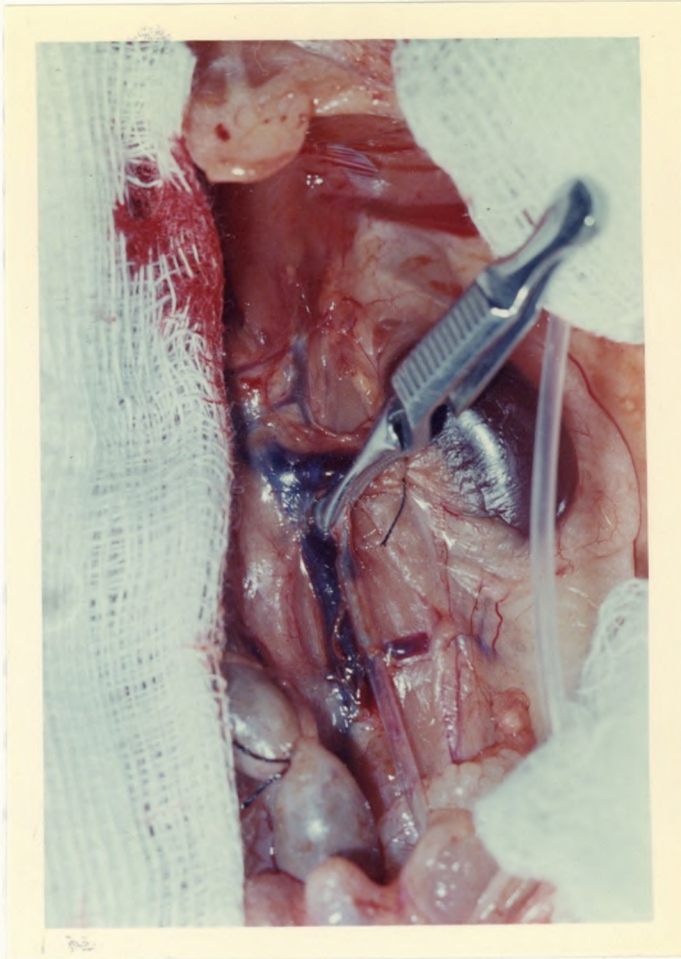


FIGURE 8

Photograph illustrating the arterial catheter in place within the aorta. The bulldog clip can be seen occluding the aorta immediately below the renal vein. A black silk ligature is seen around the left renal pedicle. Above the left renal vein both the superior mesenteric artery and the coeliac artery are well seen.

these vessels adequately resulted in considerable difficulty in subsequent dissection. It is usual at this stage to see the abdominal lymph channel overlying the aorta but it was not found necessary to sacrifice this vessel in order to secure adequate exposure of the aorta.

Thus far the circulation through the stomach, duodenum and small intestine had not been disturbed. A ligature is now placed around the aorta between the coeliac and superior mesenteric branches and drawn tight. Perfusion was immediately commenced from a reservoir of warm, oxygenated, physiological buffer solution, the fluid passing from the cannula up the superior mesenteric artery (Figure 9).

At once a second cannula was introduced between the second and third ribs one centimetre to the right of the midline, and its smooth blunt tip directed caudally into the abdominal cavity through the diaphragm to lie adjacent to the portal vein. A small oblique incision is made in the region of the bifurcation of the portal vein, and the catheter then introduced approximately 2 to 3 millilitres and secured in this position (Figure 10).

The perfusion medium then flowed through the arterial

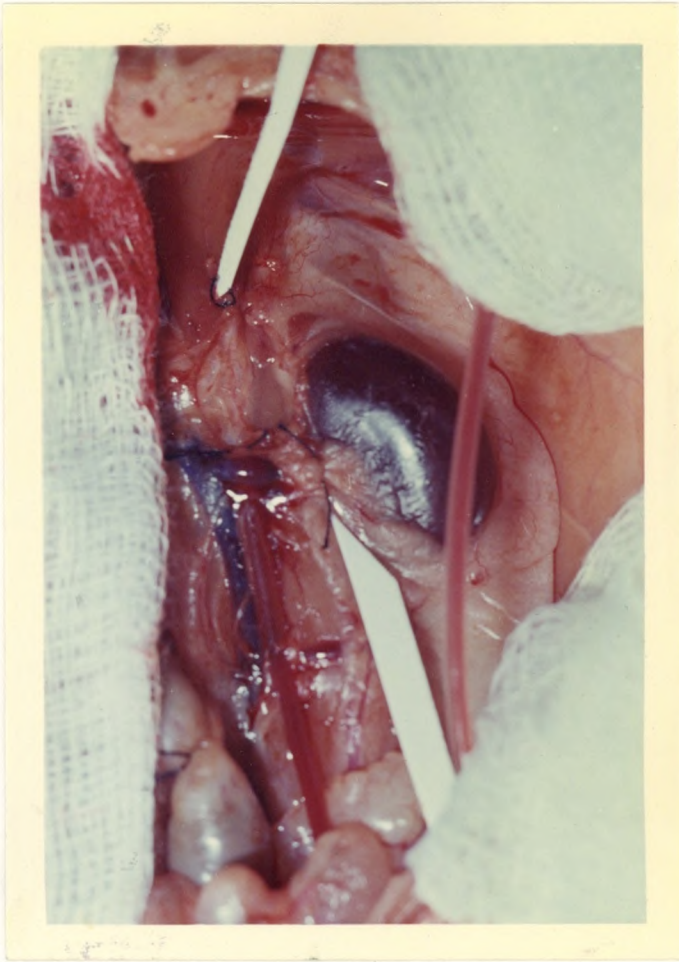


FIGURE 9

Photograph illustrating the aortic catheter in position. The ligated renal pedicle is seen (lower white arrow). Immediately above the left renal vein the ligature immobilising the catheter in the aorta can be identified. Above this (upper white arrow) the ligature occluding the coeliac artery can be identified.

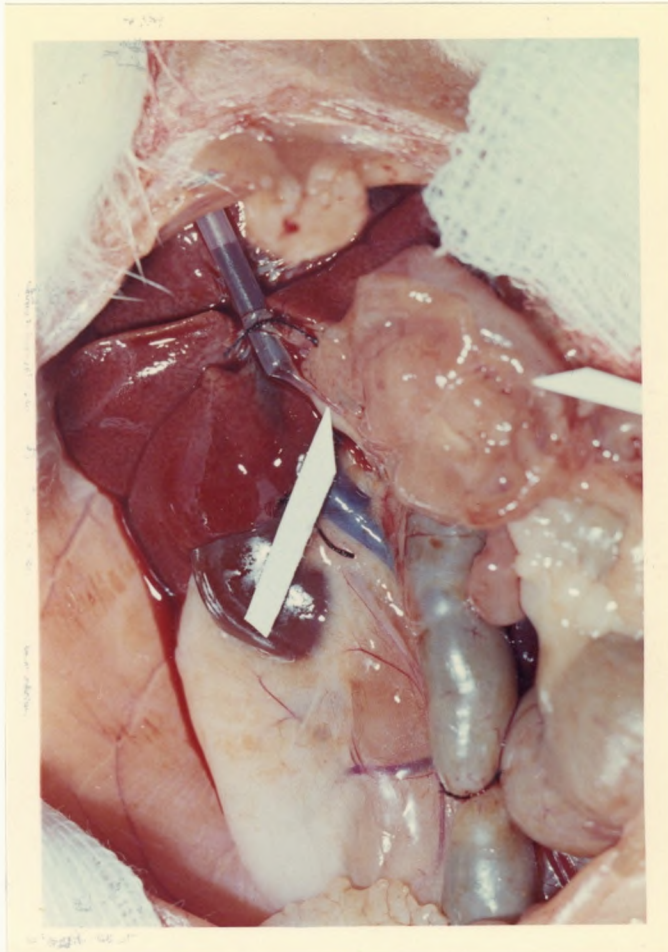


FIGURE 10

Photograph illustrating the venous catheter ligated in place within the portal vein (left hand white arrow). The white arrow on the right of the photograph indicates the position of the isolated loop in which blood vessels can no longer be recognised since perfusion with colourless dextran buffer has already commenced.

catheter, the superior mesenteric artery and its branches, the capillary bed, the portal vein and venous catheter. At this stage the animal was sacrificed by opening the thoracic compartment through the diaphragm.

The venous catheter is secured at its entry into the thoracic cage with a single silk ligature and suitably tensioned to prevent kinking or torsion of the portal vein. Considerable importance is attached to preventing mechanical occlusion in this area since this results in accumulation of perfusing medium within the bowel and associated loss of epithelial cells into the lumen.

As soon as the effluent was free of blood the perfusate was changed to a mixture of 4.7 percent dextran in the buffer solution. Once the flow-rate was satisfactory (4 millilitres per minute or more) the Fe^{59} labelled dose of iron was taken up into a 1.0 millilitre tuberculin syringe to which was attached a blunt metal cannula. The tip of this cannula was introduced into the stomach through an incision in its anterior wall, and then passed through the pylorus into the duodenum. After tensing the ligature around the pylorus, the labelled iron solution was injected into the duodenal lumen (Figure 11). The cannula was then withdrawn as the ligature was tied, so that reflux into the stomach was prevented.



FIGURE 11

Photograph illustrating the introduction of labelled iron, with a blunt needle through a stab wound in the wall of the stomach, into the isolated loop. The ligature around the pylorus is well seen while behind this the venous catheter can be identified securely ligated into the portal vein.

A stop watch was started and the venous effluent collected in a reservoir under a pressure of minus 4 centimetres of water. The whole animal was then freed from the dissecting board and immersed in 0.9 percent NaCl solution at 37 degrees Centigrade in a plastic tray, the latter lying in a water bath maintained at 40 degrees Centigrade (Figure 12).

At five minute intervals the venous reservoir was emptied into a measuring cylinder. The volume of each sample was recorded and the radioactivity of a 4 millilitre aliquot measured in a well-type scintillation counter. In this way it was possible to determine the rate at which labelled iron was transferred from the lumen to the fluid perfusing the vascular system.

With practice the preparation of this system proceeded smoothly. It was important to ensure that there was no mechanical obstruction to the flow of the perfusate. This required special attention to the venous end so that the catheter lay in the portal vein without kinking. It was also necessary to start perfusing as soon as the aortic ligature occluded the circulation to the duodenum, and to open the portal vein as quickly as possible after this to allow the perfusate to escape. The venous catheter could then be introduced at leisure.



FIGURE 12

Photograph illustrating the arrangement of the rat submerged in a bath of warm physiological saline (green tray), the temperature of the latter is maintained in a thermostatically heated water bath. The course of the arterial catheter (top right) can be seen under the water to ensure that the perfusate reaching the viscera is at 37° Centigrade. The venous catheter, secured to the anterior chest wall, is seen leaving the left hand corner of the water bath.

During the development of this technique, difficulty was not infrequently experienced in establishing an adequate rate of flow, apparently due to arteriolar spasm, but with experience the operating time was reduced to approximately 7 minutes and this problem was seldom encountered. Perfusion was usually continued for 30 minutes, during which time active peristalsis of the intestines could be seen. It was not found advantageous to continue the experiment for longer than this for two reasons: firstly, the pattern of absorption could clearly be seen by this time, and secondly, oedema of the bowel and accumulation of fluid within the lumen became significant if perfusion was continued for longer periods.

Perfusion Medium

Studies in which suspensions of erythrocytes were used (vide supra) failed to demonstrate any consistent advantage in the transfer of Fe^{59} into the venous effluent over that found when erythrocyte-free buffer solution containing 4.7 percent dextran (large-molecular*), glucose (200 milligrams per 100 millilitres) and

*"Dextran" 6 percent w/v in 0.9 percent NaCl solution supplied by Bengel Laboratories Limited, Holmes Chapel, Cheshire, England. 100 parts of this were mixed with 21 parts of 1.30 percent NaHCO_3 , 4 parts of 1.15 percent KCl, 1.5 parts of 1.22 percent anhydrous CaCl_2 , one part 2.11 percent KH_2PO_4 , and 0.5 parts of 3.82 percent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

insulin⁽¹⁾ (0.15 unit per 100 millilitres) was used. During the initial perfusion, when blood was still present in the vessels, the solution was employed without dextran.

Oxygenation was achieved by continuously bubbling a mixture of 94 percent oxygen and 6 percent carbon-dioxide through the perfusate in the reservoir. The latter was suspended above the preparation so that the level of fluid in the drip-chamber was 90 centimetres above the intestine (Figure 13). An adequate rate of flow was obtained at this hydrostatic pressure if the arterial catheter was 10 centimetres long with an internal diameter of 1.2⁽²⁾ millimetres. The polythene tubing between the drip-chamber and the arterial catheter was of larger diameter, and long enough to allow several coils to lie in the water-bath. In this way the perfusate was warmed before entering the preparation. The venous

(1) Although the absorption of glucose from the intestinal lumen is unaffected by insulin, it was included in the perfusing medium because no evidence concerning mucosal cell uptake of glucose from the plasma could be found.

(2) Intramedic Polythene tubing supplied by Clay-Adams Inc., 141 East 25th Street, New York.

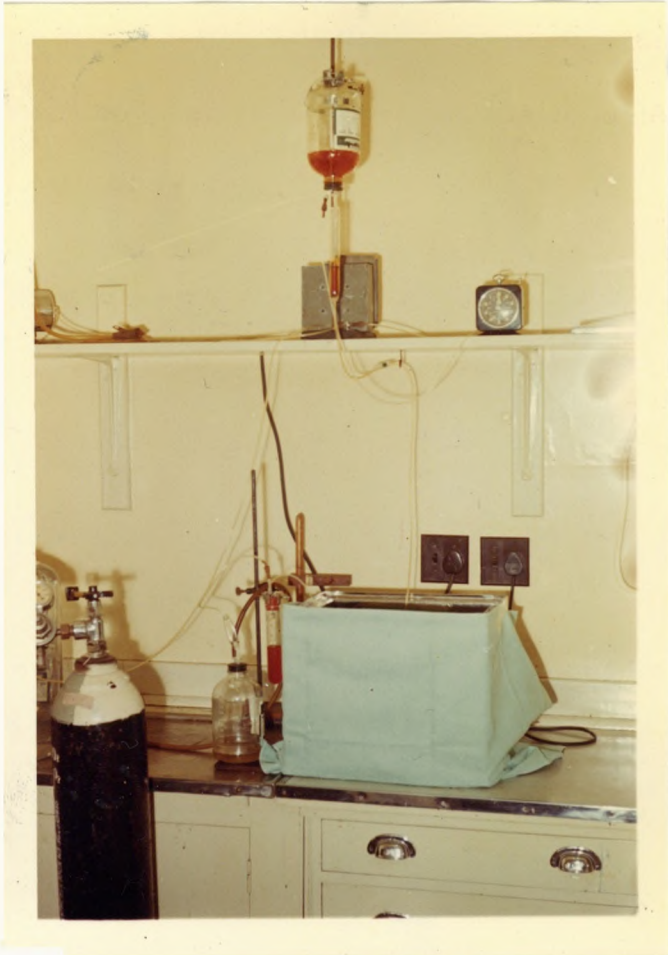


FIGURE 13

Photograph illustrating the general arrangement of the perfusing apparatus. Above, coloured red to facilitate photography, is seen the reservoir containing the physiological buffer. This is oxygenated from a cylinder (bottom left). At the front left hand corner of the water bath is seen the test tube which acts as reservoir for venous effluent. Immediately to the left of this is the suction apparatus which maintains a constant negative pressure of 4 centimetres of water

catheter was 15 centimetres long with an internal diameter of 1.7 millimetres (3).

When the effects of various metabolic substrates and poisons were studied, the substances to be tested were dissolved in the perfusate, and the pH adjusted to its previous level of 7.25 to 7.40 with either hydrochloric acid or sodium hydroxide. Anoxic conditions were produced by changing the gas mixture to 94 percent nitrogen and 6 percent carbon-dioxide.

Administration of Radioiron

Iron as ferric chloride was made up in distilled water each day in such concentration that the required quantity for each dose was present in 0.25 millilitre. The $\text{Fe}^{59} \text{Cl}_3$ was then added to give 1.5 microcuries per dose. In each experiment, 0.25 millilitre was drawn up into a tuberculin syringe and delivered into the duodenal lumen via the blunt cannula.

A standard was prepared by diluting a dose in 200 millilitres of 0.1 N hydrochloric acid in a volumetric flask. Four millilitre aliquots, representing 2 percent of the administered dose, were then

(3) Intramedic Polythene tubing supplied by Clay-Adams Inc., 141 East 25th Street, New York.

set aside to be counted together with the samples at the completion of the study.

Measurement of Absorption

In these studies absorption or transfer is defined as the appearance of radioactive iron in the venous effluent. It does not take into consideration the uptake of iron from the lumen into the mucosal cells, unless specifically stated, or the possible transfer of tracer into the lymphatics.

In each experiment the radioactivity of a 4 millilitre aliquot was counted in a well-type scintillation counter. The particular instrument used was a thallium activated sodium-iodide crystal coupled, via a photomultiplier tube, to a IDL 1700 Scaler which was calibrated for this isotope. All samples were counted in test tubes of uniform bore against a 4 millilitre aliquot of standard, suitably diluted, so that count rates were in a comparable range.

Absorption was expressed as the percentage of the original activity introduced into the loop or as absolute transfer in micrograms of iron. In either case these data were plotted as function of time.

COMMENTS

Determination of Labelled Ferritin

On a number of occasions, the Fe^{59} labelled ferritin content of the intestine was determined at the conclusion of the experiment.

i) Identification of Ferritin

Several millilitres 0.1 M EDTA (pH 7.0) was added to washed gut, and the mixture was homogenized and then heated to 70 degrees Centigrade over a period of five minutes. After centrifugation at $9,000 \times g$ for 10 minutes, the supernatant solution was collected. Alum-precipitated horse spleen ferritin was isolated by the method of Mazur and Shorr (1950), and antiserum was prepared by bleeding rabbits immunized with samples of this ferritin using a modification of the method of Proom (1943). (The cross-specificity of horse spleen ferritin and rat liver ferritin has been established (Mazur et al., 1960)). One millilitre of the pooled antiserum precipitated approximately one milligram ferritin. To a sample of the mucosal supernatant solution to be tested was added one millilitre antiserum and 0.2 millilitre carrier ferritin solution containing approximately 500 micrograms ferritin. The mixture was then incubated for one hour

at 37 degrees Centigrade. The ferritin-antiferritin complex, which appeared as a visible precipitate, was washed three times with distilled water after centrifugation at 1,500 x g for 10 minutes. The Fe^{59} activity of the precipitate provided a measure of the Fe^{59} -labelled ferritin present.

In this method carrier ferritin was added to the solution for two reasons. First, precipitation could be verified by estimating the iron in the deposit using the thioglycollic acid method after wet digestion (McCance et al., 1936), and second the measurement of minute amounts of labelled ferritin was facilitated. The specificity of the antiserum was confirmed by the demonstration that no activity was precipitated on incubation with either rat haemoglobin or rat transferrin labelled with Fe^{59} .

The purpose of the EDTA was to prevent the attachment of Fe^{59} to carrier ferritin or to unlabelled mucosal ferritin. It was shown that if the chelate were present in a solution of $\text{Fe}^{59} \text{Cl}_3$ before adding ferritin, less than 0.2 percent of the label was precipitated with the ferritin-antiferritin complex. Mucosal supernatant solutions prepared with EDTA consisted of two fractions, the first precipitable with either perchloric acid or the ferritin antiserum and the second identifiable as EDTA-iron. This indicated that the chelate had taken

up all nonferritin activity. Furthermore, the fact that only minimal ferritin labelling was observed under certain circumstances, e. g., in iron deficiency, provided added evidence to suggest that the approach was a valid one.

Finally, it was necessary to establish that EDTA did not remove activity from physiologically labelled ferritin. This was done by incubating a solution of ferritin, prepared from rat livers after the intravenous injection of 5 micrograms Fe^{59} -citrate, with 0.1 M EDTA (pH 7.0) for one hour. On adding the antiserum 94 percent of the activity was recovered in the deposit.

ii) Factors Affecting Ferritin Recoveries

Experiments were done to find out to what extent washing of the gut affected the subsequent recovery of ferritin. Ten centimetre lengths of duodenum taken from two animals 30 minutes after administration of 10 micrograms labelled iron were divided longitudinally in half, the halves being weighed. One half of each duodenum was then washed as before, after which the labelled ferritin content was determined. The remaining halves were not washed, but were homogenized directly in 0.1 M EDTA. The labelled ferritin content of the unwashed portions was 20 to 25 percent higher than that of equivalent weights of washed intestine, indicating a significant

loss of the ferritin-containing cells during the washing procedure. However, no attempt was made in subsequent experiments to correct for this loss,

In previous studies of iron absorption different centrifugation procedures have been used (Brown and Rother, 1963 a; Brown and Rother, 1963 b). It was therefore of importance to establish the extent to which the speed of centrifugation influenced the recoveries of labelled ferritin in gut supernatant solutions. Approximately 500 micrograms of carrier horse spleen ferritin was added to each of several samples of heated supernatant solution, obtained from three rats that had been given 10 micrograms radioiron 30 minutes previously. Half the samples were spun at 9,000 x g for 20 minutes and half at 105,000 x g for 60 minutes. Treatment of the supernatant solution with antiserum revealed that the labelled ferritin content of the three samples spun at high speed was between 50 and 59 percent lower than after low speed centrifugation. Further confirmation of the fact that ferritin iron can be precipitated by high speed centrifugation was obtained by analyzing chemically the iron content of the precipitate obtained after spinning a solution of pure horse spleen ferritin at 105,000 x g for 60 minutes. Eighty percent of the iron was found to be in the precipitate.

Histological Appearances of the Perfused Gut

In order to obtain direct evidence that no gross anatomical destruction was taking place under the experimental conditions defined above, a series of histological sections were taken throughout the length of the intestine.

Conventional light microscopy failed to reveal significant desquamation of epithelial cells, and the morphology of the individual cells was well maintained during the course of these studies (Figures 14 and 15). Examination using electron microscopy was unfortunately not possible. There did, however, occur minimal exudation of buffer, but oedematous areas were not demonstrated unless some degree of venous obstruction had occurred. These features agree with those described by Manis and Schachter (1962 a) and are at variance with changes described by Brown and Justus (1958).

The histological features described support one of the prime requirements expected of this system, namely, that anatomical integrity should be maintained: this, in itself, would appear to represent an advance upon some of the previous preparations (Brown and Justus, 1958).

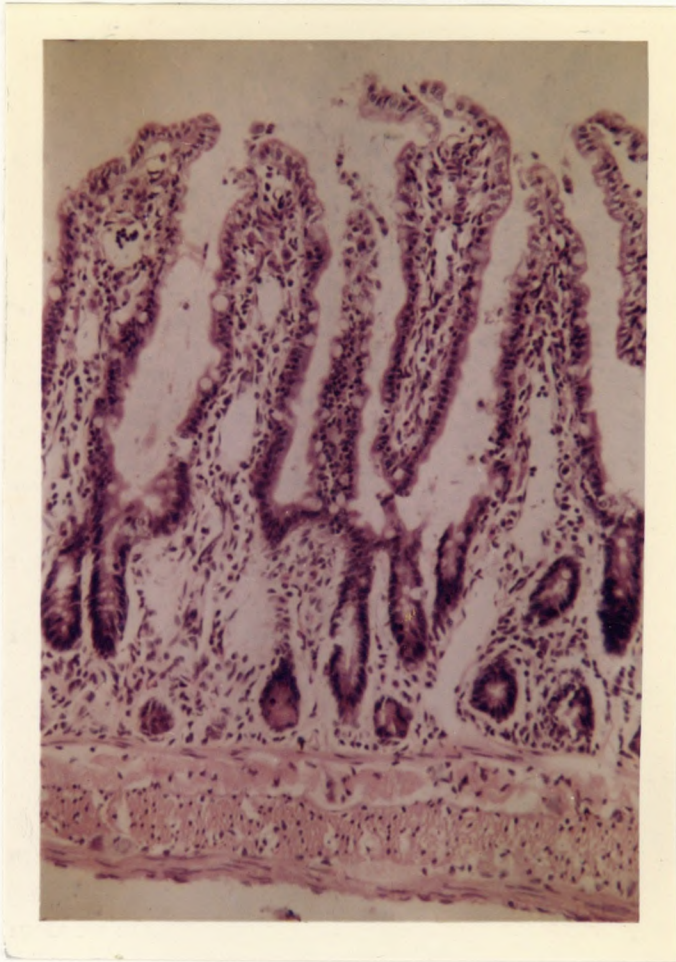


FIGURE 14

FIGURE 13

Photomicrograph of a section of gut at the commencement of
section of gut
perfusion.

