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A STUDY OF DIETARY IRON COMPLEXES AND THEIR ABSORPTION

THESIS

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SUMMARY

The world wide prevalence of iron deficiency states has led to an extensive study of iron metabolism during this century. Although much information has been amassed, the mechanism of iron absorption and factors controlling absorption, have not been clearly delineated. Furthermore, since most of the investigations have involved the use of inorganic iron salts alone, the relevance of these results to the total process of dietary iron absorption is uncertain.

The total iron content (McCance & Widdowson, 1960) and the overall iron absorption from basic foodstuffs has been quantitated by both chemical (Widdowson & McCance, 1942) and isotopic techniques (Moore & Dubach, 1951; Pirzio-Biroli, Bothwell & Finch, 1958). However, the physical and chemical nature of dietary iron remains poorly understood, and little information is currently available about the changes induced by cooking or digestion. These latter changes may cause the iron to be more or less available for absorption, and may be critical both to an understanding of deficiency states and physiological iron absorption.

This study was designed to quantitate the iron in two particular food items, namely liver and wheat; to partially

characterise the nature of the iron present, and to detect any physical or chemical changes in iron complexes induced by cooking and simulated digestion. As well, iron absorption from raw, cooked and digested foodstuffs was measured and compared between groups. Finally an attempt was made to correlate changes in absorption with observed changes in the nature of iron complexes.

Guinea pig liver was selected as a model of a dietary iron source rich in both non-haem and haem iron. Preliminary studies showed that the iron content and form of liver iron was comparable in this species with that of sheep, calf and chicken liver, which provide a rich source of dietary iron.

The guinea pig liver iron stores were labelled with Fe^{59} following depletion of body iron stores by repeated blood lettings over a period of two weeks. The iron removed was replaced, after a lapse of a further two weeks, by intra-peritoneal administration of Fe^{59} chloride together with carrier ferric chloride. This technique resulted in the localisation of sufficient radioactivity within the liver to allow detection and extraction of the various iron fractions.

Estimations of total iron in guinea pig liver showed that the concentration of iron was approximately 150 μg per g of tissue wet weight, and that this iron was uniformly

distributed throughout the organ. After the specimens had been washed repeatedly and blotted to dryness, 60 per cent of the total liver iron was found in the non-haem iron fraction, mostly as ferritin, and the remainder was haem iron. The techniques of "cooking" and digestion used in this study did not alter the distribution of iron between the haem and non-haem iron fractions.

Further characterisation of the liver iron complexes was attempted using centrifugation to measure the soluble iron fraction, and dialysis and sephadex gel filtration to allow some estimation of the physical size of the iron complexes. These estimations were done on raw liver and were repeated following simulated digestion and cooking.

Rapid, prolonged centrifugation of liver homogenate showed that two-thirds of the iron in raw liver was present in the supernatant soluble fraction, and the remainder was precipitated. Both cooking and in vitro tryptic digestion reduced the soluble iron fraction by about one-half. By contrast with a previously published study (Jacobs, Path & Greenman, 1969), this study showed that acid digestion, acid-peptic digestion, and peptic hydrolysis plus cooking did not release iron from insoluble complexes and did not increase the soluble iron component.

The results of dialysis and molecular sieving experiments indicated that various digestive procedures conducted in vitro produced a profound alteration in the size of the liver iron complexes. There was no small molecular weight dialysable fraction detected in raw and cooked liver. However following enzymatic digestion, and cooking plus acid-peptic digestion of liver, the small molecular weight dialysable iron accounted for up to 67 per cent of the total liver iron. Maximal release of small molecular weight dialysable iron complexes was achieved when liver was cooked and then acid-peptic digested. Furthermore, the increase in the dialysable iron fraction was shown to result from breakdown of the ferritin molecule rather than from disintegration of the porphyrin haem molecule.

The elution pattern of raw liver homogenate following filtration through sephadex G200 revealed two protein fractions which contained Fe^{59} activity. These were a high molecular weight fraction, which was subsequently identified as ferritin, and a fraction of intermediate molecular weight identified as haemoglobin. When liver was cooked and digested these two peaks were replaced by a single low molecular weight fraction which contained the whole of the radioactivity.