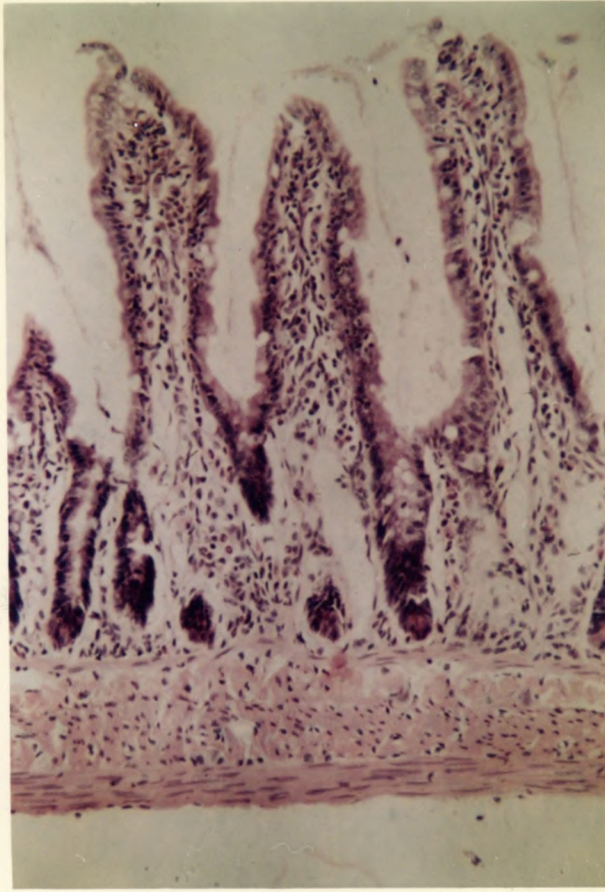


FIGURE 15

Photomicrograph of a section of gut collected at the completion
of a study.

Loss of Radioactivity

It was considered important to determine whether radioactivity was lost from the lumen other than by the usual transfer mechanism. Studies were carried out in which the saline bathing the peritoneal surface of the gut was examined for activity at the completion of an experiment. Although this particular study was repeated many times, it was not possible to identify Fe^{59} in this bath.

Additional studies were carried out in which radioiron was included in the perfusing medium, either as a simple salt or bound to transferrin. In these experiments no isotope could be recovered from the lumen of the perfused bowel or from the saline bathing the serosal surface. Analogously, when a dye was added to the perfusing buffer medium, no leakage could be demonstrated either into the lumen or into the peritoneal cavity. Conversely, radioactivity introduced into the peritoneal cavity could not be recovered either in the perfusate or within the lumen.

While these observations do not exclude the possibility that water and electrolyte shift may have taken place across the capillary endothelium, the limited facilities available made further investigation of this particular problem impossible. In any event, it appears that

labelled iron is not lost by unphysiological routes

RESULTS

Variations in Performance

Some variation in the transfer of labelled iron into the perfusate was encountered within each group of rats selected for a particular experiment. There were also differences in the results obtained with animals of different weights and ages. In addition to these variations, however, marked alterations in performance affecting all the rats in the colony were noted at times. This altered behaviour, which sometimes persisted for many weeks at a time, may have been due to changes in environmental conditions. Similar results have been reported by other workers (Dowdle et al., 1960). Because of such variations in performance it was not possible to compare results which had been obtained at different times, and in any particular set of studies care was taken to compare directly the performance of test preparations with controls in alternate experiments.

The Role of Transferrin

In these studies transfer into the venous channels took

place in the absence of iron-binding protein, while the addition of varying concentrations of transferrin to the perfusate resulted in no improvement in performance. It is concluded that, under these circumstances, transferrin is not necessary for iron transfer from the lumen; a finding which is not surprising in view of a previous clinical report (Heilmeyer, Keller, Vivell, Keiderling, Betke, Wöhler and Schultze, 1961).

The Effect of Temperature

A seasonal variation in iron absorption has been observed when groups of intact animals were studied. It was therefore of interest to examine possible alterations produced in the pattern of transfer by manipulations in the temperature of the medium surrounding the isolated loop.

Further importance was attached to this study since the temperature of homoiothermic animals is normally maintained within very narrow limits and most chemical and enzyme-catalysed reactions are markedly influenced by temperature (Baldwin, 1953).

Iron transfer was compared when the temperature of the perfusing medium and bath varied between 35 and 42 degrees Centigrade, but no difference could be demonstrated. It is not clear

what effects these variations in temperature may have had upon the formation of various iron containing intermediates within the cell, since studies of this nature were not undertaken.

Effect of Variations in Dose

The transfer of iron into the venous effluent when 5 micrograms of iron was introduced into the duodenum was compared with the results when the dose of iron was 50 micrograms. Twelve experiments were carried out with 5 micrograms and 14 experiments with 50 micrograms. After perfusing for 30 minutes, a mean of 8.4 percent (S.E. $\pm$ 1.30) of the administered dose had emerged in the venous effluent in the studies using 5 micrograms, while with 50 micrograms the mean was 4.2 percent (S.E. $\pm$ 0.82) (Figure 16).

Although a smaller percentage was transferred with the larger dose, the quantity of iron in the venous effluent was greater. Expressed in this way the mean figures were 0.42 micrograms of the dose of 5 micrograms and 2.13 micrograms with a dose of 50 micrograms (Figure 17). The same phenomenon was observed in 14 experiments when 100 microgram quantities were administered.

The Effect of Valency

The effect of valency on the transfer of iron was tested by

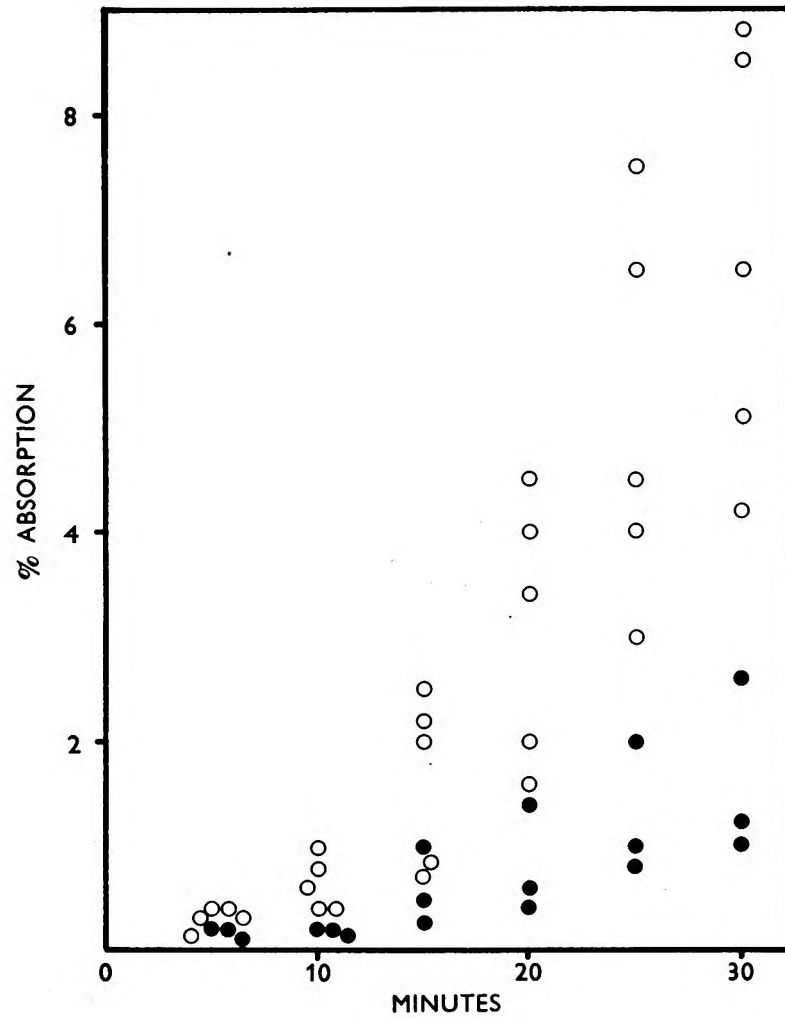


FIGURE 16

The results of a typical study illustrating the greater percentage absorption of 5 micrograms of iron (open circles), when compared to 50 micrograms (closed circles).

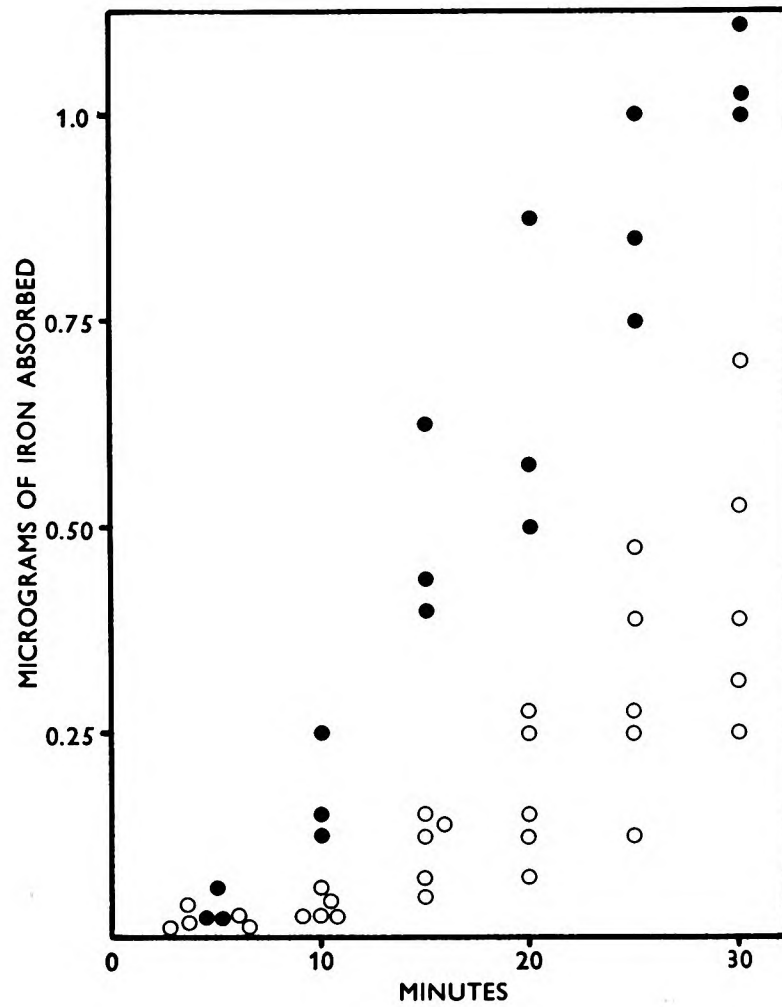


FIGURE 17

The results of a typical study illustrating the greater absolute absorption of iron from a 50 microgram dose (closed circles), when compared to a 5 microgram dose (open circles).

adding ascorbic acid in the proportion of 10:1. This produced a considerable increase in the quantity of iron transferred. In 8 experiments with 5 micrograms of iron the mean figure was 18.6 percent (S.E. $\pm$ 1.76) (Figure 18), equivalent to 0.93 micrograms. The effect was even more marked with the larger dose of iron, the mean value of 11 observations being 16.7 percent (S.E. $\pm$ 1.14) (Figure 19), equivalent to 8.35 micrograms.

The same effect was demonstrated in 20 experiments with 100 micrograms of iron (Figure 20), and also when 500 micrograms quantities were examined.

The Effect of Ligating the Common Bile and Pancreatic Duct

In all these in vitro studies care was taken to exclude the common bile and pancreatic duct from the ligature which secured the venous catheter into the portal vein, although it has been shown that ligation of this duct did not alter iron absorption (Manis and Schachter, 1962 a).

The effect of excluding bile and pancreatic secretion was also specifically studied using this perfusion system. In 17 experiments, using 5 micrograms of iron, no difference could be demonstrated whether these secretions were present or not (Figure 21).

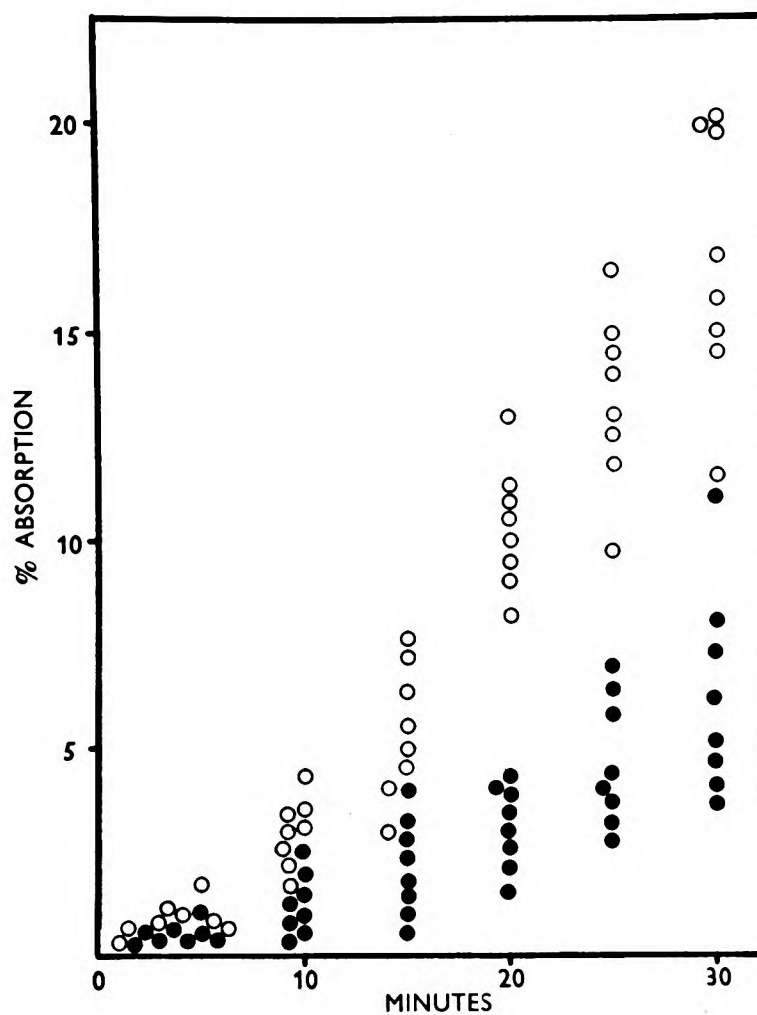


FIGURE 18

The results of 16 experiments illustrating the enhancing effect of ascorbic acid (open circles) on the transfer of a 5 microgram dose of iron (closed circles).

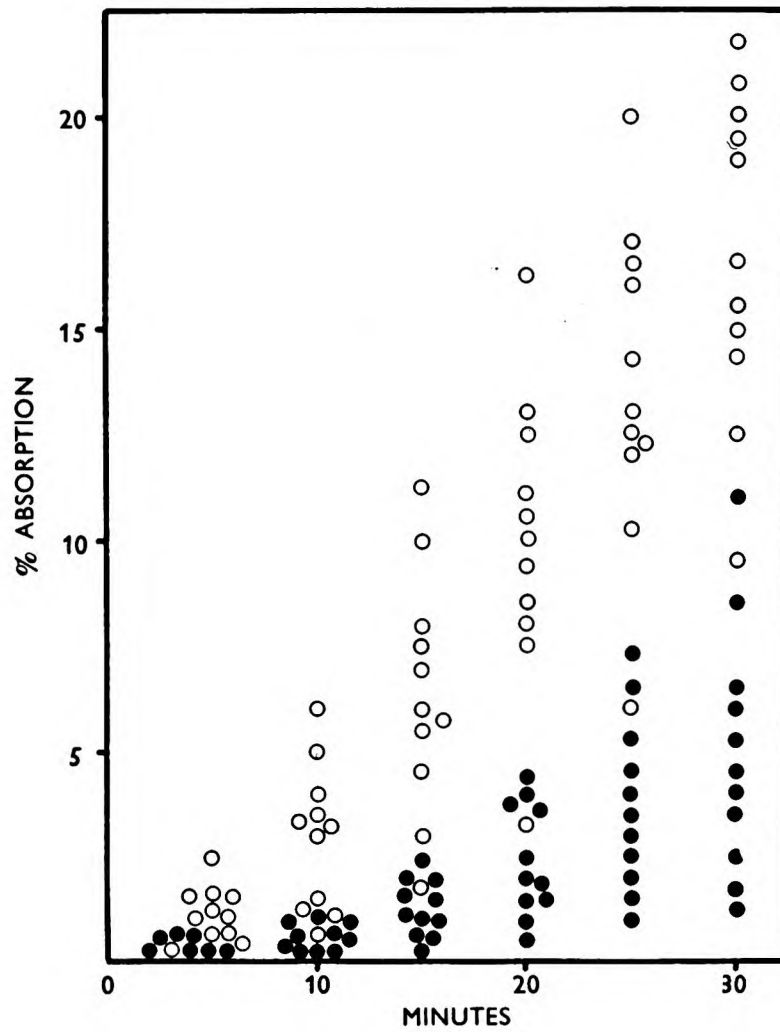


FIGURE 19

The results of 22 experiments illustrating the enhancing effect of ascorbic acid (open circles) on the transfer of a 50 microgram dose of iron (closed circles).

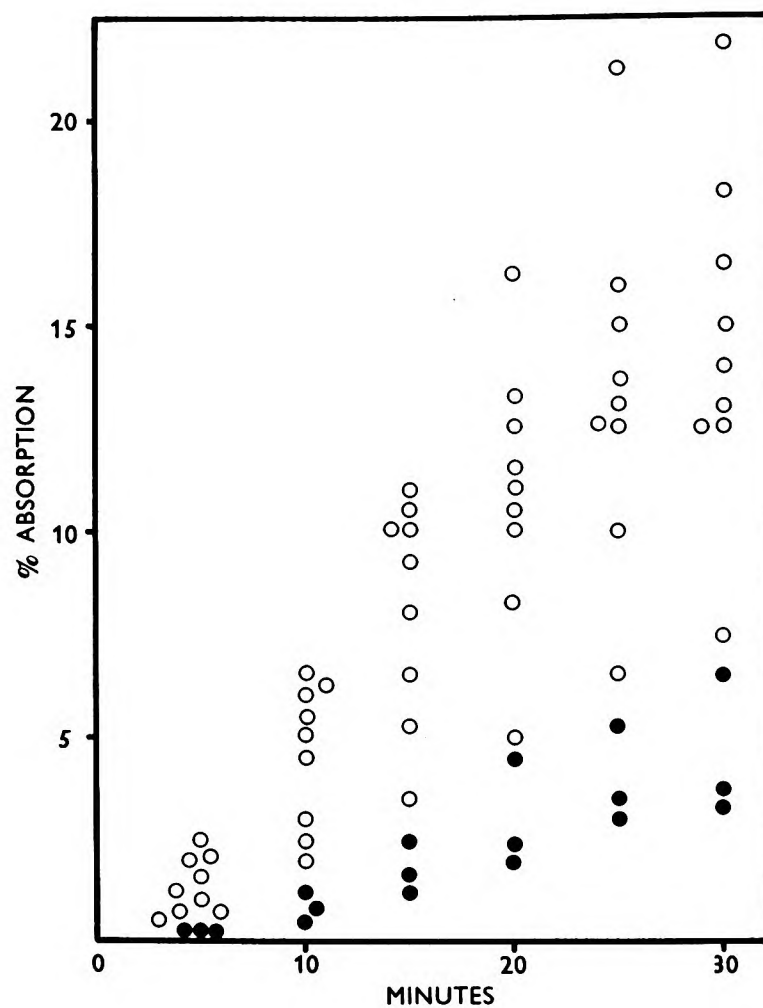


FIGURE 20

The results of a typical study illustrating the enhancing effect of ascorbic acid (open circles) on the transfer of a 100 microgram dose of iron (closed circles).

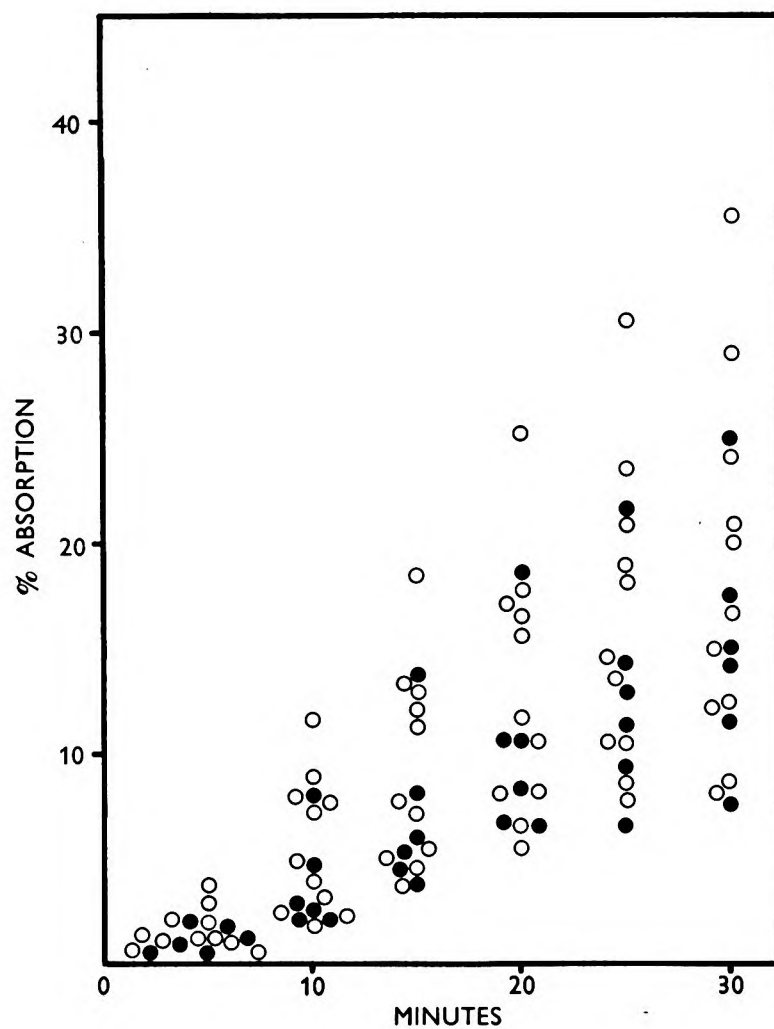


FIGURE 21

The result of 17 experiments illustrating that the transfer of 5 micrograms of iron was the same, whether bile and pancreatic secretions were excluded (open circles), or not (closed circles).

Effect of pH of Luminal Contents

Since it was possible that ascorbic acid potentiated transfer, not only by reducing the iron but also by lowering the luminal pH, further experiments were carried out. At various intervals after introducing 50 micrograms of iron alone (10 experiments) or with 500 micrograms of ascorbic acid (20 experiments), the luminal contents were aspirated and the pH determined. In these studies the changes in pH was studied over half-an-hour. Thus the mean pH five minutes after introducing the dose was 5.9 (range 4.8 to 7.0) as compared with fasting levels between 6.5 and 7.5. However, by 10 to 15 minutes the pH had returned to normal (Figure 22). The same pattern was found when 5 microgram doses were studied.

The effect of hydrochloric acid was then studied in 40 rats. When 50 micrograms of iron made up in 0.1 N HCl (0.25 millilitres) was introduced into the duodenum, a marked fall in pH occurred. Five minutes after introduction, values between 2.2 and 4.1 were observed. A steady rise in pH then took place, normal levels being reached by 25 minutes (Figure 23).