Furthermore the pattern of the dialysis and the filtration

experiments indicated that the digestion of liver homogenate with acid alone, in the absence of pepsin, was sufficient to release low molecular weight iron components.

The in vitro studies demonstrated that a reduction in the size of the liver iron complexes occurred when liver was subjected to procedures similar to those encountered during food preparation, mastication and gastric digestion. Although the physical and chemical changes induced in the iron complexes were of interest alone, their physiological importance could only be demonstrated if they produced changes in the degree of iron absorption. Accordingly the absorption of iron from these various fractions was tested in rats and compared with iron absorption from an inorganic source, ferric chloride. Iron retention was estimated using a small animal counter by determining the whole body radioactivity eight days after administration of the test dose.

The absorption of selected liver fractions was also tested in six normal male volunteers. Each subject received both cooked, and cooked and then acid-peptic digested liver. Iron^{59} retention was measured in a whole body liquid scintillation counter. In this case the iron retention was calculated from the radioactivity remaining in the body after fourteen days.

In the rat the efficiency of iron absorption from raw, cooked and enzymatically digested liver did not correlate absolutely with the size of the iron complexes. Although both peptic digestion, and cooking followed by peptic digestion produced an increase in small molecular weight iron components, retention of iron was increased only when liver was pretreated by cooking and acid-peptic digestion. Following these combined procedures liver iron was absorbed equally efficiently as the iron from inorganic ferric chloride.

It is true that differences in iron absorption from raw liver and from liver which had been peptic digested in vitro, may have been masked, to a degree, by the processes of in vivo digestion which occurred following administration of the test dose. However absorption from cooked liver remained low despite subsequent exposure to gastric juice. By contrast in vitro cooked and digested liver iron was avidly absorbed. In addition raw liver was readily absorbed even when the stomach was by-passed and the liver preparation was injected directly into the duodenum following pyloric ligation.

Although the correlation was not absolute, the study did strongly suggest that the size of the iron complexes was an important determining factor in iron absorption. Thus,

cooking combined with acid-peptic digestion released the largest percentage of low molecular weight liver iron complexes, as demonstrated by dialysis, and produced an almost two-fold increase in liver iron absorption. By contrast, iron was retained poorly from cooked liver in which all the iron was shown to occur as high molecular weight complexes. The relationship between molecular size of the iron complexes and efficiency of absorption was further suggested by the observed increase in iron absorption from the dialysable fraction when compared with both the non-dialysable fraction and the total liver iron.

In man there was no statistically significant difference between the absorption of iron from cooked liver and from liver which had been both cooked and acid-peptic digested. However, a similar trend of greater iron absorption from in vitro cooked and peptic hydrolysed liver, than from liver which had been cooked but not digested, emerged. Paired studies showed that five of the six test subjects absorbed a higher percentage of iron from cooked and digested liver than from cooked liver. The less striking difference in absorption of iron from these two preparations in man, compared with the rat, may reflect a more efficient mechanism of in vivo peptic hydrolysis in the human subject.

Although the results of this study are consistent overall in demonstrating an effect of cooking and peptic hydrolysis to reduce the size of iron complexes and so increase iron absorption, other factors cannot be excluded. Thus, cooking and peptic hydrolysis result in protein degradation and release a variety of peptides and physiologically active amino acids, as well as reducing the size of the iron complexes. Earlier investigators (Kroe, Kaufman, Klavins & Kinney, 1966; Layrisse, Martinez-Torres & Roche, 1968) have previously shown that certain amino acids do enhance iron absorption; such a stimulating effect cannot be excluded in the present study.

Gabo variety wheat was chosen as an example of a basic dietary iron component of vegetable origin. The physico-chemical form of the iron complexes and the effect on them of acid, acid-pepsin and cooking plus peptic hydrolysis was examined. This study thus allowed a limited comparison of the handling of dietary iron of animal and vegetable origin.

The wheat iron stores were isotopically labelled by allowing the ears of grain to mature in the laboratory in a sucrose medium with added radioactive ferric citrate. After ten days, at a stage when the wheat was judged to be mature, a maximum of 5 per cent of the added radioactivity was incorporated into the grain. The total iron content of the

matured, dried and threshed wheat averaged 27 μg Fe per g of wheat, and as such was in agreement with the figures published by McCance & Widdowson (1960).