When the transfer of iron from this acid medium into the

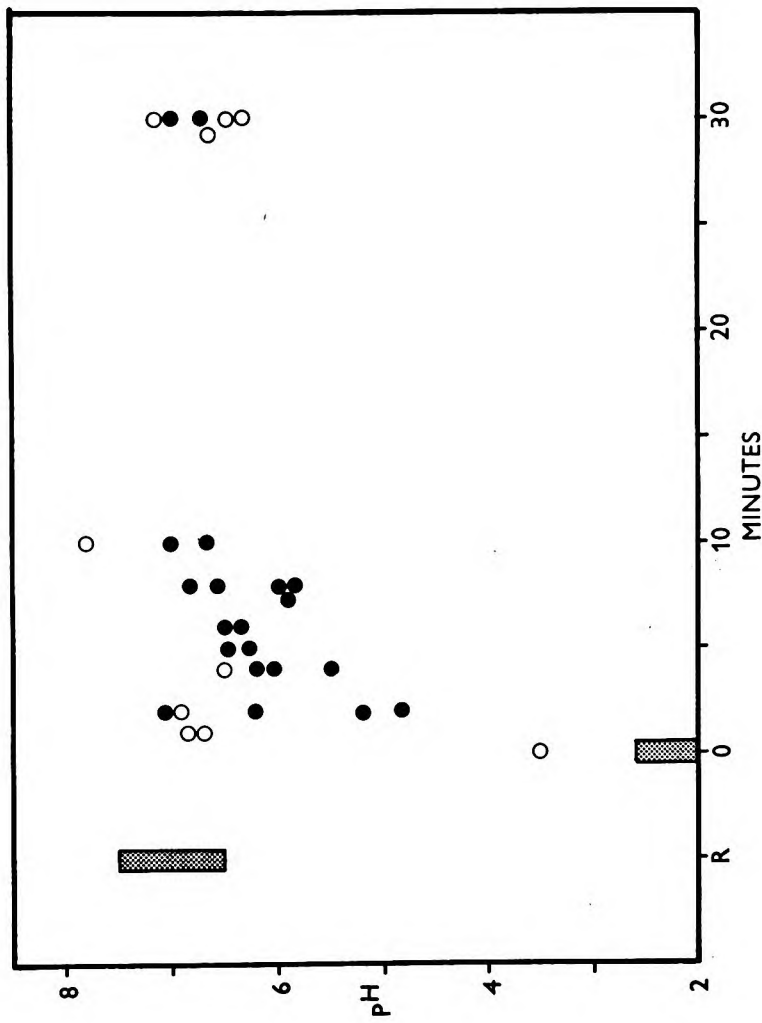


FIGURE 22: Fifty experiments illustrating the rise in luminal pH following the introduction of labelled iron alone (open circles), and with ascorbic acid (closed circles)
 R = Resting pH (10 experiments) O = pH immediately following introduction of iron (10 experiments)

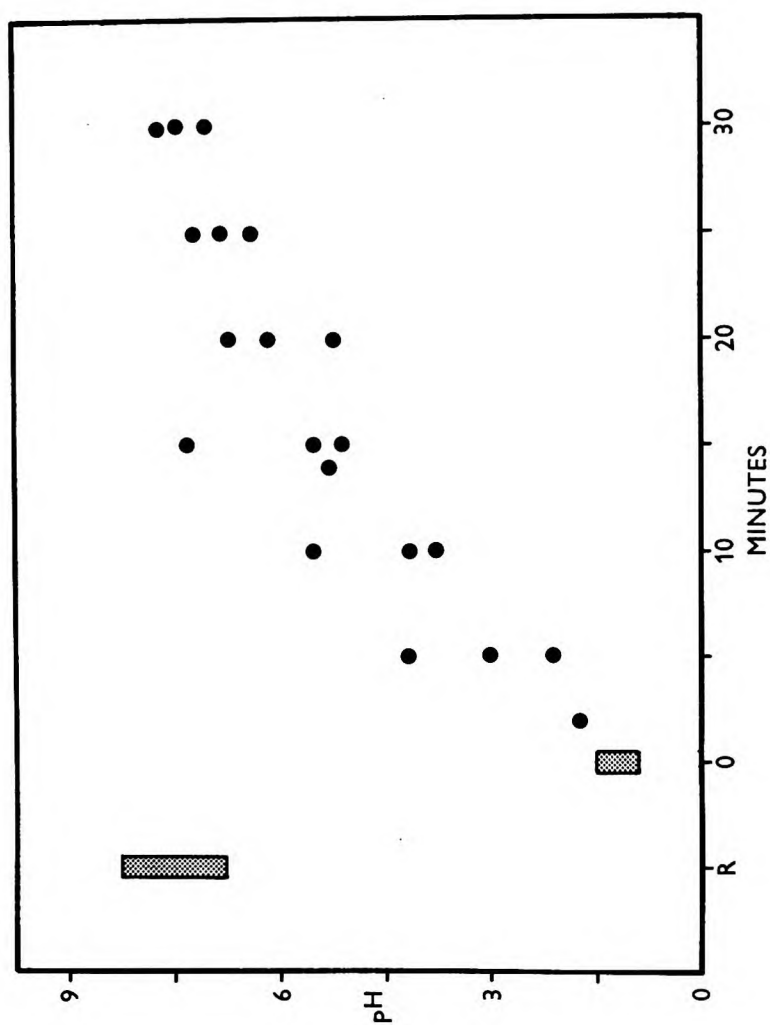


FIGURE 23: Forty experiments illustrating the change in luminal pH in the presence of 0.1 N HCl.
R = Resting pH (10 experiments) O = pH immediately following introduction of acid
(10 experiments).

venous effluent was examined, a striking increase was observed. In a series of experiments between 32 and 45 percent of the administered dose was transferred as compared with 2 percent to 6.5 percent in experiments in which iron was given with water and 9.0 percent to 25.5 percent in experiments in which 500 micrograms of ascorbic acid was added (Figure 24). The effect of 0.1 N HCl on the transfer of a 5 microgram dose of iron was also studied; a marked increase in transfer between 22 and 44 percent was noted in 5 experiments.

The same effect has been observed when 100 micrograms of iron were studied (Figure 25), and similar results were obtained with 500 micrograms of iron.

Further studies revealed that the effect of nitric acid was similar to that of hydrochloric acid (Figure 26). It was also shown that concentrations of acid greater than 0.1 N (35 experiments) did not produce an additional increase in the transfer of iron. With 0.01 N an intermediate effect was observed.

Transfer of Iron from Terminal Ileum

In 3 experiments, 5 micrograms were introduced into a 10 centimetre length of terminal ileum, and 0.75 percent, 1.25 percent and 2.25 percent of the administered dose was transferred into the venous effluent. Three control (duodenal) results obtained at the same

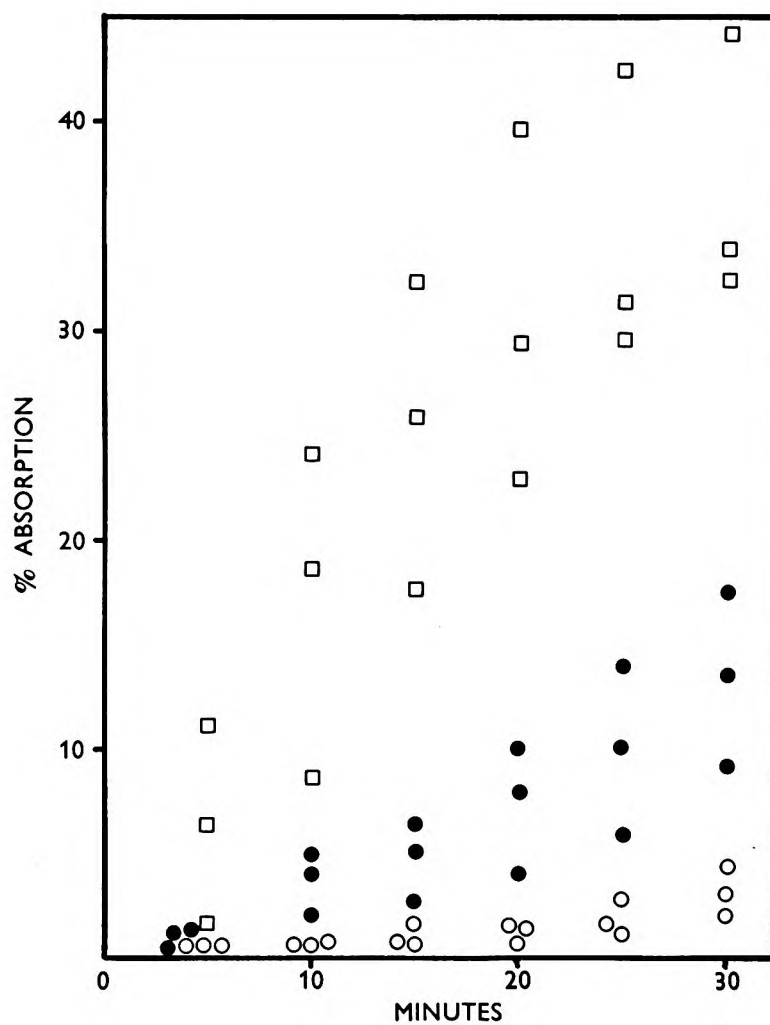


FIGURE 24

The results of a typical study illustrating the enhancing effect of 0.1 N HCl (open squares) and ascorbic acid (closed circles) on the transfer of 50 micrograms of iron (open circles).

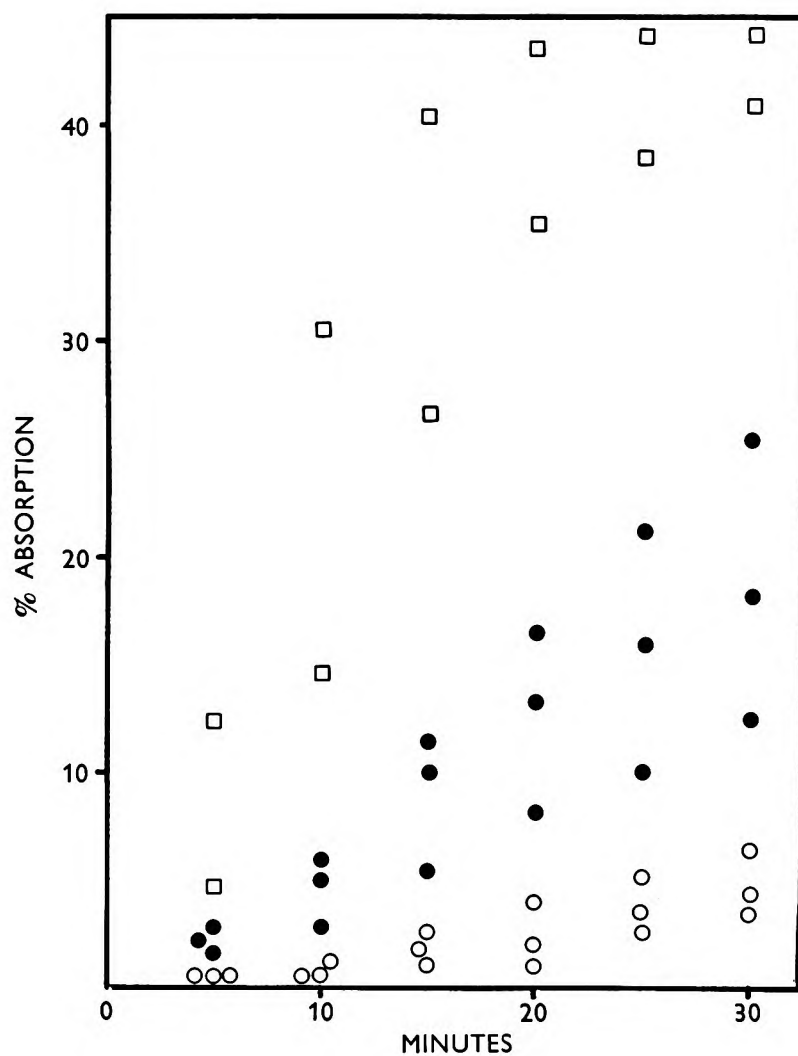


FIGURE 25

The results of a typical study illustrating the enhancing effect of 0.1 N HCl (open squares) and ascorbic acid (closed circles) on the transfer of 100 micrograms of iron (open circles).

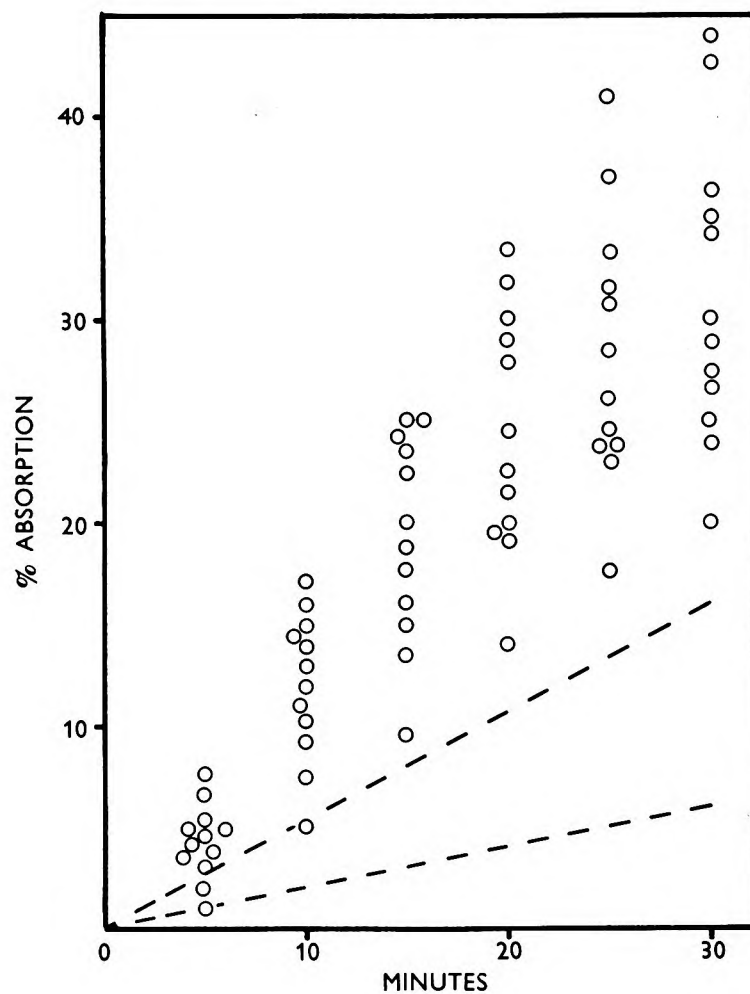


FIGURE 26

The results of 12 experiments illustrating the enhancing effect of nitric acid (open circles) on transfer of 5 micrograms of ferrous iron. Interrupted lines indicate range found in the absence of acid.

time were 5.4 percent, 6.5 percent and 8.4 percent. The addition of 50 micrograms of ascorbic acid resulted in an increase rate of transfer from the ileum (3.5 percent, 5.0 percent, 7.0 percent and 8.5 percent in 4 experiments).

When the iron was given in 0.1 N HCl there was an even greater increase in the amount transferred, with figures of 13.0 percent, 19.8 percent and 21.5 percent (Figure 27).

Absorption of Haemoglobin Iron

Studies could not be satisfactorily carried out using haemoglobin iron, since absorption of this organic molecule under these circumstances was negligible. Absorptions measured simultaneously in vivo indicated that the retention of 5 microgram, 50 microgram and 100 microgram quantities was very much less than that found for various inorganic salts.

Incorporation of Labelled Iron into Mucosal Ferritin

We have recently demonstrated (Charlton et al., 1965) that even with small doses of iron a significant proportion entering the mucosa is incorporated into ferritin when studied in vivo. However, it was discovered that the percentage of mucosal activity

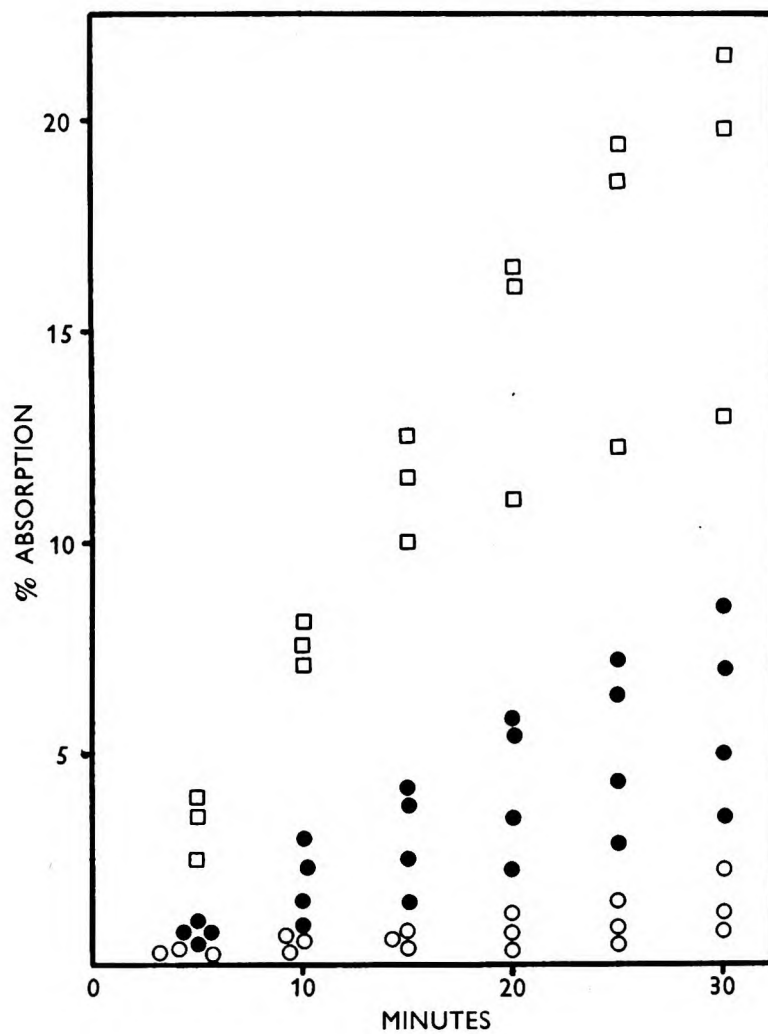


FIGURE 27

The results of a typical study illustrating the enhancing effect of 0.1 N HCl (open squares) and ascorbic acid (closed circles) on the transfer of 5 micrograms of iron (open circles) from the terminal ileum.

attached to ferritin was considerably smaller with this artificial preparation than at a comparable stage of absorption in vivo. An experiment was therefore devised to determine the reason for this difference.

Nineteen fasting rats were randomly assigned to three groups. The first was given the labelled dose of iron via an oesophageal cannula without anaesthetic. The second received the iron directly into an isolated 10 centimetre loop of duodenum at laparotomy under anaesthesia. Artificial preparations were manufactured from the remaining animals. The dose of iron was 5 micrograms without ascorbic acid in each case.

After 30 minutes groups 1 and 2 were killed, and the entire gastrointestinal tracts were removed without contaminating the carcasses with luminal contents. Absorption was then assessed by measuring the activity present in the carcasses using a small animal whole-body counter.

The proximal 10 centimetres of duodenum was freed from the remainder of the gastrointestinal tract in all three groups, and opened longitudinally. This segment was washed twice by shaking in a test tube with 0.01 M EDTA (pH 7.0) in 0.9 percent NaCl solution and then centrifuging.

The activity present in the washed duodenal segments was determined and labelled ferritin content was then assessed after homogenisation.

The results are set out in Table XII. In the two in vivo groups, the amount of labelled ferritin was similar, but this formed a smaller fraction of the mucosal activity in the animals in which a duodenal loop was isolated, because a much greater percentage of the administered dose was present in the washed gut. Some of this extra Fe^{59} was probably attached to mucosal cell membrane, since it was present in the centrifuged deposit after homogenisation. However, even though a mean mucosal activity in this group was almost three-fold greater, absorption (represented by the radio-activity present in the remainder of the carcasses) was similar to that of group one.

The findings in the artificial preparations resembled those in group two, except that both absorption and mucosal ferritin labelling was decreased. This presumably reflects the imperfect duplication of physiological conditions. As in group two, however, the major reason for the low percentages of mucosal activity attached to ferritin was the high proportion of the labelled dose of iron present in a washed duodenal segment.

TABLE XII

Incorporation of Fe⁵⁹ into Mucosal Ferritin During Absorption Under Different Conditions

Group	% dose absorbed	% dose in washed duodenum	% dose in deposit from homo-genised duodenum	% dose in duodenum as ferritin	Ferritin as % washed duodenum
1	30	6.8	1.4	3.0	44
iron administered	15	6.1	1.2	2.0	33
by oesophageal	2	1.5	0.4	0.5	33
catheter to	15	11.4	2.6	6.2	54
anaesthetised	2	2.6	0.5	1.0	38
animals	21	4.7	0.9	1.7	36
	2	3.8	0.7	1.4	37
Means	12.4	5.3	1.1	2.3	39
2	24	11.2	2.7	2.5	22
Iron introduced	15	15.3	4.4	2.8	18
into isolated	14	12.9	6.1	0.9	7
duodenal loop	15	16.3	4.3	0.7	4
at laparotomy	3	10.3	2.5	2.1	20
under anaesthesia	11	21.7	6.2	3.5	16
Means	13.7	14.6	4.4	2.1	14.5
3	5	17.2	6.4	1.9	11
Artificial	10	13.7	2.2	0.9	7
preparation	0,5	5.8	1.2	0.8	14
	12	14.6	3.7	1.1	8
	8	15.2	2.2	0.3	2
	8	20.4	6.8	0.8	4
Means	7.2	14.5	3.8	1.0	8

Effect of In Vivo Manipulations on Subsequent Performance

Eight animals were randomly assigned in two groups, one of which was fed the stock diet and the other a diet with a very low iron content. After 14 days the transfer of 5 micrograms ferric iron was assessed. The figures in the control group were 4.8 percent, 10.0 percent, 11.7 percent and 15.5 percent, while those in the low iron diet group were 13.0 percent, 19.0 percent, 20.7 percent and 34.6 percent (Figure 28).

Transfer was also studied in animals venesected 4 and 2 days previously. The figure in 11 control animals was 1.9 percent (range 0.3 to 4.7 percent) compared with 7.4 percent (range 1.9 to 12.7 percent) in the 12 venesected animals (Figure 29). A similar effect has been noted in 15 animals made anaemic with phenylhydrazine (Figure 30).

The converse effect has been demonstrated in 24 animals who have previously been loaded with an iron-sorbitol-citric-acid complex (Figure 31).

The Effect of Anoxia

The transfer of labelled iron into the venous effluent was

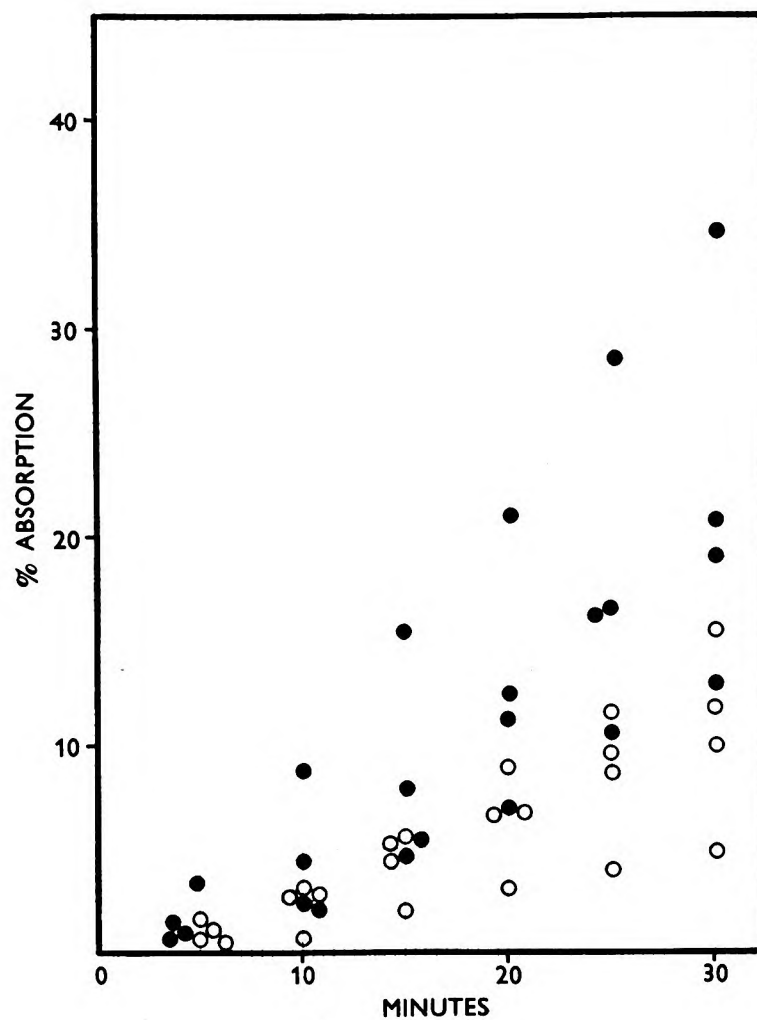


FIGURE 28

The results of a study illustrating the greater transfer of 5 micrograms of iron by animals previously on low iron diet (closed circles) compared to those fed a stock diet (open circles).

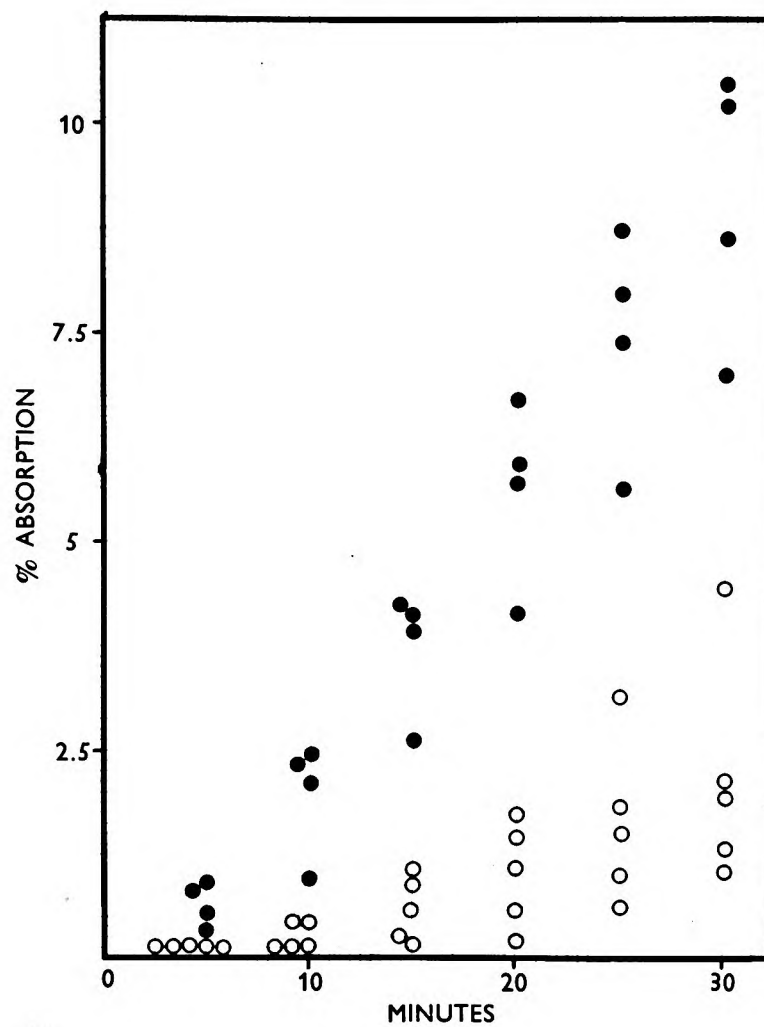


FIGURE 29

The results of a typical study illustrating the greater transfer of 5 micrograms of iron in animals previously venesected (closed circles) compared to normals (open circles).

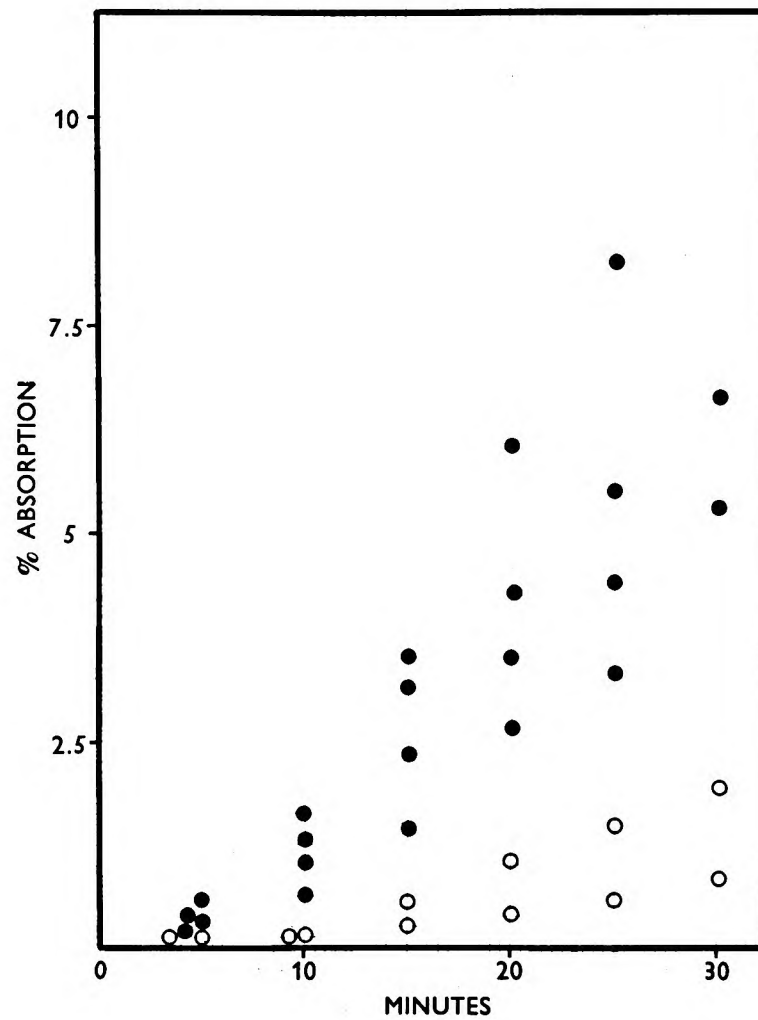


FIGURE 30

The results of a typical study illustrating the enhancing effect of acute haemolysis (closed circles) on the transfer of 5 micrograms of iron (open circles). Note that two experiments were terminated at 25 instead of 30 minutes.

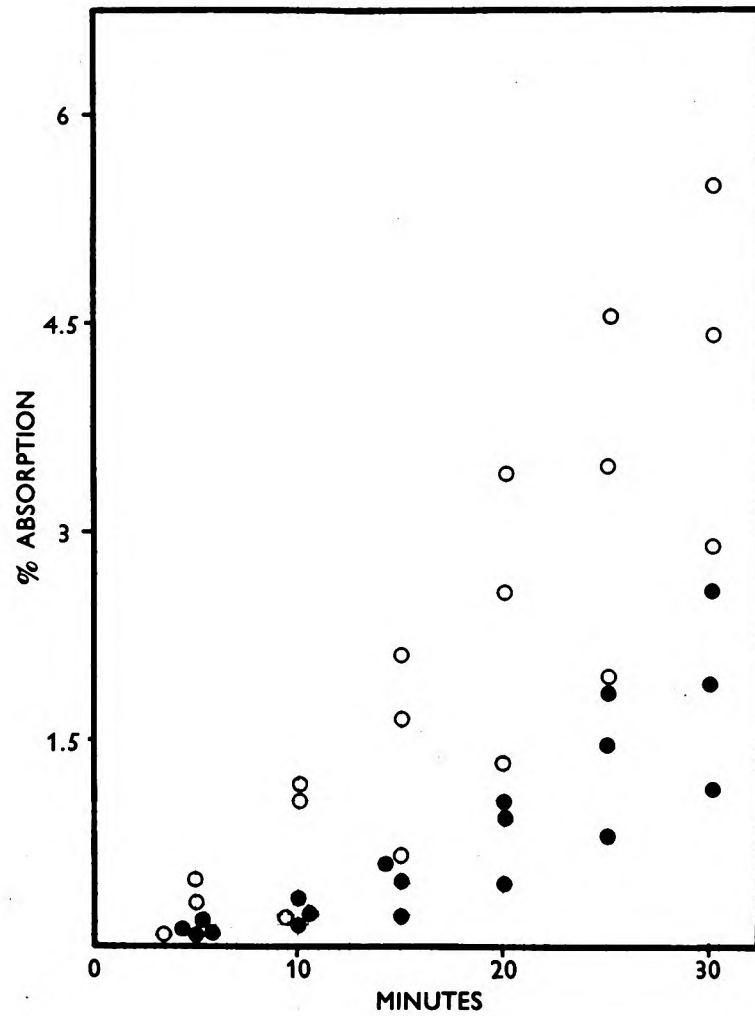


FIGURE 31

The results of a typical study illustrating the effect of previous iron loading (closed circles) on transfer of 5 micrograms of iron (open circles).

always depressed under anoxic conditions. With 50 micrograms of iron plus 500 micrograms of ascorbic acid, the mean percentage transferred in 5 anoxic experiments was 4.9 percent (range 4.0 percent to 6.8 percent), as compared with 14.5 percent (range 10.7 percent to 19.6 percent) in 6 control experiments (Figure 32). A similar effect has been noted with 5 microgram doses (Figure 33) and also with ferric iron.

When the transfer of 50 micrograms iron with ascorbic acid in 0.1 N HCl was studied, anoxia once again resulted in a considerable depression, with little or no further increase in the amount transferred after 15 minutes (Figure 34). In 4 experiments the figures were between 6.0 percent and 14.5 percent, compared with control results of 34 percents to 41 percent.

The Effect of Metabolic Poisons

The transfer of 50 micrograms of iron given with 500 micrograms of ascorbic acid was found to be considerably depressed by azide, cyanide and iodoacetamide. When 10^{-3} M sodium azide was present the mean percentage transferred in 9 experiments was 4.5 percent (range 1.0 percent to 7.7 percent), compared with the mean control figure of 15.3 percent (range 11.5 percent to 20.0 percent) (Figure 35).

With 10^{-3} M sodium cyanide the mean figure for 7

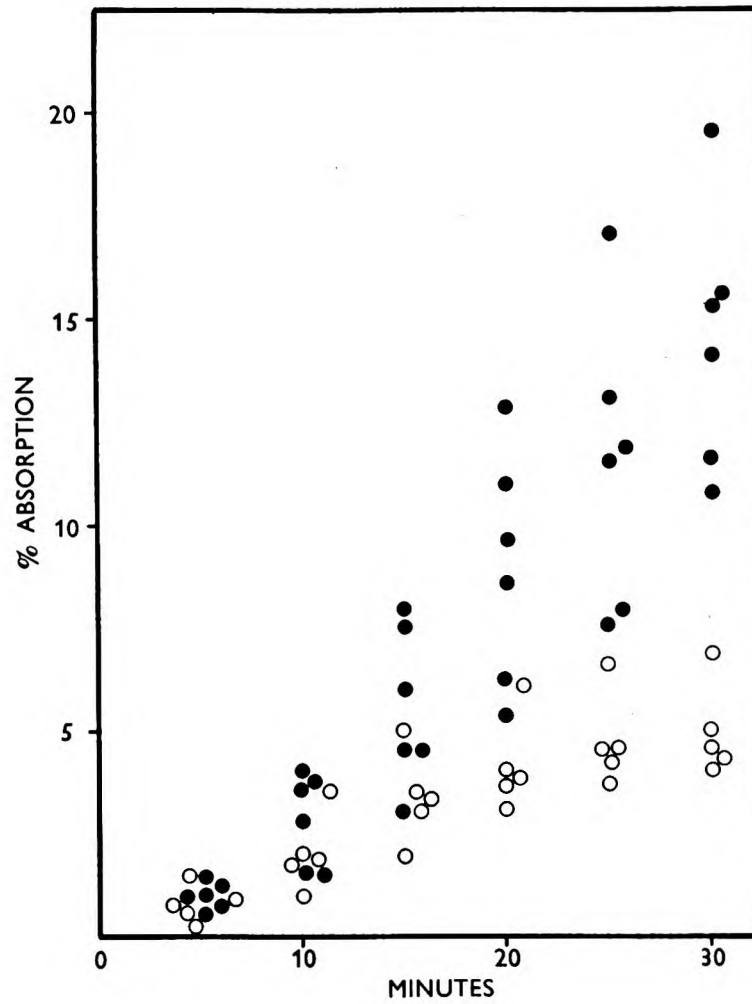


FIGURE 32

The results of 11 experiments illustrating the depressing effect of anoxia (open circles) on the transfer of 50 micrograms of ferrous iron (closed circles).

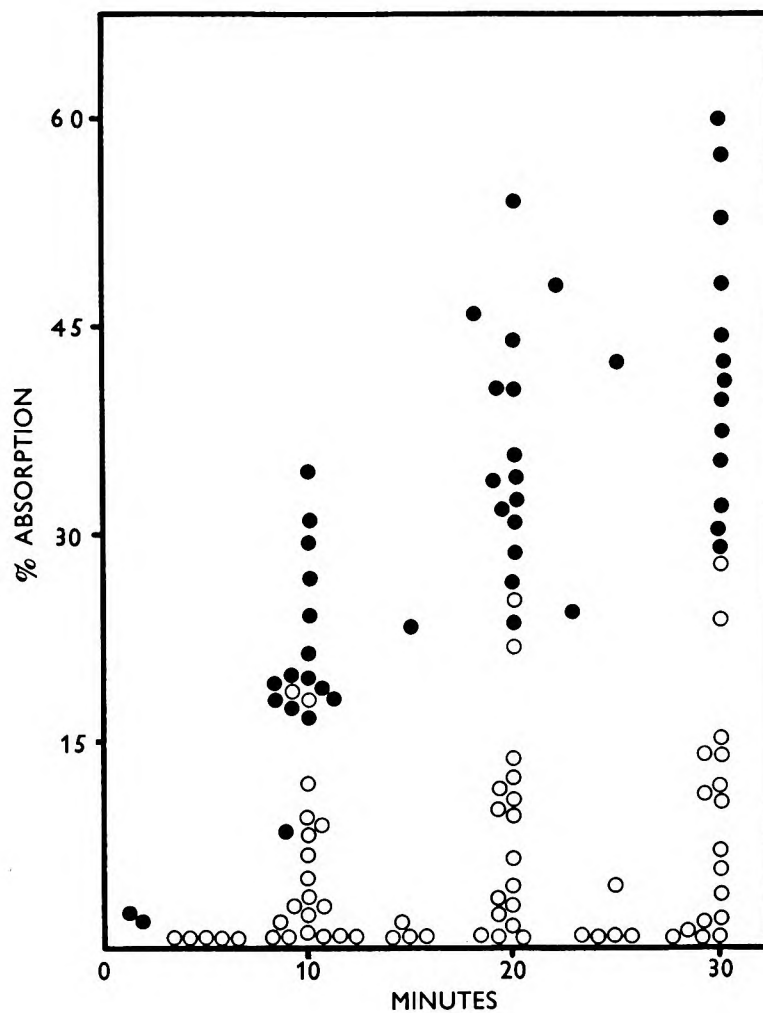


FIGURE 33

The results of 30 experiments illustrating the uniform depression brought about by anoxia (open circles) on the transfer of 5 microg of iron (closed circles).

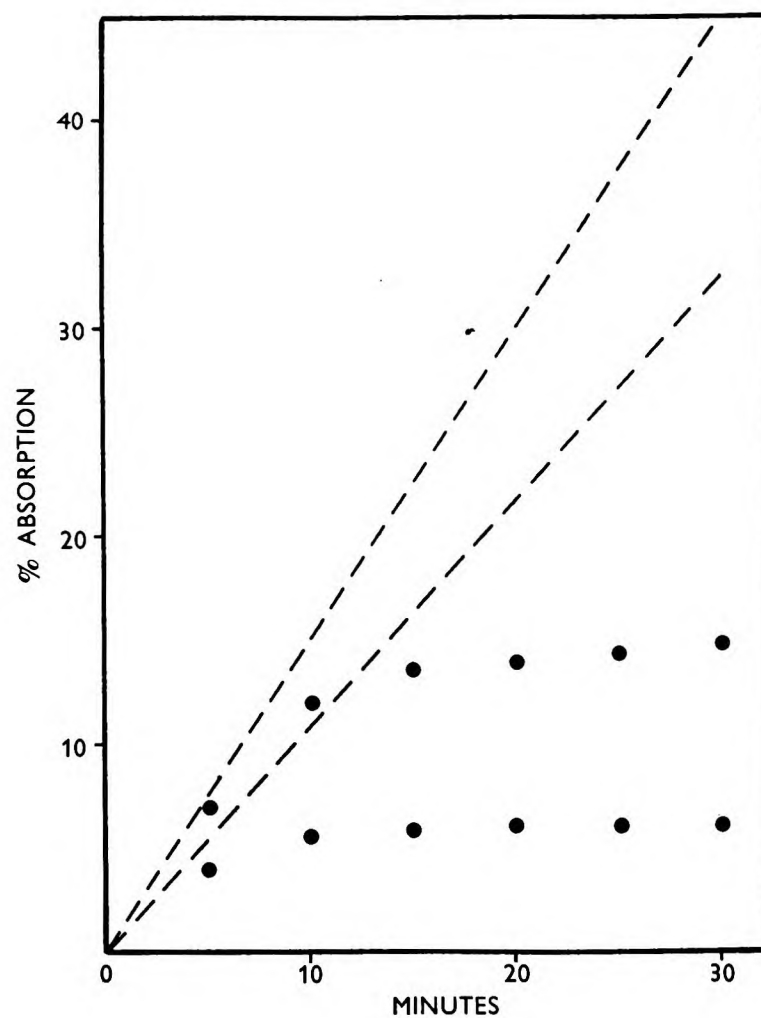


FIGURE 34

The results of a typical study illustrating the depressing effect of anoxia (closed circles) on the transfer of 50 micrograms of iron in the presence of 0.1 N HCl. Interrupted lines indicate range found when buffer was oxygenated.

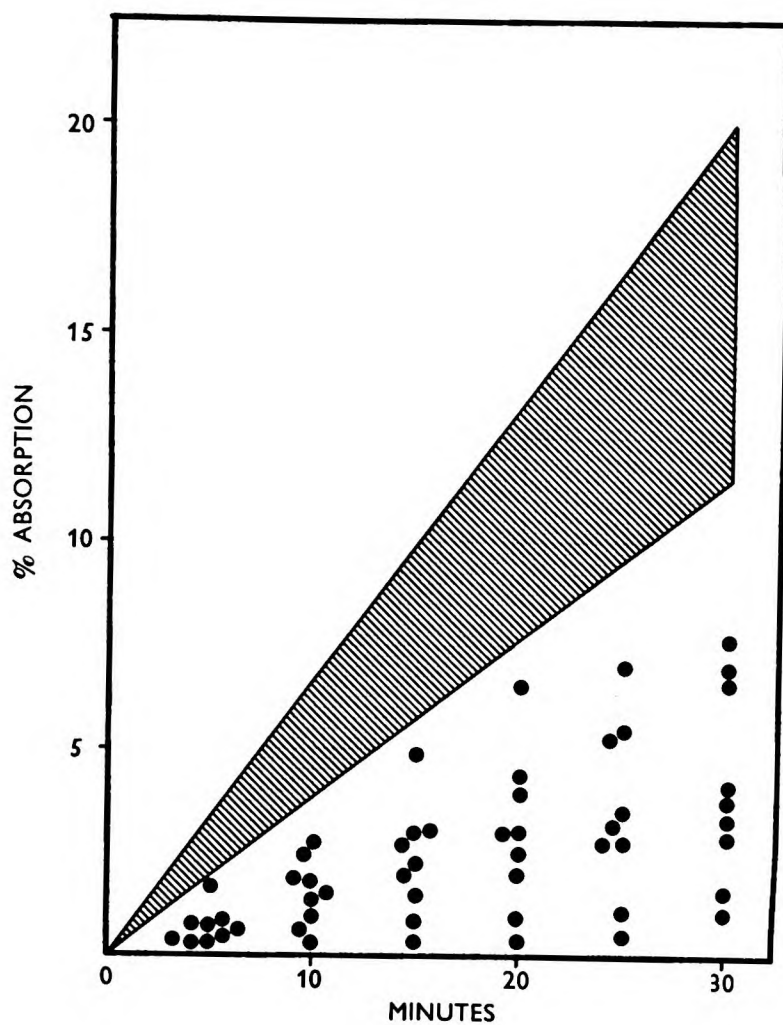


FIGURE 35

The results of 9 experiments illustrating the depressing effect of 10^{-3} M sodium azide (closed circles) on the transfer of 50 micrograms ferrous iron (hatched area).

experiments was 3.9 percent (range 1.0 to 9.5 percent)(Figure 36).

When the effect of 5×10^{-4} M iodoacetamide was assessed, the transfer in 5 control experiments was greater than previously, the figures being 12.0 percent, 29.7 percent, 35.3 percent, 36.0 and 55.0 percent. In this context, the percentages obtained with iodoacetamide were considered to be reduced (5.5. percent, 10.5 percent, 11.5 percent, 15.5 percent and 16.0 percent) (Figure 37). Further confirmatory figures were obtained at a later date. At this time, control results were 7.0 percent, 9.1 percent, 10.3 percent and 16.9 percent, and those with the iodoacetamide 0.9 percent, 4.1 percent and 5.2 percent (Figure 38).

When 2:4 dinitrophenol was studied, only a concentration of 10^{-4} M could be tested because of its low solubility. No clear-cut difference was noted; control figures were 25.5 percent, 29.4 percent, 35.6 percent, 37.5 percent, 39.5 percent, 43.0 percent and 55.0 percent, while with dinitrophenol the percentages were 14.2 percent, 22.2 percent, 23.4 percent, 26.4 percent and 38.2 percent (Figure 39).

Effect of Metabolic Substrates and Methylene Blue

Although glucose and insulin were present in the perfusing

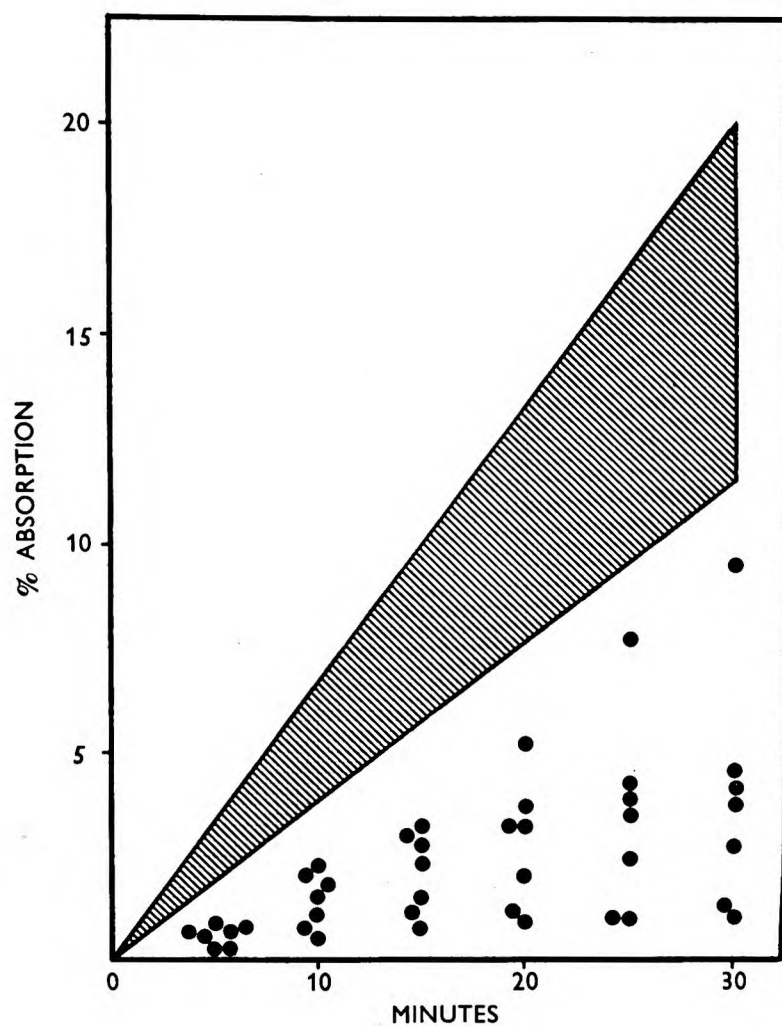


FIGURE 36

The results of 7 experiments illustrating the depressing effect of 10^{-3} M sodium cyanide on the transfer of 50 micrograms of ferrous iron (hatched area).