Untreated wheat contained only a minimal soluble iron fraction, and following centrifugation over 90 per cent of the total iron was located in the precipitate. By contrast with liver, cooking, acid and enzymatic digestion all produced alterations in the soluble wheat iron fraction. Both acid digestion and to a greater degree acid-peptic hydrolysis increased the soluble iron component. Cooking alone and cooking in conjunction with peptic hydrolysis reduced the percentage of soluble iron when compared with raw wheat and peptic hydrolysed wheat respectively.

The changes in soluble iron detected following various digestive procedures were accompanied by an expected pattern of changes in the small molecular weight components detected by dialysis experiments. Raw wheat contained a very small quantity of dialysable iron complexes, and cooking reduced this to barely detectable levels. However when wheat was digested, and both cooked and digested, more low molecular weight complexes were released. Overall the maximum effect was considerably less than in liver homogenates, and the small dialysable components approached only 4 per cent of the total wheat iron.

Sephadex gel filtration of the iron compounds in the supernatant of the wheat homogenate largely substantiated the results of the dialysis experiments. An elution curve for raw wheat was unattainable due to very low concentrations of soluble iron complexes. Following acid digestion a small intermediate iron protein peak and a larger low molecular weight iron protein peak were detected. After exposure to pepsin only a single low molecular weight iron fraction was present. Following cooking and pepsin digestion both a high and a low molecular weight protein fraction were demonstrated. To this point there were certain similarities in the handling of liver and wheat in that dialysis and molecular sieving experiments confirmed release of small molecular weight iron components following digestion. The patterns of release were, however, different. In liver the maximum release of low molecular weight iron complexes was achieved by cooking combined with peptic hydrolysis, whereas with wheat acid-peptic digestion alone achieved the maximum release.

It was possible to measure wheat iron absorption only in rats and thus no comparison is possible with the liver iron absorption studies in human subjects. The absorption of iron from raw, acid digested, acid-peptic digested and cooked as well as peptic hydrolysed wheat preparations did not differ significantly. However, following cooking, iron absorption

from wheat was markedly increased. In direct contrast to the situation with liver, the wheat preparation which had the highest concentration of high molecular weight iron complexes, and the lowest proportion of soluble iron complexes, was the most efficiently absorbed.

The physico-chemical studies and absorption patterns of wheat iron, therefore, did not indicate any critical relationship between the maximal size of iron complexes and absorption. Thus, factors other than molecular size need to be considered in wheat iron absorption. Phosphorus in the form of phytate compounds occurs in wheat and is known to inhibit iron absorption (McCance & Widdowson, 1935). It may be that the processes of cooking and digestion alter the chemistry of the phytate complexes naturally occurring in the aleurone layer of the grain, or other iron-binding substances within the grain, and so affect iron absorption.

The work in this thesis has shown using a single animal source of iron, guinea pig liver, that cooking, acid and enzymatic digestion alter the physico-chemical nature of the iron complexes and also iron absorption. Increased iron absorption correlates best with decreasing size of the iron complexes; with vegetable wheat iron no such dependence on molecular size has emerged. In this case iron absorption is

probably profoundly affected by iron-binding materials within the grain.

No information has been obtained concerning interactions between various articles of diet. Extension of work in this field would be directed towards similar physico-chemical characterisation of a broad variety of foodstuffs and examination of their absorption, both singly and in combination.

DECLARATION

I hereby declare that this thesis is of my own composition and is a true record of original work which has not been submitted for the award of any degree or diploma in any university. To the best of my knowledge and belief the thesis contains no material previously published or written except where due reference to such material is made in the text.

September, 1972

Rane Naish

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CHAPTER I

INTRODUCTION

The body is maintained in iron equilibrium by the balance of iron loss and iron absorption. Iron loss from the body is small. McCance and Widdowson (1937) reviewed the literature and concluded that no mechanism for the excretion of iron existed. This theory was later confirmed by chemical iron balance studies (McCance and Widdowson, 1938) and by long term balance studies using radio-active isotopes of iron (Dubach, Moore and Callender, 1955; Finch, 1959). Using the latter method iron losses were shown to be of the order of 1 to 2 mg per day. The low, relatively fixed rate of iron excretion suggested that variations in iron absorption were critical in maintaining iron balance. In iron deficiency absorption is increased (Hahn, Bale, Ross, Balfour and Whipple, 1943; Bothwell, Pirzio-Biroli and Finch, 1958; Conrad and Crosby, 1963) while absorption falls when iron stores are replete (Hallberg and Sölvell, 1967). The mechanism of iron absorption has not been clearly eludicated. Most of the current concepts on iron absorption have been derived from studies on the absorption of iron salts and not food iron. Consequently, they may not reflect the mechanism involved in the absorption of iron in the diet.

The daily dietary iron intake is variable throughout the world, and levels ranging from 6 mg to 405 mg per day have been reported (World Health Organization, 1970). Daily dietary iron requirements are difficult to determine because the dietary iron is only partially absorbed. An iron intake of 10 to 12 mg per

day is widely quoted (National Research Council, 1948; Moore and Dubach, 1956; Coons, 1964) as being sufficient to maintain a normal adult male in iron balance. However in some parts of the world iron deficiency may develop while taking a diet, high in vegetable content, even in the absence of blood loss (Foy and Kondi, 1956; Patwardhan, 1962; Apte and Venkatachalam, 1962).

Staple foodstuffs such as cereals and black beans provide most of the dietary iron on a global basis (Ramalingaswami and Patwardhan, 1949; Apte and Venkatachalam 1962; Layrisse, Martinez-Torres and Roche, 1968), while in Western society a very considerable contribution is made by meat (Walsh, Kaldor, Brading and George, 1955; McCance and Widdowson, 1960). It is known that marked differences exist in the absorbability of these forms of iron (McMillan and Johnston, 1951; Moore and Dubach, 1951; Layrisse et alii, 1968). At present it is not understood which physical and chemical forms of iron are preferentially retained by the body.

Ionic iron has been measured in many foods on the assumption that this is more readily available for absorption (Lottrup, 1934; Moore, Dubach, Minnich and Roberts, 1944). In 1934, Sherman, Elvehjen and Hart showed that 60 per cent of iron in liver and heart muscle, less than 50 per cent of beef skeletal muscle iron, and less than 25 per cent of the iron in oysters and spinach was

in the ionic state. However they found that correlation between the available ionic iron and the absorption in rats was poor. Later workers (Kaldor, 1957; Sandford, 1960) demonstrated that the amount of ionic iron in other food substances ranged from 0.1 per cent to 4.7 per cent in blood, liver, flour, silver beet and sultanas. They also extended the study to assess the effects of cooking and acid-peptic digestion on the iron complexes. Both cooking and peptic hydrolysis released ionic iron from silver beet, sultanas, blood and liver. In a further study involving twenty-five common foodstuffs, soluble and ionic iron was found to be released by both acid-peptic digestion and cooking prior to peptic hydrolysis (Jacobs, Path and Greenman, 1969). By contrast, cooking alone reduced both the soluble and ionic components in most of the foodstuffs.

The amount of food iron, and iron added to food, converted to the reduced form during artificial gastric digestion (Kirch, Bergeim, Kleinberg and James, 1947) and intra-gastric digestion (Bergeim and Kirch, 1948) has also been measured. Fresh fruits and vegetables were found to have the greatest reducing properties. Almost all of the total iron was reduced by addition of these foods. Protein rich foods also had a significant effect in that 25 to 40 per cent of the iron was shown to be reduced. From these results the authors postulated that ascorbic acid, proteins and their breakdown products may facilitate iron

absorption by their reducing properties.

The most extensively studied form of dietary iron is that bound in the porphyrin ring of myoglobin and haemoglobin. It has been shown (Conrad, Cortell, Williams and Foy, 1966a; Brown, Hwang and Nicol, 1966) that haemoglobin is split in the gut lumen into haem and globin and that the haem moiety is absorbed into the mucosal cell where the iron is released from the porphyrin ring before entering the blood stream (Conrad et alii, 1966a); Wheby, Suttle and Ford, 1970). Furthermore its absorption is not affected by ascorbic acid and iron chelating agents such as desferrioxamine (Callender, Mallett and Smith, 1957; Turnbull, Cleton and Finch, 1962; Hwang and Brown, 1963; Hallberg and Sölvell, 1967).