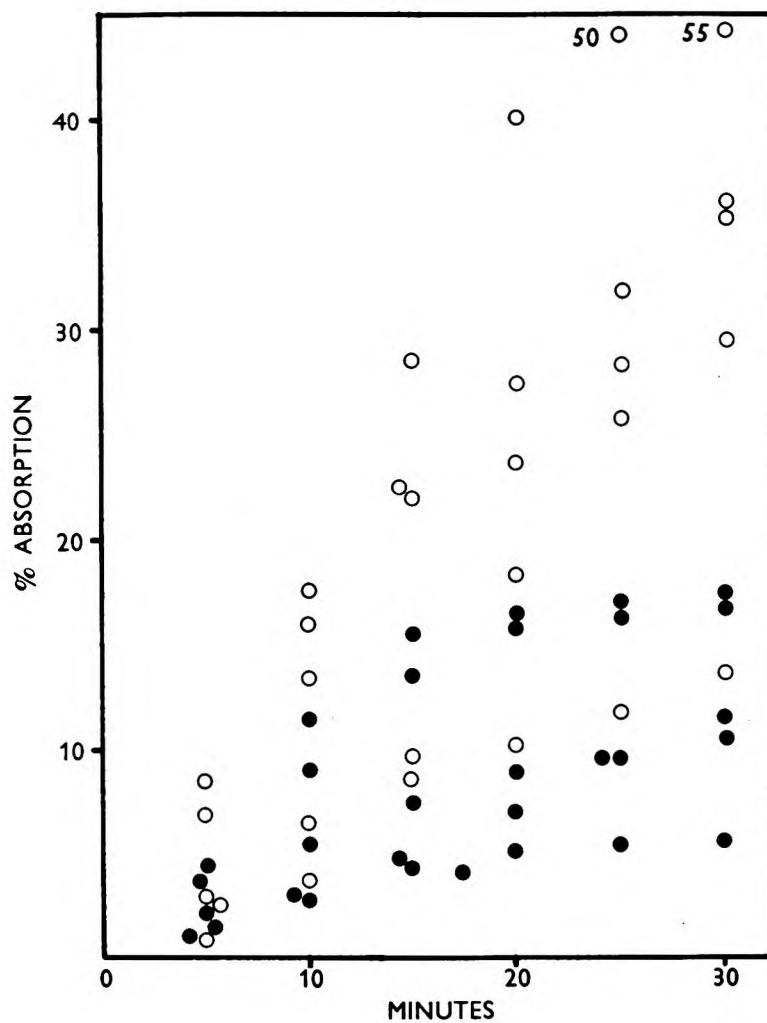


FIGURE 37

The results of a typical study illustrating the depressing effect of 5×10^{-4} M iodoacetamide (closed circles) on the transfer of 50 micrograms ferrous iron (open circles).

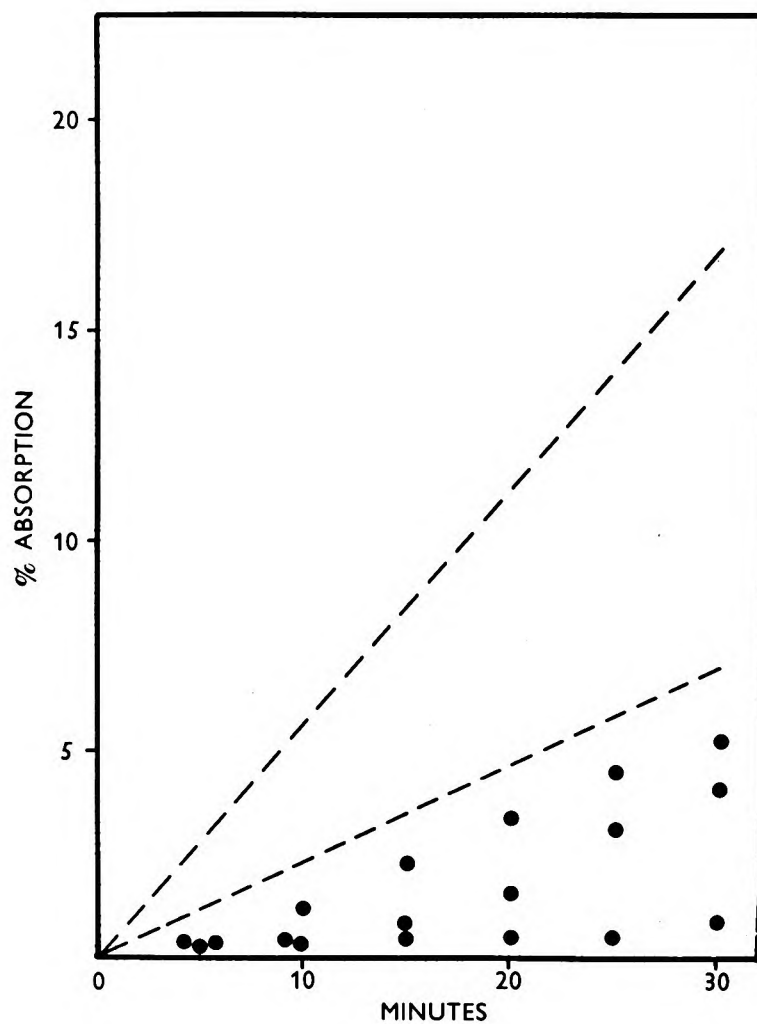


FIGURE 38

The results of 3 additional experiments illustrating the depressing effect of 5×10^{-4} M iodoacetamide (closed circles) on the transfer of 50 micrograms ferrous iron. The interrupted lines represent the range for absorption in the absence of iodoacetamide.

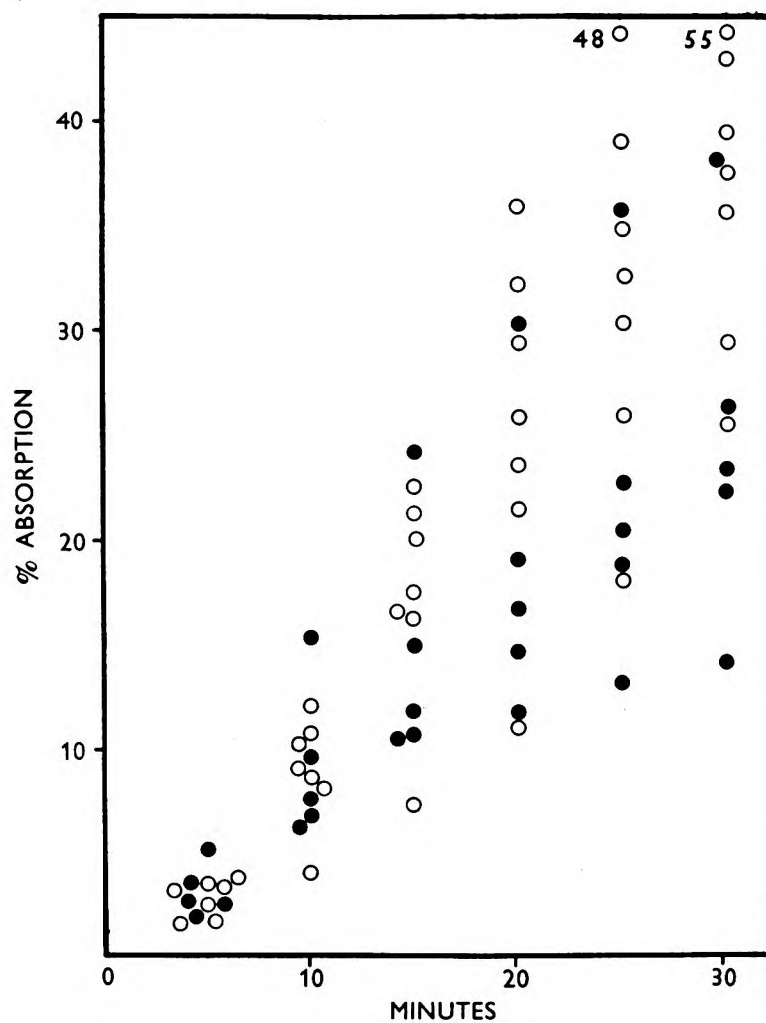


FIGURE 39

The results of 12 experiments illustrating that 2:4 dinitrophenol (closed circles) did not significantly influence the absorption of 50 micrograms of ferrous iron (open circles).

medium in all the experiments, diminished transfer of iron over 30 minutes could not be demonstrated when they were omitted.

In a study, using 50 micrograms of iron without ascorbic acid, the mean transfer in 7 experiments with glucose and insulin was 3.4 percent (range 2.2 to 4.5 percent) as compared with 3.7 percent (range 1.3 to 6.1 percent) in 7 experiments in which they were not present (Figure 40). Furthermore, no difference was obtained in the presence of ascorbic acid or when studies were prolonged to 90 minutes.

These results are interesting because, while no direct effect of insulin upon iron transfer has been reported, glucose is thought to influence this process (Manis and Schachter, 1962 a); in addition, these workers have suggested that mannose is more effective than glucose in this respect. However, when mannose in the same concentration was substituted for glucose, transfer was not enhanced using this system. Thus, in 3 experiments with mannose the figures were 1.9 percent, 4.1 percent and 6.8 percent, compared with control figures of 2.9 percent, 4.6 percent and 5.1 percent.

The addition of 10^{-3} M alpha-ketoglutaric acid increased

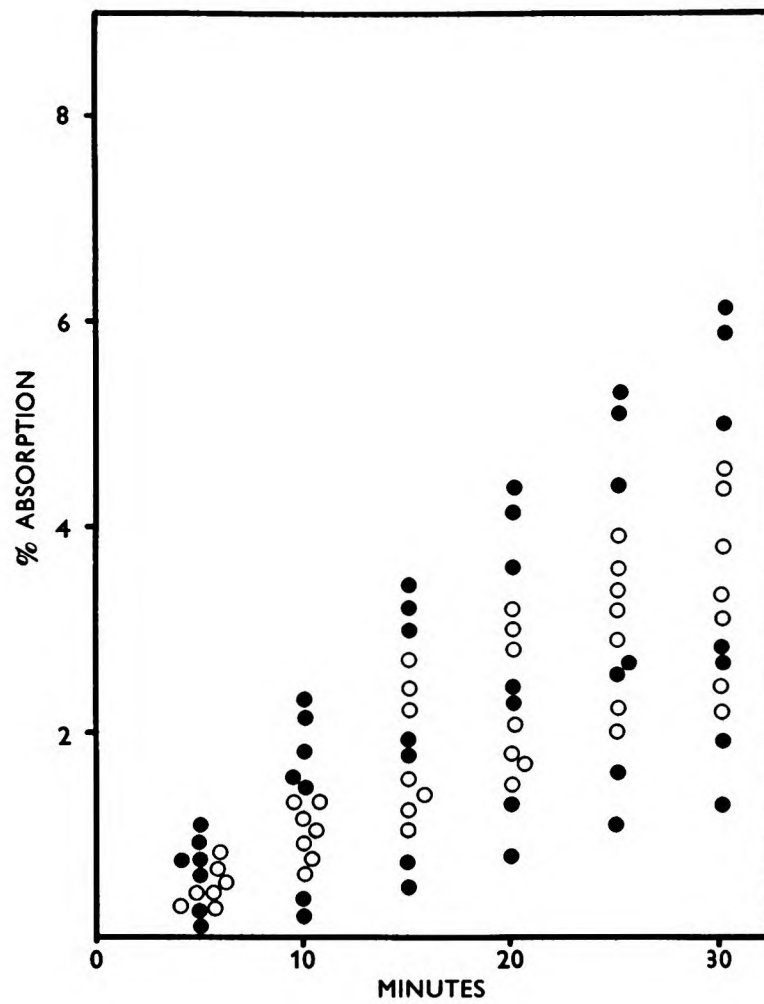


FIGURE 40

The results of a study illustrating that the transfer of 50 micrograms of iron was unaffected by the presence of glucose and insulin (open circles), or its absence (closed circles).

the percentage transfer when 5 micrograms of iron were studied without ascorbic acid. The mean of 7 experiments was 5.2 percent (range 3.1 percent to 8.2 percent), compared with the figure of 2.8 percent (range 0.9 percent to 4.3 percent) in 7 controls (Figure 41). A similar effect was noted with 50 micrograms of iron (Figure 42).

Further studies with other substrates such as oxaloacetate (Figure 43), succinate (Figure 44) and pyruvate (Figure 45) in concentrations of 10^{-3} M provided equivocal results. However, transfer was studied in 24 animals and found to be accelerated in the presence of 10^{-3} M citrate (Figure 46).

When methylene blue was present in the perfusing fluid at a concentration of 10^{-4} M, the transfer of 50 micrograms ferric iron was found to be enhanced. The mean of 9 experiments was 9.3 percent (S.E. $\pm$ 0.6) compared with control figures of 4.2 percent (S.E. $\pm$ 0.8) in 7 experiments (Figure 47).

DISCUSSION

In the present study it was found that the alteration of conditions within the lumen of the intestine produced effects upon the transfer of iron into the perfusing fluid resembling those observed in vivo. When the quantity of iron in the lumen was increased, the

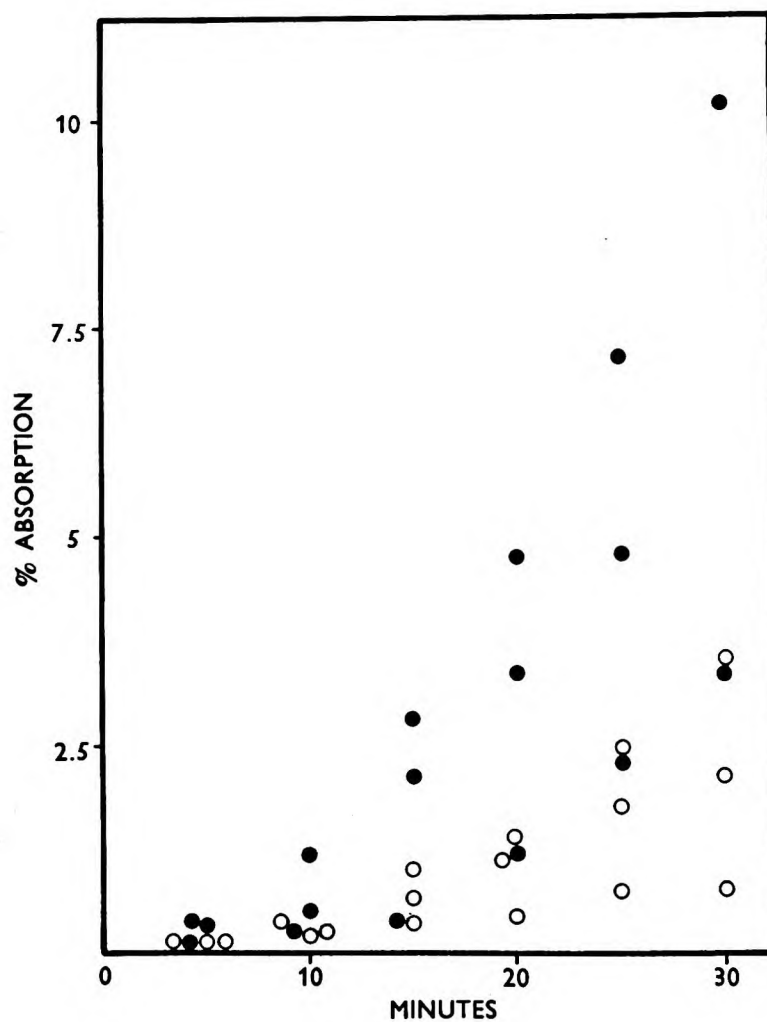


FIGURE 41

The results of a typical study illustrating the enhancing effect of 10^{-3} M alpha-ketoglutaric acid (closed circles) on the transfer of 5 micrograms of iron (open circles). Note that one experiment was terminated at 25 instead of 30 minutes.

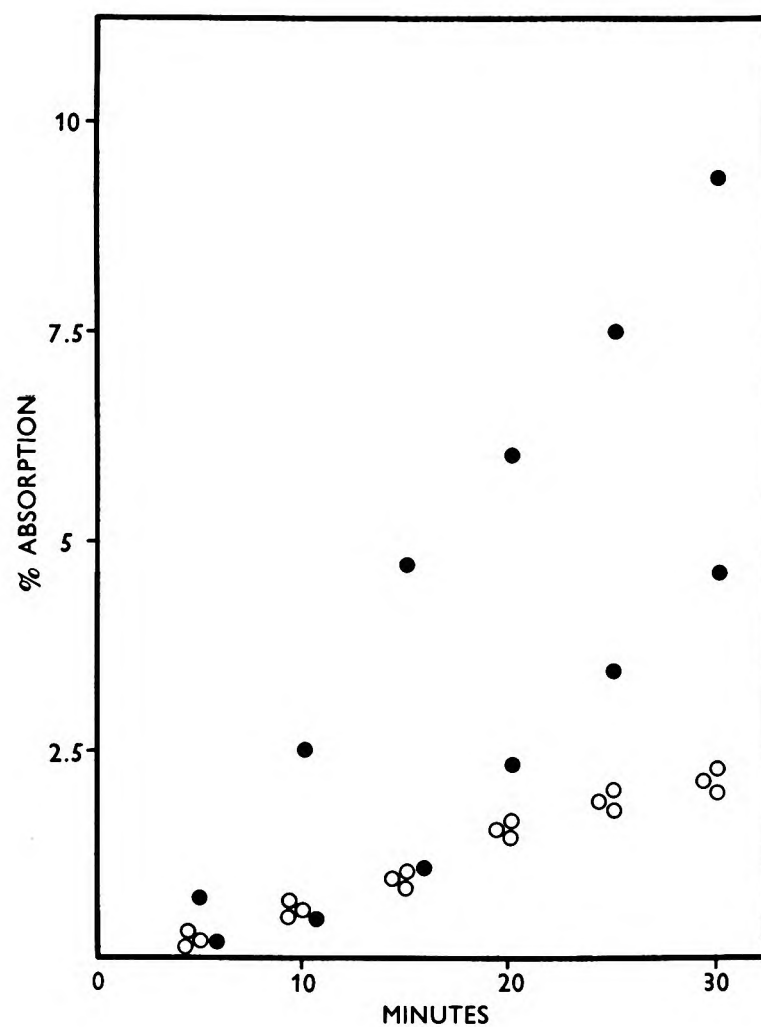


FIGURE 42

The results of a typical study illustrating the enhancing effect of 10^{-3} M alpha-ketoglutaric acid (closed circles) on the transfer of 50 micrograms of iron (open circles).

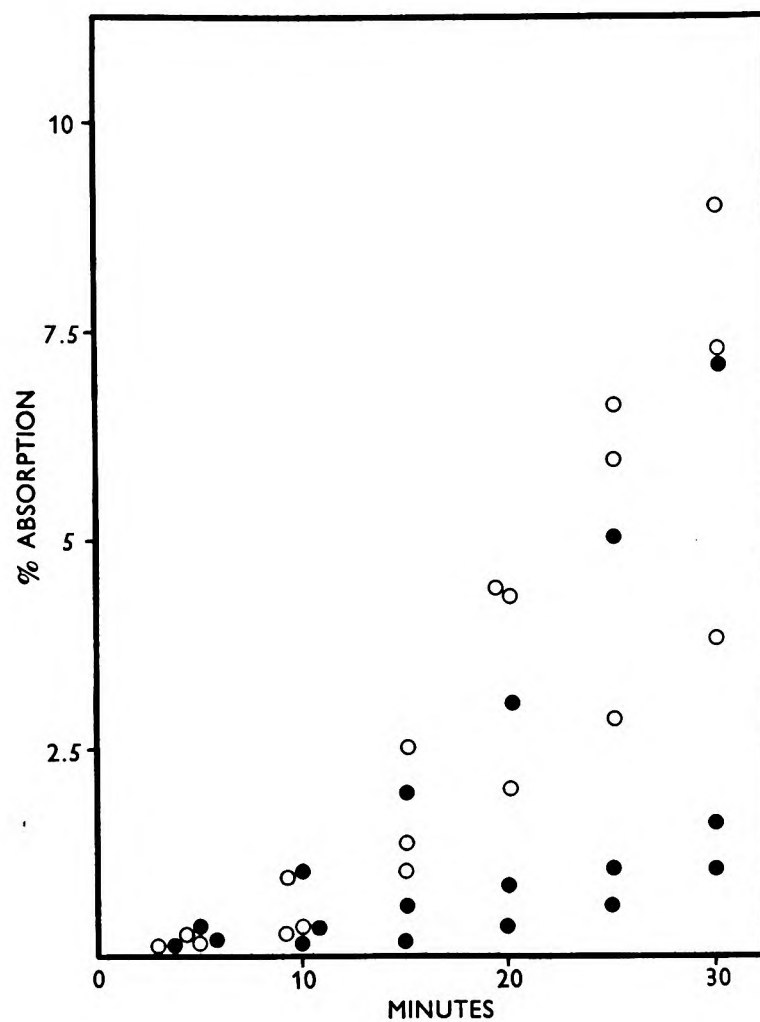


FIGURE 43

The results of 6 experiments illustrating that 10^{-3} M oxaloacetate (closed circles) did not significantly influence the transfer of 5 micrograms of iron (open circles).

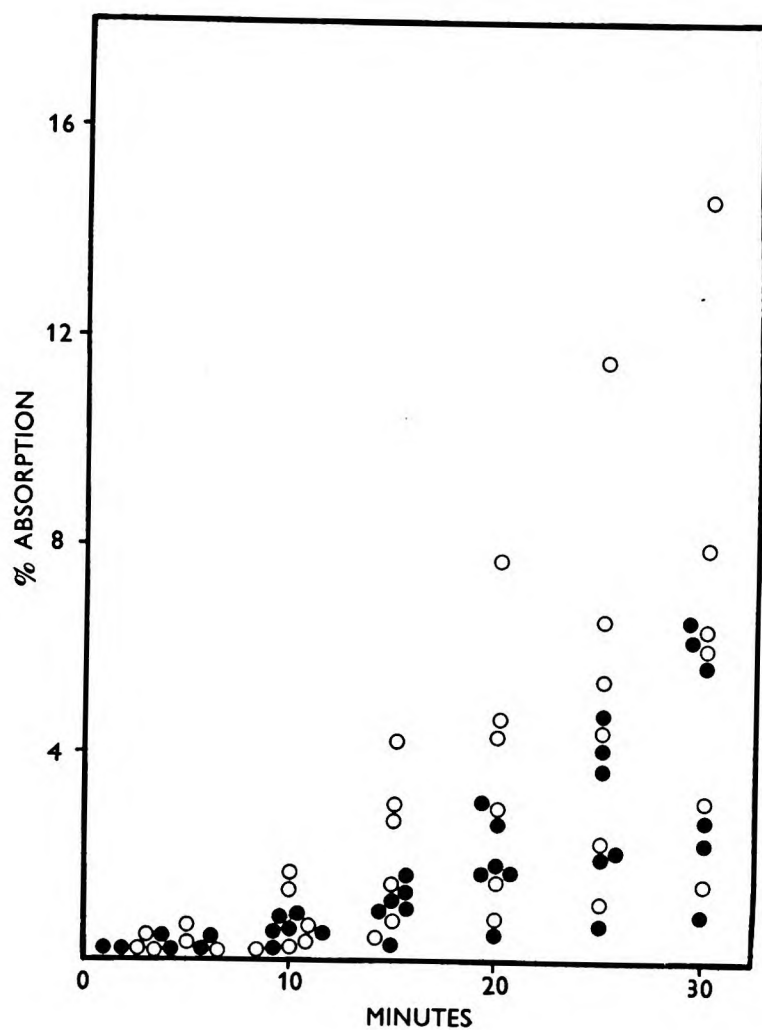


FIGURE 44

The results of 12 experiments illustrating that 10^{-3} M succinate (open circles) did not significantly influence the transfer of 5 micrograms of ferric iron (closed circles).

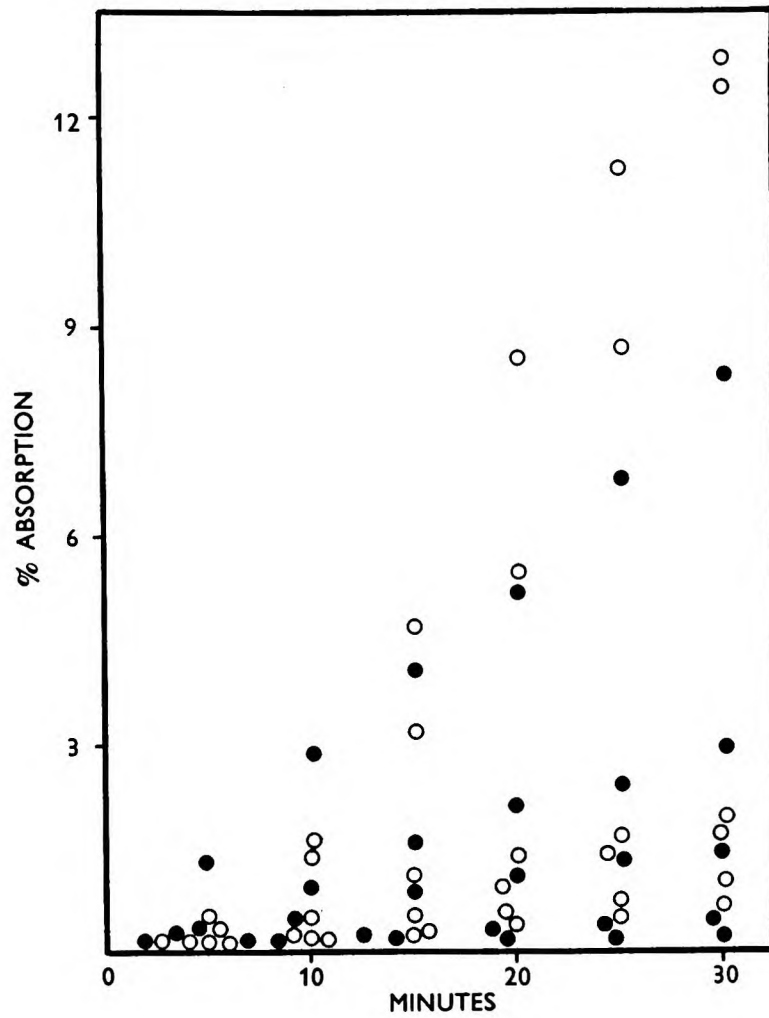


FIGURE 45

The results of 11 experiments illustrating that 10^{-3} M pyruvate (closed circles) did not significantly influence the transfer of 5 micrograms of iron (open circles).

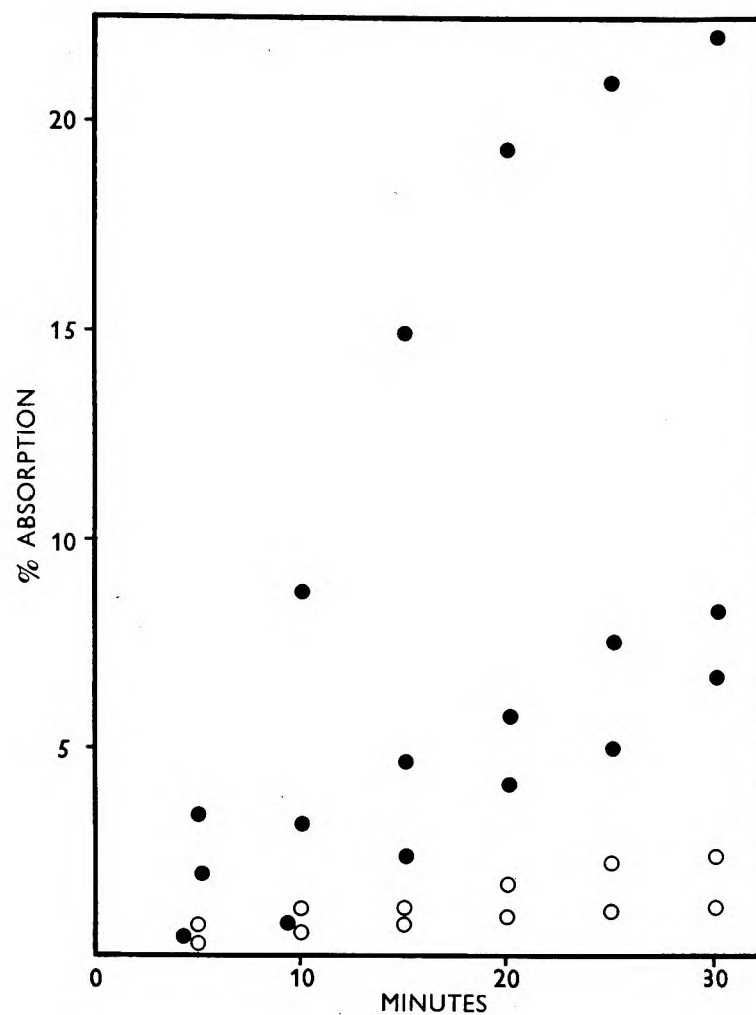


FIGURE 46

The results of a typical study illustrating the enhancing effect of 10^{-3} M citrate (closed circles) on the transfer of 5 micrograms of iron (open circles).

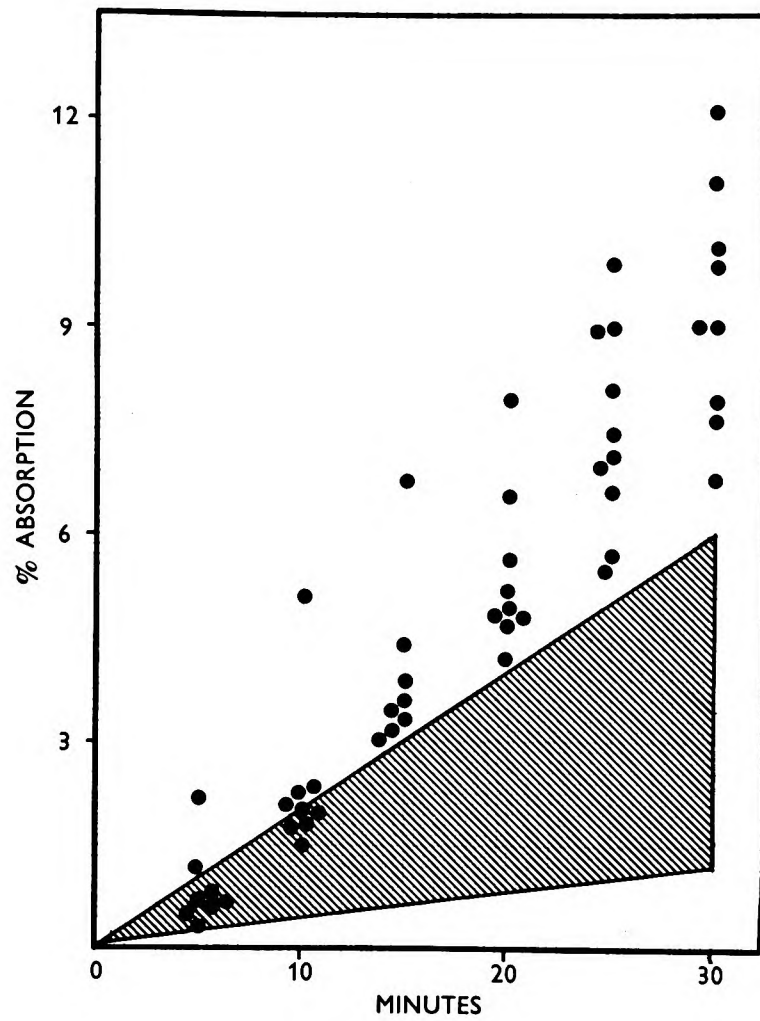


FIGURE 47

The results of 9 experiments illustrating the enhancing effect of methylene blue (closed circles) on the transfer of 50 micrograms of iron (hatched area).

amount transferred rose although the percentage fell. This contrasts with the experience of Manis and Schachter (1962 a) using the isolated pouch technique. These workers reported that transfer to the serosal surface did not continue to increase when the iron concentration was raised above 0.1 micromole per millilitre, i.e. a 14 microgram dose of iron.

The addition of ascorbic acid markedly enhanced the transfer of iron. While there is an abundance of evidence that iron in the ferrous form is more freely absorbed than ferric iron, it seemed possible that some of the enhancement might have been due to the reduction in pH of the luminal contents produced by the ascorbic acid, since the transfer of iron was found to be considerably increased when the pH was lowered with hydrochloric or nitric acids. Results in keeping with the present observations have been obtained in an in vivo study on the effects of hydrochloric acid in iron absorption (Chapter IV). It was found that the absorption of ferric iron was enhanced when given with acid to achlorhydric subjects. The effect with ferrous iron was much less marked while the absorption of haemoglobin iron was not increased by acid at all.

When the labelled dose of iron was introduced into the terminal ileum instead of the duodenum, transfer was much less

efficient. Similar results have also been reported by other workers (Manis and Schachter, 1962 a). It was noteworthy, however, that the addition of ascorbic acid or hydrochloric acid increased the amount transferred, suggesting that luminal factors, such as pH, account for some of the difference between the absorbing capacity of these regions of the intestine.

It was of interest that the transfer of iron by the artificial preparation could be modified by various procedures, such as venesection, carried out in vivo before hand. Since the fluid circulating during the iron transfer was the artificial perfusing medium, this suggests that the altered handling of iron by the preparation was not a function of any constituent of the plasma. The changed behaviour might therefore be due either to alterations in the luminal conditions, or to modified mucosal cell function. The second possibility seems the more likely one since Manis and Schachter (1962 a) have demonstrated similar changes using an isolated gut sac preparation in which the bathing luminal fluid was artificial.

This evidence adds further weight to previous in vivo findings in rats which suggest that the mucosal epithelium is able to exert some control over iron absorption. It has been shown that under certain circumstances most of the iron entering the cells of the

absorbing mucosa does not reach the plasma, but is held within the cells until they exfoliate (Conrad and Crosby, 1963; Wheby and Crosby, 1963; Charlton et al., 1965). Evidence has been presented that this incarcerated iron is incorporated into the storage compound ferritin (Charlton et al., 1965). On the other hand, when additional iron is needed by the body (e.g. if body stores are depleted), nearly all the iron taken up from the lumen rapidly crosses into the plasma, and little is incorporated into ferritin within the mucosa.

When ferritin labelling was studied in the artificial system, however, it was noticed that the proportion of mucosal activity present in this form was considerably less than would have been anticipated at a comparable stage of absorption in vivo. On investigation it was shown that this was largely due to the presence of a much greater proportion of the administered iron in the washed mucosa than that found during normal absorption (Table XII). A similar phenomenon was observed in vivo when the labelled dose of iron was introduced into an isolated duodenal loop under anaesthesia. Some of the additional iron in the mucosa in both groups was probably attached to cell membranes, since it was present in the centrifuged deposit after homogenization. It is possible that the extra supernatant activity was also non-specifically attached, and was freed by the

homogenization procedure. On the other hand, it may be that there was a true increase in the amount of iron entering the mucosal cells without a concomitant increase in either the amount crossing the mucosa into the plasma or the amount of ferritin formed. If this is so, then it suggests that the mucosal mechanism for controlling absorption may not reside in the absorbing cells' ability to incorporate unwanted iron into ferritin.

For a number of years there has been controversy as to whether the movement of iron across the mucosa and into the plasma during absorption is an active metabolic process or merely a passive diffusion. The evidence obtained with the present artificial preparation supports the concept that it is an active process. This evidence includes the increased transfer observed in the presence of methylene blue, the diminution which was noted with poisons such as cyanide, azide and iodoacetamide, and the effects of anoxia. Not only was there a consistent reduction in the amount of iron transferred during 30 minutes perfusion under anoxic conditions, but also a progressive slowing of the rate of transfer as the period of anoxia lengthened.

The metabolism of rat intestinal epithelial cells has recently been studied, and a high rate of aerobic and anaerobic glycolysis found (Stern and Reilly, 1965). It was shown that oxygen

consumption was increased by the addition of succinate, pyruvate, citrate, fumarate and lactate, although glutamate and acetate did not produce this effect. Iodoacetate in a concentration of 5×10^{-3} M decreased the consumption of oxygen by 50 percent, and abolished the production of lactic acid. While the relevance of these observations to iron absorption remains to be elucidated, there is limited evidence to suggest that the transfer of iron across the intestine is affected by certain substrates. For example, Dowdle et al. (1960) found that iron transfer by the everted gut sac preparation is enhanced by the substitution of fructose for glucose in the medium bathing the mucosal and serosal surfaces, and Manis and Schachter (1962 a) reported that mannose is even more effective than fructose. It has also been shown that absorption in vivo is enhanced by the administration of fructose with the labelled dose of iron (Pollack, Kaufman and Crosby, 1964 c; Stitt et al., 1962). However, in the present study the substitution of mannose for glucose in the perfusing fluid did not increase the transfer of iron. When other substrates were investigated, transfer appeared to be increased by the addition of alpha-ketoglutaric acid and citric acid, but results were equivocal with oxaloacetic acid and succinic acid. No impairment in transfer could be shown, however, when all substrates were omitted from the perfusate. A possible explanation for the failure to demonstrate substrate dependence may

be the relatively brief period during which transfer was observed.

SUMMARY

A method is described for studying iron absorption in isolated perfused segments of rat intestine using an artificial circulation. After cannulating the aorta and the portal vein in rats, buffered physiological solution containing dextran was circulated through the intestinal vascular system. Iron labelled Fe^{59} was introduced into an isolated duodenal loop, and the transfer into the venous effluent studied.

The effect of varying the dose and valency of the iron in the lumen was found to be similar to previously reported observations upon iron absorption in vivo.

When the pH of the luminal contents was lowered, transfer was markedly increased. Anoxia and several metabolic poisons depressed iron transfer, while methylene blue, alpha-ketoglutaric acid and citric acid increased it. It was therefore concluded that iron transfer across the intestinal mucous membrane is an active metabolic process.

Previous in vivo manipulation of the test animals, such as

venesection or alteration of the iron content of the diet, influenced the behaviour of the artificial preparation. These observations may, therefore, be added to the existing evidence indicating that a degree of control over iron absorption resides in the mucosal cells of the upper small intestine.

CHAPTER VIII

CONCLUDING REMARKS

The experimental work which forms the basis of this thesis is presented in two parts. The first section is devoted to a discussion of the luminal factors which have been investigated in vivo. These studies were carried out in man using a twin isotope technique. This approach was employed to reduce as far as possible the variation in absorption which is known to occur between individuals and also to try to stabilise local factors prevailing within the gastrointestinal tract. The technical details of the method, and a review of the other techniques which are currently available to assess iron absorption, have been set out respectively in two appendices.

The first of the luminal factors to be examined was the role of the gastric secretions in iron absorption. Initially, an attempt was made to resolve some of the controversy surrounding the effects of hydrochloric acid per se; these studies were carried out in subjects with proven histamine-fast achlorhydria. It was demonstrated that this acid enhanced ferric iron absorption but that the effect was much less marked with ferrous salts. Little influence at all could be demonstrated upon the absorption of haemoglobin iron. These results are probably related to differing solubilities of the two salts rather

than to mucosal factors, while the failure to alter haemoglobin absorption significantly suggests that this particular organic compound may follow a different pathway.

In view of the complex nature of these secretions this problem was further investigated in another group of achlorhydric subjects. Thus, the availability of iron administered with gastric juice obtained from normal subjects, was contrasted with that available from an equal volume of hydrochloric acid of matched acidity. In these experiments no clear-cut difference was noted on the two occasions suggesting that it was the hydrogen ion, rather than any other substances in the gastric juice, which was the important factor.

In an extension of this investigation it was demonstrated that both whisky and brandy facilitated the absorption of ferric iron in normal subjects, but had little effect upon ferrous ascorbate or haemoglobin iron. This pattern of absorption suggests that the results obtained with alcohol may reflect stimulation of gastric hydrochloric acid secretion. These findings are of interest since it is possible that they may have some relevance for the association which exists between excessive alcohol consumption and the development of iron overload.

Once an association had been established between alcohol consumption and iron absorption, a third in vivo study was carried out to document the availability of iron from Bantu beer. This investigation was of particular local interest because of the high iron content of this beverage, the drinking habits of the adult Bantu population living in South Africa, and the notable incidence of siderosis in this group of people. The evidence accumulated in this study indicated that the iron in Bantu beer behaved as a simple ferric salt and that the overload present in many of these middle-aged subjects could be explained simply on their high iron intake. It did not appear likely that the relatively low alcohol content of this beer stimulated iron absorption. However, it remains possible that the much higher alcohol content found in some of the adulterated drinks consumed by these people may, in fact, enhance iron absorption.

The second part of this thesis describes the results of investigations which have been carried out in vitro. Since particular interest lay in further investigating the role of metabolic energy in iron transfer and in defining a possible site of a cellular regulating mechanism, a technique for the perfusion of isolated rat intestine was developed. Evidence has been presented that this process is

depressed by anoxia and certain metabolic inhibitors and that it can be stimulated, under certain defined circumstances, by substrates. Experimental data obtained in this phase of the investigation also indicated that previous in vivo manipulations of the test animals could modify the subsequent behaviour of the preparation. From these observations it was concluded that iron transport is an active metabolic process, and that a degree of control over this transfer mechanism resides within the mucosal cells of the upper small intestine.

In summary, this thesis reports experimental work which has been carried out over the last five years into various aspects of iron metabolism. In the first instance a number of luminal factors have been investigated in man and, where relevant, complementary observations have been carried out using an in vitro technique. These combined studies have indicated a possible mechanism whereby hydrochloric acid influences the absorption of iron and they have provided some evidence that the enhancing effect of alcohol operates through the same mechanism; further observations support a possible role of Bantu beer in the pathogenesis of iron overload in the drinking Bantu population in the Republic of South Africa. Finally, evidence has been presented which supports previous suggestions that iron

transfer is an active metabolic process and that "conditioned" cells, residing within the absorbing mucosa of the upper gastrointestinal tract, may modify iron absorption quantitatively.

A P P E N D I X I

METHODS OF DETERMINING IRON ABSORPTION

INTRODUCTION

The scientific documentation of iron absorption and the various factors which influence this process has been rendered difficult by the small exchange of this metal which takes place between man and his environment under physiological conditions. Furthermore, meaningful comparisons between the various published studies has been complicated by the development of a number of alternative techniques, all providing information on slightly different aspects of the same process. The fact that so many methods exist implies that no single one is perfect. Accordingly, each method must be weighed against the technical difficulties in execution and the value of the data so collected; the final choice reflecting the particular type of problem which is to be studied. Because of these problems it was considered essential to review briefly the more important techniques and to emphasize their relative merits in order that the various studies could be evaluated. It should be noted that some reports have only historical interest, being based on methods which can no longer be regarded as reliable.

Broadly speaking, investigations have employed either chemical methods or radioactive isotopes of iron. The actual measurements of absorption have been variously carried out on blood, plasma, faeces and by whole body counting while frequently combinations of these methods have been used (Table XIII).

IN VIVO METHODS

I. CHEMICAL METHODS

a) Balance Experiments

Prior to the introduction of radioiron a large number of studies were done using this method, and it remains the oldest of the techniques currently in use to assess iron absorption. In this type of study retention, or net balance, is given by the difference between dietary iron intake and faecal iron content. It is generally accepted that little actual secretion, or excretion, of iron takes place into the lumen of the bowel (McCance and Widdowson, 1937 a).

Chemical balance studies have certain obvious advantages. Thus, food iron is distributed over the usual three daily meals although the choice of diet remains arbitrary. Secondly, such studies may be continued over long periods of time and so tend to "smooth out" variations resulting from changes in bowel motility and altering

TABLE XIII
METHODS OF DETERMINING IRON ABSORPTION

IN VIVO METHODS

I. CHEMICAL METHODS

- a) Balance experiments
- b) Excretion studies
- c) Plasma Iron Tolerance Test
- d) Increase in Red Cell Iron in Iron Deficiency

II. RADIOACTIVE ISOTOPIC METHODS

- a) Balance Experiments
- b) Excretion Studies
- c) Plasma Radioiron Absorption Curves
- d) Red Cell Utilization Studies
- e) Double Isotope Methods
- f) Whole Body Counting

IN VITRO METHODS

- a) Isolated Everted Loops
- b) Perfusion Technique

secretory patterns. Further merit lies in the fact that repeated investigations may be carried out in the same patient, without the inconvenience of correcting for previously administered radioactivity.

On the debit side is the difficulty inherent in complete stool collections, the accurate analysis of large quantities of food and faeces, and the amount of time and meticulous technique necessary to prevent contamination from extraneous sources. All these factors would then tend to reduce the number of investigations which could satisfactorily be carried out at any one time and make each study relatively costly. In addition, it has been suggested that this method may not be sufficiently quantitative to have practical values (Bothwell and Finch, 1962), since a disparity exists between some of the data obtained using this approach and results from isotopic techniques (Smith and Pannacchiulli, 1958; Bothwell et al., 1958). Similarly, the retention of iron from therapeutic doses, when predicted on the basis of balance studies, does not correlate with subsequent recovery by phlebotomy (Haskins, Stevens, Finch and Finch, 1952). It is, however, acknowledged that the latter criticism may not be valid at lower levels of iron intake (Bothwell and Finch, 1962) since in this range absorptions are similar to those obtained using isotopic methods (Josephs, 1958).

Nevertheless, provided stringent attention is devoted to minute detail a great deal of useful information can be derived from such studies. They have, in fact, been instrumental in establishing the general limits of iron absorption from the diet (Bothwell and Finch, 1962). Furthermore, Josephs (1958) is of the opinion that chemical balance studies retain a very definite place in the study of iron metabolism and have a much wider application than is generally appreciated.

b) Excretion Studies

From time to time attempts have been made to assess the loss of iron in faeces by chemical methods (McCance and Widdowson, 1938; McCance and Widdowson, 1943). Such studies have generally proved unrewarding since losses are small and extraneous contamination markedly affects results.

However, it has been possible to modify this method to some extent by manipulating the iron content of the diet. Thus, attempts have been made to evaluate the role of the gastrointestinal tract in contributing to daily iron loss by providing a diet which is very low in iron. The application of this technique to a study of patients with idiopathic haemochromatosis has demonstrated that even under conditions of overload, iron loss is low (Jacobs, personal

observation). This finding accords well with the earlier report of McCance and Widdowson (1937 a), and with that of Crosby (1963) who believes that the capacity of such an excretory mechanism would be limited.

c) Plasma Iron Tolerance Test

Since absorbed iron is transported in the plasma (Moore et al., 1937), attempts have been made to assess absorption by monitoring changes of the serum iron and the unsaturated iron binding capacity at various times after the administration of large doses of iron to fasting subjects.