The iron in egg yolk has also been characterised. It has been shown to exist in the ferric form strongly complexed to the phosphate of the yolk phosphoproteins (Chodos, Ross, Apt, Pollycove and Halkett, 1957). The chemical nature of egg iron may explain its poor absorption.

Absorption of iron from individual foods and from mixtures of several foodstuffs has been measured by both chemical (McMillan and Johnston, 1951; Apte and Venkatachalam, 1962) and radio-isotopic techniques (Pirzio-Biroli, Bothwell and Finch, 1958; Layrisse et alii, 1968). However the isotopic labelling of a

complete diet is almost impossible and so little information is available about interactions between various iron containing foods. Nevertheless certain observations have gained acceptance. Striking differences exist in the absorption of iron from animal and vegetable foods (Moore and Dubach, 1951; Layrisse et alii, 1968). Absorption from vegetable foods was shown to range from 1 to 8 per cent while absorption from animal foods has generally been of the order of 20 per cent (McMillan and Johnston, 1951; Martinez-Torres and Layrisse, 1970). In addition Layrisse and his co-workers (1968) demonstrated that animal foods enhanced vegetable iron absorption, whereas vegetables decreased animal food iron absorption.

Ascorbic acid has been shown to enhance absorption of iron from all the food studied (Steinkamp, Dubach and Moore 1955; Höglund and Reizenstein, 1969) with the exception of haem iron (Hallberg and Sölvell, 1967) and soya bean iron (Kuhn, Layrisse, Roche, Martinez-Torres and Walker, 1968). The effect of citrus juice has been studied on a smaller scale, and was shown to have an enhancing effect on iron absorption (Moore and Dubach, 1951) which has been attributed to its ascorbic acid content.

The amino-acids, cysteine and methionine were demonstrated to enhance iron absorption from black beans; cysteine also increased egg iron absorption (Moore and Dubach, 1956; Layrisse et alii, 1968; Martinez-Torres and Layrisse, 1970). Desferrioxamine and

phytate have been shown to decrease food iron absorption, probably due to the formation of insoluble iron complexes (Sharpe, Peacock, Cook and Harris, 1950; Apte and Venkatachalam, 1962; Kuhn et alii, 1968).

Limited studies have been performed to determine the effect of luminal factors on food iron absorption. Ferric chloride labelled with Fe^{59} and baked into bread was equally well absorbed by normal subjects as by those who had undergone gastric resection (Choudhury and Williams, 1959). However absorption in anaemic subjects was greater in those with an intact stomach. Absorption of Fe^{59} administered with a standard meal was not reduced in achlorhydric subjects without anaemia (Jacobs, Rhodes, Peters, Campbell and Eakins, 1966). By contrast normal gastric juice was found to increase the absorption of iron in the anaemic achlorhydric patients. These studies suggest that a component of gastric juice may have a role in food iron absorption, and from the latter investigation it would appear that gastric acid was the important enhancing factor.

Evaluation of the food fortification programme in the United States showed that metallic powdered iron added to flour as sodium ferric pyrophosphate, as ferric orthophosphate, as reduced iron, or as ferrous sulphate, and then baked into bread was equally absorbed by human subjects (Steinkamp et alii, 1955). The equal utilisation of these vastly differing iron preparations suggests that the various

iron forms are changed during the baking process so that all the forms are equally effective.

It is apparent that many authors have studied single parameters pertaining to dietary iron, such as the content of ionic or reduced iron, the effect of acid-peptic digestion, and the absorption of single test substances. As yet no comprehensive correlative study has been performed to relate the form of iron in food to absorption, or to assess the effects of cooking and enzymatic digestion on the nature of food iron and its subsequent retention by the body.

The present work was designed -

1. to determine the total iron content of food substances;
2. to partially characterise the nature of the iron;
3. to estimate the absorption of the various fractions of food iron; and
4. to determine the effects of cooking and enzymatic digestion on the nature and absorbability of the iron.

Guinea pig liver and Gabo variety wheat were the test foods studied.