It would appear that this method is able to contribute to an understanding of some of the aspects of iron absorption, but exact interpretation of each curve remains a matter of conjecture. Hence, the pattern of response to these unphysiological doses of iron is significantly influenced by factors such as the rate and quantity of absorption, the availability of transferrin and the clearance of iron from the plasma; the latter reflecting both erythropoietic activity and the avidity of tissue receptors. Cognisance must also be taken of the fact that all the iron absorbed may not necessarily appear in the plasma as has been assumed in the past. Many workers have given only scant consideration to those changes taking place within the mucosal

cells of the gut or in the liver.

This test would then appear to be of value as a means of indicating increased absorption in subjects who have adequate iron binding protein available, and are not suffering from any condition likely to accelerate iron clearance from the plasma such as infection, iron deficiency or haemolysis. As such, the plasma iron tolerance test, or "absorption curves" as they are sometimes known, has its major application in the diagnosis of depleted stores before the patients become anaemic (Bothwell and Finch, 1962). However, unless the variables affecting these curves are clearly understood it is likely that too much value may be read into the results obtained (Josephs, 1958).

d) Increase in Red Cell Iron in Iron-deficiency
 Anaemia

The demonstration that absorbed iron is almost completely utilized for haemoglobin synthesis (Whipple and Rabscheit-Robbins, 1936; Balfour, Hahn, Bale, Pommerenke and Whipple, 1942) forms the basis for this test, in which the maintenance of a constant haemoglobin level by phlebotomy has been used to assay the yield of iron from different diets (Whipple and Rabscheit-Robbins, 1936).

Similarly, this method has been applied to determining iron absorption in polycythaemic patients (Finch, Haskins and Finch, 1950 b). In much the same way the amount of iron available from various diets can be judged by haemoglobin response in iron deficient animals (Frost, Potter, Elvehjem and Hart, 1940; Ruegamer et al., 1946; Pye and MacLeod, 1946; Blumberg and Arnold, 1947).

At the present time the major application of this particular test would be in defining the absorption of medicinal iron. Interpretation should be carefully considered in the light of unphysiological load, the completeness of iron deficiency and attention be paid to the ability of the bone marrow to remove large loads of iron (Bothwell and Finch, 1962).

II. RADIOACTIVE ISOTOPIC METHODS

a) Balance Experiments

In a manner analogous to chemical balance techniques, absorption is again given by the difference between the radioiron administered by mouth, and that recovered in the faeces. This method has the advantage of greater specificity and simpler analytical techniques. Its efficiency has been multiplied by improved counting methods and the availability of higher specific activity isotopes, both

tending to elevate the accuracy of this method to a level at which it is useful, even when absorption is low.

Ideally, all the articles in any test diet should be labelled, but this is currently not possible. Alternatively, single items are tagged or inorganic radioiron given with a meal (Dubach et al., 1948; Moore, 1955). Faeces are collected for approximately 7 to 10 days at which time less than 0.5 per cent of the administered dose is normally likely to be present in the stool. The radioactivity is then determined on either homogenised or digested aliquots, or by counting the complete stool in a ring of Geiger-Müller tubes or suitable scintillation counter.

Early theoretical objections (Garby and Sjölin, 1959) that some degree of bi-directional transfer may take place across the intestinal barrier, resulting in data which failed to reflect true absorption, cannot be supported (Chappelle et al., 1955; Pollack, Kaufman and Crosby, 1964 a).

The apparent discrepancy between data obtained using stool excretion methods and red cell mass radioactivity studies has recently been reviewed (Bothwell and Finch, 1962). A consistently higher level obtained using the former method (approximately 50 percent) could not be explained even when red cell utilization values

were corrected to allow for storage. The fact that twin isotope studies and total body counting accorded well with corrected red cell values, stimulated these workers to investigate the situation further. They report that significant submucosal deposits of radioiron could not be demonstrated (Bothwell and Finch, 1962) and raise the possibility that such balance studies may have inherent technical difficulties. Nevertheless, in a study designed to test this point reasonable agreement between stool absorptions and red cell radioactivity was obtained, provided the former were carried out with meticulous care. These studies, in addition to drawing attention to possible shortcomings in this approach, have also underlined a necessity for critical comparison of the various methods, perhaps by means of total body counting.

b) Excretion Studies

Isotopic methods are likely to provide more meaningful information on excretion than chemical techniques largely because extraneous contamination can be excluded. Two distinct approaches have been used in the measurement of radioiron excretion. Both techniques require that a tracer with a long half-life (Fe^{55}) be used and this has the disadvantage that samples must, at present, be digested and electroplated prior to counting.

In the first approach (Dubach, Moore and Callender, 1955) the radioactivity in excreta, hair, nails and sweat is measured and compared to a standard. In the second method (Finch, 1959) excretion is reflected by changing specific activity of the blood. The fact that results obtained by these two methods agree well, suggest that they are based on valid assumptions.

c) Plasma Radioiron Absorption Curves

In this method, changes are measured in the level of plasma radioactivity following the oral administration of a dose of labelled iron. The particular merit of this technique, in which physiological quantities of iron can be employed, is that it may be used to analyse early movements of iron from mucosal cells to the plasma. Furthermore, the effect of such variables as plasma iron concentration and transferrin levels on inflow can be determined (Bothwell and Finch, 1962).

Early studies in dogs, using this method (Yuile et al., 1950), demonstrated a poor correlation between iron absorption and the shape of these curves. However, more recently attempts have been made to refine the accuracy of this method by determining the rate at which the same isotope is cleared from the plasma on a different day (Bothwell, Mallett, Oliver and Smith, 1955), or by the

simultaneous use of two isotopes, giving one by mouth and the other intravenously (Hallberg and Sölvell, 1960 a). This latter method is discussed in more detail subsequently.

The appreciation that absolute retention, calculated from such data, is about 50 percent lower than by other techniques (Hallberg and Sölvell, 1960 b) suggests that there may be two phases in the absorptive process; a fast initial component and a later slow one (Bothwell and Finch, 1962).

d) Red Cell Utilization Studies

These methods depend upon observations that small quantities of radioiron injected intravenously were nearly completely utilized for haemoglobin synthesis in iron deficiency anaemias. Utilization was between 70 and 85 percent in normal subjects after approximately two weeks (Dubach, Moore and Minnich, 1946; Finch, Gibson, Peacock and Fluharty, 1949), an important observation which has subsequently been confirmed (Huff, Hennessey, Austin, Garcia, Roberts and Lawrence, 1950; Elmlinger, Huff, Tobias and Lawrence, 1953; Bothwell, Callender, Mallett and Wits, 1956; Giblett, Coleman, Pirzio-Biroli, Donohue, Motulsky and Finch, 1956) .

This approach has the great advantage of simplicity in that the radioiron, in the desired dose and form, is given by mouth; an aliquot being retained for the preparation of standards. Two weeks later a blood sample is collected and both it and the standard are analysed for radioactivity in a suitable counter (Hahn, Bale, Lawrence and Whipple, 1939; Hahn et al., 1940; Balfour et al., 1942; Hahn et al., 1943; West, Jackson, Elliott, Hahn, Patterson and Anderson, 1952). Blood volume can be determined either from body weight, surface area or by other techniques (Gregersen and Rawson, 1959) and circulating counts thus determined. From these data utilization can be calculated, since it has been demonstrated that the radioiron is incorporated into haemoglobin and does not leave the red cell and exchange with plasma during the erythrocyte life-span (Miller and Hahn, 1940; Hahn et al., 1940).

Once utilization is known, absorption can be determined in both iron deficient and normal subjects. It should, however, be appreciated that these data will be misleading in disease states, such as haemochromatosis and aplastic anaemia, where utilization is low or absent.

e) Double Isotope Techniques

The availability of two different radioactive isotopes of iron and practical methods for their detection, have rendered possible significant improvement in studies based upon red cell utilization. Two techniques are currently employed.

In the first instance, comparative studies can be made in the same subject where test doses, each tagged with a different isotope, are given on consecutive days (Bothwell et al., 1958). The levels of the two isotopes in a blood sample collected two weeks later give a measure of their relative absorption. A further refinement has been introduced by Hallberg, Brise and Sölvell (1958), in which the two isotopes are administered on alternate days for a consecutive 10-day period, so increasing sensitivity and reducing day to day variations. Such an approach has application in studies designed to test the efficiency of different therapeutic preparation of iron, or to document the effects of such factors as reducing agents or gastric juice upon absorption. This method is discussed in some detail in Appendix II.

The two isotopes of iron may be used simultaneously in a second way. The one isotope is given orally while tracer quantities of the second are administered intravenously (Saylor and Finch,

1953). Blood samples are collected in the usual way when circulating activity has reached a stable level, aliquots are digested, electroplated and the activity determined by suitable differential counting (Bothwell et al., 1958; Pirzio-Biroli et al., 1958; Pirzio-Biroli and Finch, 1960). The ratio of the two isotopes appearing in circulating haemoglobin is a function of the relative amounts in which they were administered and the percentage absorption of the oral dose. Absorption can thus be calculated, provided that suitable aliquots have been retained for the preparation of standards (Bothwell and Finch, 1962).

The latter method, which automatically corrects for tissue distribution and obviates any error from blood volume calculation, assumes that iron from the bowel behaves in the same way as the intravenous dose in terms of distribution (Saylor and Finch, 1953; Bothwell et al., 1958). It is considered that this is the most accurate of the various isotopic techniques thus far described and has a methodological error of about one percent when absorption is at the 10 percent level (Bothwell and Finch, 1962).

Recently this method has been modified (Hallberg and Sölvell 1960 c) in that one isotope is given intravenously to determine basal iron turnover and blood volume, while the second isotope is

given by constant intragastric infusion starting one hour later. Monitoring the amount of change in the turnover rate of the injected isotope, produced by the oral dose, will provide kinetic data on total iron entering and leaving the plasma. It is, however, not clear to what extent iron being removed by the liver from the portal blood influences these figures.

f) Whole Body Counting

The ready availability of Fe^{59} , which emits a strong gamma radiation, makes whole body counting the most obvious and convenient method of determining iron absorption both in intact experimental animals and man. This technique is probably the best method currently available for determining retention provided the expensive apparatus is available, correctly calibrated (Bothwell and Finch, 1962) and provided that enough time has been allowed for the elimination of iron from the gastrointestinal tract (van Hoek and Conrad, 1961). This approach has been tested in experimental animals over a wide range of dosage (Chappelle et al., 1955; Krantz et al., 1959) and its validity has been demonstrated.

IN VITRO METHODS

a) Everted Pouch Techniques

Certain of the phenomena which influence iron absorption cannot, for one reason or another, be studied in intact animals. Early workers (Wilson and Wiseman, 1954) described a technique of everting a pouch of intestine and suspending this in a physiological solution. By placing radioactive isotopes either within the pouch or in the solution bathing the mucosal surface it is possible to study various facets of iron transport, such as transfer against a gradient. A number of subsequent studies have been carried out using this method, but these have yielded conflicting results (Brown and Justus, 1958; Dowdle et al., 1960) and recently the validity of the method itself has been questioned (Job and Howell, 1962).

b) Perfusion Method

In an attempt to overcome the obvious criticism of everted pouches, namely the gross distortion of the anatomy during their preparation, a perfusion technique has been elaborated. In this method (described in Chapter VII), the gastrointestinal tract of the rat is perfused with warm physiological buffer via the superior mesenteric artery and the venous effluent from the portal

vein collected. Radioactive iron is then introduced into a loop of small bowel, which is isolated between the ligatures, and transport phenomena are characterised in terms of iron movement across the mucosal cells and into portal vein. This contrasts with movement of tracer from mucosal to serosal surfaces - an unphysiological pathway at the best of times.

SUMMARY

There are currently available a number of chemical and isotopic methods for the determination of iron absorption in vivo. It would appear (Bothwell and Finch, 1962) that when these are critically evaluated, the most accurate methods are:

- (1) Measurement of erythrocyte radioactivity in subjects with normal red cell utilization,
- (2) Measurement of the increase of red cell iron in iron deficient subjects with anaemia,
- (3) Double isotope technique, and
- (4) Total body counting.

Under certain specific circumstances studies are

better carried out in vitro and then perfusion methods appear preferable to the use of everted gut sac preparations.

APPENDIX II

THE TWIN ISOTOPE METHOD

INTRODUCTION

The ready availability of radioactive isotopes of iron and their increasing application to tracer studies in various biological investigations, has led to the development of a number of specialised procedures for their detection; these have in turn facilitated more sophisticated experimental design. While sensitive methods are available to determine single absorptions they have definite disadvantages, for example, the wide variation normally found between individuals often necessitates the study of large groups of subjects before the data collected attains statistical significance. The elaboration of a twin isotope technique in which valid comparisons of absorptive phenomena can be made in the same individual, thus obviating the use of large numbers, has therefore given considerable impetus to the study of iron absorption. This is particularly true in the investigation of uncommon pathologies, where limited clinical material may be available.

However, there exists a number of other situations where the simultaneous use of two iron isotopes in the investigation of metabolic problems is a great advantage, a point first appreciated by Peacock and his colleagues (Peacock, Evans, Irvine, Good, Kip

Weiss and Gibson, 1946). These workers described a method in which the isotopes were administered to the patient; samples of blood were then collected, digested and the iron contained therein electroplated onto copper discs. The radioactivity in the samples was measured using two types of Geiger-Müller tubes, so constructed that they could discriminate against either isotope, depending upon the characteristics of each individual tube. This method has the disadvantage of intrinsically low efficiency and has subsequently been modified by Hallberg and Brise (1960) who assayed the plated iron in a gas-flow counter equipped with suitable platinum and beryllium absorbers. These workers obtained counting efficiencies of 5.4 percent for Fe^{55} and 5.0 percent for Fe^{59} (Katz, Zoukis, Hart and Dern, 1964).

In attempts to improve further the sensitivity of this approach, liquid-scintillation counting techniques have been applied to the detection of beta and x-ray emitting isotopes. In some cases these methods have greatly simplified their measurement but problems, such as the relative insolubility of the substances in the usual scintillator solvents, are inherent in this approach as well (Katz et al., 1964). Furthermore, while inorganic iron can form stable compounds with a variety of organic agents, these complexes are generally strongly coloured; although Fe^{59} may be detected with fair

efficiency in a liquid scintillation system, colour-quenching precludes its application to the assay of Fe^{55} (Leffingwell, Melville and Riess, 1959; Leffingwell, Riess and Melville, 1962). Dern and Hart (1961 a; 1961 b) have overcome this difficulty by developing a method in which the iron is converted to its colourless perchlorate salt, which is then dissolved in ethanol. These workers report high counting efficiencies and good resolution of the two isotopes using this method.

More recently Katz and his colleagues (1964) have described a method for the simultaneous assay of Fe^{55} and Fe^{59} in a liquid scintillation system, employing the formation of stable emulsion of an aqueous solution in thixotropic gel. This method has particular merit in that special electroplating equipment is not necessary; the iron being precipitated from the blood digest, converted to a ferrous chloride in an excess of hydrochloric acid and emulsified in the gel. High counting efficiencies and minimal cross-counting are readily obtained and the samples remain stable for at least two weeks after preparation.

It is clear from the foregoing comments that considerable progress has been made in the development of methods for the simultaneous detection of Fe^{55} and Fe^{59} in a single sample.

However, at the time these studies were carried out, such techniques were not locally available. Consequently the method described by Peacock et al., (1946) has been used with the modification described in detail below.

METHOD AND COMMENTS

1. Preparation of the Patients

The absorption of a standard dose of iron varies not only between individuals, but also in the same individual at different times. In order to minimize variations produced in this way, all subjects were fasted of food and water from 10 p.m. the night preceding the study and for a further 4-hour period following the administration of labelled iron. All oral doses were administered at 8 a.m. on the morning of the examination and wherever practical the test dose was contained in a fixed volume of fluid. In studies where the form of iron was unpalatable a thin nasogastric tube was passed, the substance administered, and the tube immediately withdrawn. Isotopic studies using Fe^{59} demonstrated that the amount of tracer adherent to the apparatus during administration by this route was seldom in excess of 1 percent of the dose administered.

2. The Radioactive Isotopes of Iron

a) Fe^{59} , having a half-life of 45 days (The Radiochemical Manual, 1962; Katz et al., 1964), has enjoyed the greatest popularity in the investigation of iron metabolism, largely because of its presence is easy to determine.

While this isotope may be produced in a cyclotron by deuteron bombardment of cobalt 59 (Gile, Garrison and Hamilton, 1951), the isotope used in these studies was obtained from the Oak Ridge National Laboratory and was prepared by an (n, gamma) reaction in a pile resulting in neutron capture by an Fe^{58} enriched target. Although the purity of the Fe^{59} supplied was greater than 99 percent, exclusive of Fe^{55} which was always less than 10 percent, the latter method actually is capable of giving Fe^{59} unaccompanied by Fe^{55} (Moore and Dubach, 1955).

Fe^{59} decays in a complex manner to Co^{59} emitting beta spectra of 0.13 Mev. (1 percent); 0.27 Mev. (46 percent); 0.46 Mev. (53 percent); and 1.56 Mev. (0.3 percent), as well as gamma quanta of 0.14 Mev. (0.9 percent); 0.19 Mev. (2.4 percent); 0.34 Mev. (0.3 percent); 1.29 Mev. (44 percent); and 1.10 Mev. (56 percent) ("Radioelements artificiels", le Commissariat a l' Energie atomique - France, April, 1962; Radiochemical Manual, Part I, 1962).

Until recently this isotope was detected by counting the beta rays. However, since these emissions are of relatively low energy, and therefore readily absorbed, this method involved separating the iron and electroplating it onto copper discs which were then counted under fixed geometric conditions by a thin window Geiger-Müller tube (Good, Kipp and Brown, 1946). More recently the availability of various types of scintillation counters has made possible the detection of this isotope by gamma ray counting directly, without special preparation (Katz et al., 1964).

b) Fe^{55} has a half-life of 2.7 years and may be produced in a cyclotron by deuteron bombardment of Mn^{55} . The isotope used in these studies was obtained from the Oak Ridge National Laboratories and prepared by an (n, gamma) reaction; it had a purity of greater than 99 percent exclusive of Fe^{59} which was always less than 10 percent (Oak Ridge National Laboratory Catalogue, 1962).

Fe^{55} decays by K-capture with emission of manganese characteristic x-rays with an energy of 0.0059 Mev. (Brownell and Maletskos, 1950).

c) Fe^{52} has recently been produced and having a half-life of 8 hours is well suited to studies where repeated observations are required (Francois and Szur, 1958; Bothwell and Finch, 1962).

This isotope decays by K-capture or by positron emission - the branching ratio being 43 percent electron capture to 57 percent positron emission, all disintegrations giving rise to gamma rays of 0.165 Mev. and 0.51 Mev. respectively. This isotope is, however, not yet generally available for study (The Radiochemical Manual, 1962).

3. Isotope Dosage

The ethical considerations involved in experimentation have recently received attention in the literature (Brit. med. J., 1962; Mitchell, 1964). Of particular importance in this regard are studies in which radioactive isotopes are administered to patients, and for this reason every reasonable care was taken to insure that no risk to subjects resulted from careless use of these substances.

It was furthermore necessary to balance the dose of radioactive isotopes administered so that the count rates from suitably diluted standards, and from both isotopes in an aliquot of blood, were in the same order. Accordingly, the dose of Fe^{55} varied between 80 and 125 microcuries and the dose of Fe^{59} between 1 and 5 microcuries at the time of administration. These doses are presently regarded as being within the life time permissible dosage recommended as safe by the National Committee on Radiation Protection (1959), and the International Commission on Radiological Protection (1963).

The problem of radiation to the patient has also received theoretical consideration from Bothwell and Finch (1962). These authors have pointed out that the weak energy of Fe^{55} is all expended within a distance of 1 to 2 millimeters in tissue. They conclude, calculating dosage on a formula for beta-irradiation, that a dose of one microcurie per kilogram body weight will result in exposure of 3.34 rads per millilitre blood. Total marrow irradiation is likely to be in the region of 1.1 rads per millilitre of marrow with a maximum level of marrow irradiation occurring during the first 4 days amounting to 4×10^{-2} rad per millilitre of marrow.

Similarly beta-irradiation from a dose of Fe^{59} of one microcurie per kilogram of body weight will result in a dose of 4.2 rads per millilitre of blood and a total of about 2.0 rads of beta irradiation to the marrow, one third of which is delivered in the first 4-day period.

The contribution of gamma irradiation is calculated slightly differently because the gamma rays are not completely absorbed within the tissue concerned. For this reason total gamma-irradiation from a dose of one microcurie per kilogram is 1.5 roentgens per gram of body weight; this increases the total irradiation to the marrow to about 3.5 rads, the fractional dose during the first 4 days becoming

0.76 + 0.02 or 0.8 rad (Bothwell and Finch, 1962).

In considering radiation to tissues it should be appreciated that in certain pathological states the distribution of the isotope alters, and under these conditions the figures given above are likely to be incorrect.

4. Collection of Blood Samples

Venous blood was collected into heparinised iron-free apparatus 14 days after the administration of the isotopes. At this time optimum red cell utilization of the iron could be expected. The final concentration of heparin was 10 units per millilitre.

Haemoglobin was determined as cyanmethaemoglobin (King and Wootton, 1956), serum iron was determined according to Bothwell and Mallett (1955) and the unsaturated iron binding capacity according to Bothwell, Jacobs and Kamener (1959). Duplicate 10 millilitre aliquots of blood were transferred to 800 millilitre Kjeldahl flasks for digestion.

5. Preparation of the Apparatus

All glassware was soaked in freshly prepared chromic acid overnight, washed in hot running tap water, then thoroughly rinsed in

iron-free distilled water and allowed to dry at room temperature. Any apparatus which could be damaged by this rough treatment was rinsed in A. R. grade concentrated hydrochloric acid and scrubbed in hot running tap water containing a detergent (Teepol - Shell product), before rinsing in iron-free water and drying as above.

Particular care was taken, where practical, to ensure that any piece of apparatus was used exclusively for Fe^{55} or Fe^{59} and that these were kept separate at all times.

6. Solutions

Only analytical grade reagents were used and iron-free water was prepared by passing glass redistilled water over a mixed bed ion exchange resin.

a) Digesting Mixture

This was freshly prepared by adding one part of 55 percent nitric acid (specific gravity 1.502) to two parts 95 percent sulphuric acid (specific gravity 1.834).

b) Perchloric Acid

70 - 72 percent HClO_4 (specific gravity 1.764).

c) Sulphuric Acid

6 N H_2SO_4 was prepared by suitable dilution from concentrated acid.

d) Brom-cresyl Purple Indicator

0.1 percent in 0.01 N sodium hydroxide

e) Carrier-iron Solution

A stock solution was prepared by cleaning iron wire in 30-32 percent hydrochloric acid (specific gravity 1.16), washing this in ion-free water and carefully drying it at 80 degrees Centigrade. The clean wire was cut up into small pieces, since these dissolve easier, and 10 grams weighed into 75 millilitres of N hydrochloric acid. The wire was allowed to dissolve, and when solution was complete this was diluted volumetrically to 1,000 millilitres with N hydrochloric acid. After standing 2 to 3 days any precipitate which may have formed was filtered off and the iron content finally checked colorimetrically against a standard solution, using bathophenanthroline as an indicator.

A working standard containing 5 milligrams of iron per millilitre was prepared by suitable dilution with iron-free water and the final concentration again checked by comparison to a standard solution.

f) Ammonium Oxalate Solution

Eight grams of ammonium oxalate were dissolved in 100 millilitres of iron-free water and heated before use to completely dissolve the salt.

7. Wet Ashing of the Sample

The digestion of blood samples was in each case carried out by adding 10 millilitres of the digesting mixture to the 800 millilitre Kjeldahl flasks containing the aliquot of blood. These were allowed to stand for several hours after which time enough standard carrier iron solution was added to obtain a total iron content of 7.5 milligrams. The amount of carrier iron required was calculated from the haemoglobin concentration of each particular blood sample, on the assumption that there are 34 milligrams of iron in each 10 gram of haemoglobin (Bernhart and Skeggs, 1943).

Example Calculation

Haemoglobin of sample = 16 grams percent (i.e. 1.6 grams of haemoglobin are contained in the 10 millilitre aliquot of blood being digested).

But 100 grams of haemoglobin contain 340 milligrams of iron

Thus $1.6 \text{ grams} = \frac{340}{100} \times 1.6 = 5.424 \text{ milligrams of iron}$

(as contained in the aliquots)

It is thus necessary to add $7.5 - 5.424 = 2.076$ milligrams carrier iron

Stock solution contains 5 milligrams carrier iron per 1.0 millilitre i. e. add 0.4152 millilitres of carrier iron solution

A single chemically clean glass bead, which was neither cracked nor chipped, was added to each digest to prevent bumping during digestion. The flasks were then transferred to a Macro-Kjeldahl apparatus and heated with bunsen flames, adjusted between low and medium. The flasks were constantly rotated and as combustion of the organic constituents proceeded the contents became thick and occasionally began to foam. If an inadequate amount of the digesting mixture had initially been added, the contents often charred at this stage, necessitating the addition of further small volumes of the acid mixture. This step was repeated until complete digestion had been obtained. At this time the mixture appeared oily and very dark brown, or even black in colour and pulled away from the sides of the flask in many tiny bubbles.

The flask was then removed from the burners and allowed to cool. When all fuming had subsided 3 millilitres of concentrated perchloric acid were added very carefully to each digest, under a hood, allowing the acid to flow slowly down the side of the flask. Perchloric acid was never added unless the contents were liquid since a highly

explosive compound may be formed by the addition of this acid to the solid digest.

Following gentle rotation, the flask was returned to the burner for further periods of 5 to 10 minutes at a time. Where necessary, additions of perchloric acid were made, as detailed above, until the contents became colourless. It was seldom found necessary to add more than 1 or 2 aliquots of this acid when digesting 10 millilitres of blood.

Boiling was continued for a further 10 to 15 minutes to drive off any residual perchloric acid and the flask then allowed to cool. The contents, which were originally black or dark brown, change colour through orange to pale lemon yellow and finally become colourless on cooling. Occasionally small quantities of white salts were formed but these dissolved on the addition of 5 millilitres of iron-free water and reboiling of the flask. This last step had the additional effect of ridding the digest of traces of nitric and perchloric acids (Katz et al., 1964).

Once the flask and its contents had cooled, ammonium hydroxide was added cautiously down the sides while the digest was gently swirled. The alkali was added slowly until the first reddish

brown floccules of ferric hydroxide had just begun to precipitate; the volume required was usually in the region of 25 millilitres of ammonium hydroxide. At this stage the contents were transferred quantitatively to 90 millilitre heavy duty pyrex centrifuge tubes, with several washings of water totalling about 4 millilitres.

8. Precipitation of the Iron

Using chemically clean glass rods, the contents of each tube was stirred while further ammonium hydroxide was added dropwise until the characteristic heavy brown floccules of ferric hydroxide appeared. The rods were removed and rinsed with iron-free water from a wash bottle, the washings being collected into the appropriate centrifuge tubes. Where uncertainty existed as to the completeness of precipitation the process could be controlled by the prior addition of 3 or 4 drops of 0.1 percent brom-cresyl purple indicator to each tube.

The tubes were centrifuged for 10 minutes at 3,000 r.p.m. and the supernatant, which was usually clear and colourless, removed by aspiration. Occasionally the floccules did not "spin-out" quantitatively and a few drops of 1:100 aqueous Triton were then used to settle the precipitate. The latter was dissolved by adding 6 drops

of 6 N sulphuric acid and the tubes gently rotated so that none of the iron hydroxide adhering to the sides of the tubes was lost. After standing for 20 minutes the samples were ready for electroplating.

9. The Electroplating Apparatus

In the early part of this work an older type of machine was used in which poor thermodynamic design required that the anode was rotated mechanically to cool the mixture in the cell (Ross and Chapin, 1942; Peacock et al., 1946). However, in later studies an apparatus modified from that described by Hallberg and Brise (1960) was employed. The base of each electrolysis cell is screwed onto a heavy steel plate and this, in addition to forming a common cathode, helps considerably in dissipation of the heat developed during electrodeposition of the iron; so eliminating the need for constant mechanical agitation.

The electrolysis cell (Figure 48) is made up of two parts - a base and a cup that screws into it. The heavy base is machined from acid-resistant stainless-steel and has a polished surface in close contact with the copper planchet onto which the iron is to be deposited. The top part of the cell consists of a quarter inch thick acrylate plastic cylinder. This is three inches tall with an internal diameter of two inches and has a hole bored in the bottom slightly smaller than a copper planchet. In assembling the unit, the planchet

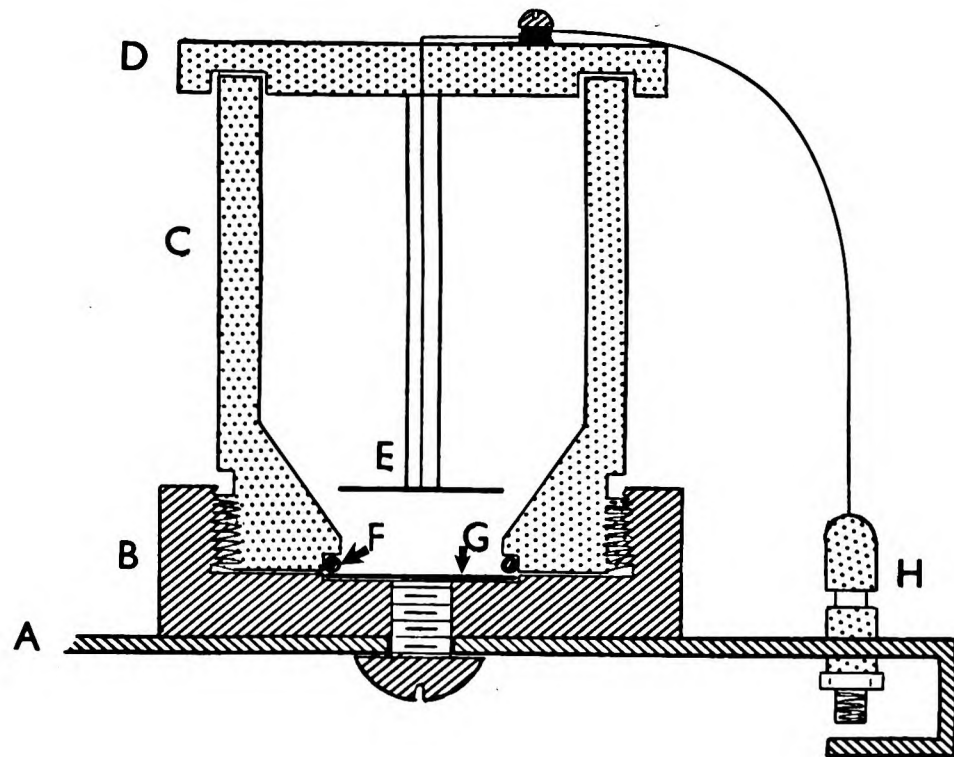


FIGURE 48

Diagram of Electrolysis Cell

- | | | | | | |
|---|---|------------------|---|---|------------------------|
| A | - | base plate | D | - | anode support |
| B | - | heavy steel base | E | - | nodal platinum spiral |
| C | - | acrylate cell | F | - | O ring (cross section) |
| | | | G | - | planchet |

is placed in the shallow groove in the centre of the base and the cup firmly screwed down; the union between the base of the cup and the planchet being rendered water-tight by a rubber "O" ring.

A satisfactory anode was constructed from a platinum spiral having a diameter of two centimetres, melted into a pyrex glass tube. Initially this was filled with mercury (Hallberg and Brise, 1960) but it was later found possible simply to lead copper wire through the centre of the hollow glass rod (Figure 49).

The design of the apparatus is such that units of 4 cells are mounted on the stainless steel base and connected in series. It was possible to run each unit independently or to couple these units in parallel, the base plate forming the common cathode between the units (Figure 50). Occasionally it was found necessary to run blanks to make up numbers in each unit, in which case 7.5 milligrams of carrier iron were electroplated in place of a sample. Alternatively, the number of samples may be distributed to form units of 3 instead of 4.

A constant voltage power pack was designed and constructed by Mr. V.W. McBride of the Department of Physiology at the University of the Witwatersrand. This apparatus has particular versatility in that both current and voltage regulation are carried out on the high

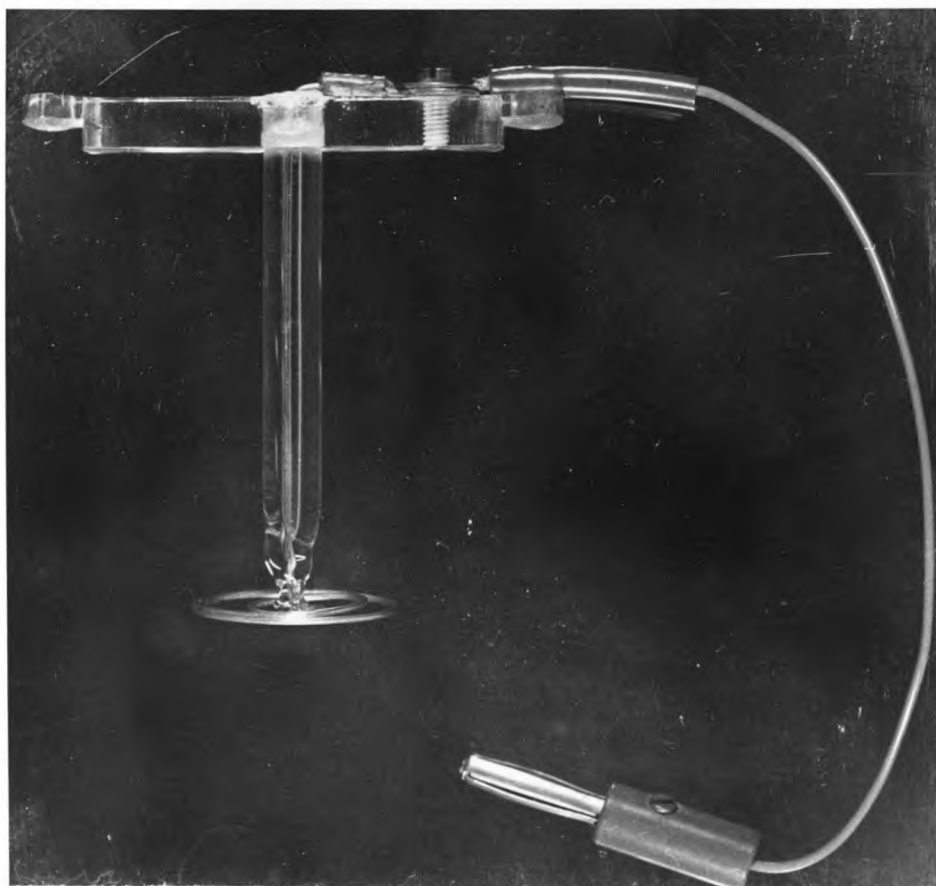


FIGURE 49

Photograph of Anode Assembly



FIGURE 50

General arrangement of electroplating apparatus. Foreground - 4 units, each of four cells on the common metal cathode. Behind is seen the voltmeter (left) and power pack containing ammeter.

voltage side of the power supply, in this way enabling smooth and easy setting of both parameters. The voltage was regulated with a rheostat and an ammeter was incorporated to monitor the current supplied to each cell. The voltage drop was constantly observed by means of a voltmeter.

10. The Electroplating Procedure

The copper planchets were thoroughly cleaned prior to use by washing them in 30 percent nitric acid until fumes of nitrous oxide evolved. They were then only partially rinsed in cold iron-free water, allowing occasional bubbles to form on the surface and so preventing corrosion while the plates were standing. Planchets should be bright and shiny while surface scratches should not be present. Any discs not fulfilling these criteria were discarded. The planchets were left standing in a beaker under this weak acid until required: they were only handled on the edge with forceps when they were mounted in the electrolysis cells.

The assembled cell, with its mounted planchet, was screwed onto the base plate. The sample, which had been previously dissolved in 6 N sulphuric acid was transferred to the cells with 35 millilitres freshly prepared ammonium oxalate. The beads and centrifuge tubes were rinsed with oxalate and the washings pooled with the contents in

the cell.

Electrolysis was started immediately and continued for a standard period of 150 minutes. During electrolysis a current density of 0.45 amperes was maintained through each cell and under the conditions described, a potential drop of 4 to 5 volts was recorded.

When electroplating was complete the planchets were removed from the base of the cell, rinsed in cold running distilled water and after immersion in absolute alcohol, dried with a fan. The plates, which ideally had a uniform silver-grey surface, were covered with a few drops of a solution containing 1 percent "Three-in-one" oil made up in benzene.

11. Factors Influencing the Electrodeposition of Iron

a) The Electrolysis Cell

In order to obtain an even distribution of the electro-deposited iron, good anode-cathode geometry has to be observed (Hallberg and Brise, 1960). The ideal anode-cathode distance depends to some extent upon the design of the former, but is usually between 1 and 2 centimetres. A larger distance increases the resistance of the cell; this produces a rise in temperature of solution and cathode which results in a dark soft plate. On the other hand, large anodes

often interfere with electrolyte circulation and in this way also adversely effect the electrolytic deposition of iron. Early workers, using the rotating propeller type of anode or a nitrogen bubbler, improved electrolyte mixing, but this was not always sufficient to obtain reproducible results (Hallberg and Brise, 1960).

Resulting from these observations the present cell was chosen in which adequate mixing has been demonstrated to take place without mechanical agitation. In addition, the size of the electrolyte compartment, coupled with the heavy base plate, assists in heat dispersion during electrolysis. The latter prevents local heating of the cathode, a factor known to interfere with the quality of the deposit. Free electrolyte circulation has been ensured by the spiral nature of the anode.

Further advantages of this type of cell include the ease with which it can be kept clean and its simplicity in assembly. The anode units, after cleaning, are stored in a special rack to prevent their distortion.

b) Current Density

In 1960, Brise and Hallberg reported that beyond an optimum period of electrolysis, there is a tendency for the electroplated iron

to redissolve. This particular point could not be confirmed in these studies.

In Fe^{59} tracer studies, 7.5 milligrams of iron have been demonstrated to be maximally deposited at two hours, using a current density of 450 milliamperes per cell. Accordingly, electroplating in the present studies was standardized at a current of 450 milliamperes for a period of $2\frac{1}{2}$ hours.

c) pH of the Solution

Much importance has previously been attached to the pH of the electrolyte from which the iron is deposited. Thus it has been stated -

"The more acid the solution the less iron is deposited and the greater the hydroxide ion concentration in the solution, the more the risk of formation of ferric hydroxide precipitate". (Hallberg and Brise, 1960).

Earlier, Peacock et al., (1946) had also indicated the importance of maintaining the pH at 6.0.

However, the electrodeposition of iron from 8 percent ammonium oxalate solution (Bothwell and Finch, 1962) has been found entirely satisfactory. This method has considerably simplified

the electroplating procedure, eliminating the need for continual observation and pH correction with ammonium sulphate.

d) Temperature and Electrolyte Circulation

These problems were limited to the earlier machines having poor anode design and thermodynamic construction; with the introduction of better apparatus these factors, resulting in unsatisfactory plates, have not proved troublesome.

12. The Efficiency of the Plating Method

The iron content of the electrolysis cell was routinely determined during electroplating. It was seldom possible to detect free iron in the supernatant after 120 minutes plating when this was examined using the sulphonated derivative of 4,7-Diphenyl-1-10-phenanthroline (Bathophenanthroline).

Complementary studies, using Fe^{59} labelled carrier, showed that less than 1 percent of the original tracer was in solution after 120 minutes. Recovery of the iron from the plate after this period was compared with the activity of the original aliquot of tracer which had been added to the electrolyte. These studies confirmed that a significant loss of labelled iron had not occurred.

It was therefore concluded that quantitative removal of both iron and tracer took place from the electrolyte onto the planchet under the controlled conditions described above.

13. The Differential Counting of the Samples

The counting of the characteristic radiations emitted by Fe^{55} and Fe^{59} from the planchets used in these studies was achieved by using two Tracerlab Geiger-Müller tubes. These were so constructed as to favour the counting of one or other isotope. Thus, beta emission of the Fe^{59} was measured in the Helium filled Geiger tube while a second tube filled with Argon, and having a beryllium filter, was used to count the x-rays of the Fe^{55} (Peacock et al., 1946). The count rates were recorded on a Phillips Scaler (Figure 51).

The Fe^{55} and the Fe^{59} standards, as well as each plate, were counted by both tubes, and in this way, appropriate corrections could be made for cross-counting (Bothwell and Finch, 1962). The divergence was usually not more than 3 percent in either direction, as reported previously (Saylor and Finch, 1953).

14. Validation Experiments

In order to quantitate the loss, if any, of isotopes during the process of digestion and electroplating, aliquots of biosynthetically labelled haemoglobin iron were counted in a scintillation counter. These samples were digested and electroplated and the planchets from each were then crushed and counted against the original sample



FIGURE 51

The general arrangement of the counting system. The two Geiger-Müller tubes are housed in lead castles (left). The scaler (centre) also has a scintillation counter (right) attached.

in the same counter. Other plates were dissolved in nitric acid and the activity compared to the original aliquot of blood. The results of these experiments are given in Table XIV. It can be seen that a mean recovery of 94.07 percent was obtained, and this agrees well with the results quoted by Hallberg and Brise (1960).

A similar study was carried out using aliquots of labelled inorganic iron salts in which the recovery rate was 94.11 percent (Table XV).

15. Reproducibility Studies

Finally, before applying this particular method to the investigation of absorptive phenomena, experiments were carried out to compare the absorption of identical doses of iron salts on consecutive days in human subjects. The results of these studies are given in Tables XVI, XVII, XVIII, XIX, XX, XXI, XXII).

It can be seen that, under the defined experimental conditions, close agreement was obtained over a practical dosage range using both ferric and ferrous salts. These data support the validity of this method.

TABLE XIV

Redovery of Haemoglobin Iron

	Aliquot of Blood C.P.M.	Planchet C.P.M.	%Recovery
1.	1064	1002	94.1
2.	1682	1637	97.3
3.	349	329	94.2
4.	762	747	98.0
5.	5302	5061	95.4
6.	6219	6102	98.1
7.	342	327	95.6
8.	1489	402	82.2
9.	681	627	92.0
10.	312	293	93.9

Mean 94.07

TABLE XV

Recovery of Iron Salts

	Aliquots of Standard C.P.M.	Planchet C.P.M.	% Recovery
1.	6054	5721	94.4
2.	5452	5032	92.2
3.	4231	4092	96.7
4.	5982	5709	95.4
5.	8727	8193	93.8
6.	6432	6047	94.0
7.	5871	5329	90.7
8.	6231	6019	96.5
9.	7129	6872	96.3
10.	5837	5321	91.1

Mean 94.11

TABLE XVI

Haematological Data and Results of the Absorption of 5 Milligrams Iron as FeCl_3 Studied in Normal European Subjects

Sex	Haemoglobin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption Day I %	Day II %
M	15.6	52	67	278	18.9	0.8	0.6
M	19.0	62	81	335	19.5	5.7	3.6
M	13.3	40.5	44	270	16.3	0.8	0.5
M	16.5	54	171	185	48.0	5.7	3.6
Mean	16.1	51.6	90.8	267	25.7	3.3	2.1

TABLE XVII

Haematological Data and Results of the Absorption of 5 Milligrams Iron as FeCl_3 Studied in Normal Bantu Subjects

Sex	Haemoglobin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption Day I	Iron Absorption Day II
M	13.0	45.0	70	192	26.7	0.61	0.38
M	15.1	46.0	143	246	36.7	0.34	0.87
M	14.3	44.5	117	125	48.3	0.5	0.25
Mean	14.1	45.2	110	108	37.2	0.49	0.50

TABLE XVIII

Haematological Data and Results of the Absorption of 5 Milligrams Iron as Ferrous Sulphate Studied in
Normal European Subjects

Sex	Haemoglobin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption Day I %	Day II %
F	14.2	44.0	73	249	21.7	30.4	19.5
F	14.2	44.5	85	209	28.8	1.3	1.7
F	14.9	46.0	83	326	20.3	6.9	6.7
F	12.8	40.0	75	244	23.3	2.2	1.3
F	16.1	51.0	94	256	36.9	0.4	6.7
F	12.4	40.0	97	237	29.0	5.2	9.9
M	15.6	50.0	79	224	26.1	1.8	3.4
M	15.2	48.0	69	280	19.8	3.4	2.9
M	14.3	43.0	80	306	20.7	29.8	24.2
Mean	14.4	45.2	81.7	259	24.2	9.05	8.5

TABLE XIX

Haematological Data and Results of the Absorption of 5 Milligrams Iron as Ferrous Ascorbate in Adult Bantu Subjects

Sex	Haemoglobin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption Day I %	Iron Absorption Day II %
F	12.0	42.0	64	249	20.4	13.1	10.9
F	14.1	48.0	50	285	14.9	65.0	60.0
F	13.3	40.0	49	256	16.0	27.6	27.4
F	14.9	50.0	63	156	28.7	84.0	70.0
F	14.4	40.5	77	162	32.2	9.8	7.7
Mean	13.7	44.0	60.6	222	22.4	39.9	35.0

TABLE XX

Haematological Data and Results of the Absorption of 30 Milligrams Iron as FeCl_3 Studied in
Normal European Subjects*

Sex	Haemoglobin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption Day I %	Day II %
M	16.7	56.5	94	231	28.9	0.73	1.46
M	13.1	42.0	37	149	19.9	0.0	0.0
M	12.2	43.5	75	244	23.6	0.77	0.10
Mean	14.0	47.3	69	209	26.1	0.50	0.86

TABLE XXI

Haematological Data and Results of the Absorption of 30 Milligrams Iron as Ferrous Ascorbate Studied
in Normal European Subjects

Sex	Haemoglobin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption	
						Day I %	Day II %
F	14.3	46	96	143	40.0	15.0	12.1
F	20.3	65	178	309	37.0	101.0	122.0
F	16.4	51	197	94	67.5	13.0	17.0
M	12.2	43	32	119	21.0	0.35	1.19
F	12.8	39	82	70	54.0	42.7	35.0
Mean	15.2	49	117	147	43.9	34.4	37.5

TABLE XXII

Haematological Data and Results of the Absorption of 30 Milligrams Iron as Ferrous Sulphate in
Normal European Subjects

Sex	Haemoglobin Grams %	P.C.V. %	Plasma Iron micrograms %	U.I.B.C. micrograms %	Transferrin Saturation %	Iron Absorption Day I %	Day II %
M	15.2	47.0	51	296	14.6	7.1	6.0
M	14.0	44.0	68	115	37.1	6.5	6.6
M	14.8	47.5	100	197	33.6	3.0	4.5
F	14.1	44.5	193	261	42.5	2.6	4.6
Mean	14.5	45.8	103	217	32.0	4.8	5.4

SUMMARY AND CONCLUSIONS

A twin isotope technique based upon the original method of Peacock and his co-workers (1946) is described. In this method, two different radioactive isotopes of iron were employed to study phenomena of iron absorption on consecutive mornings in the same subject. The measurement of absorption on each occasion was made by differentially counting an aliquot of blood for each isotope after digestion and electroplating the iron onto a copper planchet.

Studies are presented to demonstrate the validity of the electroplating procedure per se. Further experimental data confirm the reliability and reproducibility of the complete method. It is clear that while the approach may be painstaking, it is simple, and provides a practical means of examining iron absorption in a single patient under varying experimental conditions.

It is concluded that this method is reliable under the conditions defined above, and it is believed that patients are exposed to little or no risk from the administration of isotopes. This approach was accordingly applied to the investigation of a number of physiological problems related to iron transport in both man and experimental animals.

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