CHAPTER II

LITERATURE REVIEW

IRON IN BIOLOGICAL SYSTEMS

Iron is necessary to most if not all living organisms and is an integral part of a large variety of biological molecules. Probably the most important group of iron compounds are the haemoproteins in which the coordinating groups around the iron are remarkably similar. The function of the haemoproteins includes the binding of molecular oxygen as in haemoglobin and myoglobin, the transfer of electrons as in the cytochromes, and the cleavage of the peroxide structure as in catalase and peroxidase. An explanation of how these haemoproteins carry out such diverse tasks is given by Eichran (1964) and will not be elaborated on in this review.

Iron is also active in forms other than haem. It is essential for the activity of certain enzymes such as aconitase which catalyses the interconversion of citric, isocitric and aconitic acids (Dickman and Cloutier, 1951) and phenolytic oxygenases which catalyse the cleavage of the aromatic rings of phenolic compounds in the presence of molecular oxygen (Mehler, 1962). Iron is not an integral part of the above enzymes. However, iron is incorporated into the structure of ferredoxin, an enzyme essential for a number of electron transfer reactions and especially in the separation of light and dark reactions of photosynthesis (Mortensen, Valentine and Carnahan, 1962).

In man iron is an essential component of haemoglobin, myoglobin and a number of tissue enzymes. It is required for the important physiological functions of oxygen transport and cellular respiration. The normal human adult body contains 3 to 5 g of iron, which is transported throughout the body bound to a protein transferrin. The main bulk of the iron, approximately 55 per cent, is in the form of circulating haemoglobin. Ten to 20 per cent is present in myoglobin, a small amount occurs in the cellular enzymes and 20 to 30 per cent is stored as ferritin and haemosiderin in the liver, spleen, and bone marrow.

Body iron losses are limited to between 1 and 2 mg per day (McCance & Widdowson, 1937, 1938; Dubach, Moore & Callender, 1955; Finch, 1959). Therefore in the normal situation 1 to 2 mg of food iron must be absorbed daily to replace the losses and maintain the body in iron balance. This represents about 10 per cent of the average daily intake of iron in a Western community (National Research Council, 1948).

DIETARY IRON INTAKE

Iron deficiency is a very common and widespread problem both in under-developed and prosperous countries. Some indication of the extent of the problem is given by the FAO/WHO survey which showed that in 1970 up to 90 per cent of infants in Asia were iron deficient. In Western groups between 3 and 6 per cent of

adult Scandinavian men were affected and between 21 and 80 per cent of all pregnant women were iron deficient. The most vulnerable groups throughout the world were, predictably, pregnant women and young children.

The amount of iron present in most simple food substances has been determined and tabulated in detail by several workers (McCance & Widdowson, 1960; Watt and Merrill, 1963). Two methods have been used to measure daily iron intake. The first method has estimated the dietary iron content from existing food composition tables (Leverton & Roberts, 1937; Bransby, Daubney & King, 1947). In the second method, the same investigators calculated the iron intake after chemical analysis of mixed diets. These early workers found poor correlation between the two methods, but a recent report (Monsen, Kuhn & Finch, 1967) suggested close agreement between the two parameters.

A recent analysis of dietary surveys conducted during the past 25 years has been published (WHO Expert Group 1970, Addendum 1). Although the majority intake varies only between 10 and 20 mg per day, there are extremely high intakes of iron in some areas. In particular the South African Bantu was shown to have an iron intake of up to 200 mg per day (Walker & Arvidsson, 1953); this high dietary iron level was attributed to the type of cereals in the diet and the iron cooking utensils.

ADDENDUM 1. INTAKE LEVELS OF IRON IN VARIOUS PARTS OF THE WORLD IN MG PER PERSON PER DAY

REGION	EXTREME RANGE*	MAJORITY RANGE**
Africa	8 - 405	14 - 19
Asia, including the Far East	6 - 80	10 - 25
Near East	6 - 80	10 - 25
Latin America	10 - 20	12 - 18
Europe		10 - 16
U.S.A.		12 - 19.5

*Average lowest and highest per capita intakes reported in the surveys.

**Lowest and highest per capita intakes of more than 80% of the population reported in the surveys.

The actual daily iron requirement is difficult to determine because dietary iron is only partially absorbed and little is understood of the factors which affect absorption of the various iron complexes. The recommended levels of dietary iron intake vary from 12 to 18 mg daily (National Research Council, 1948; United States, Agricultural Department, 1969). It is accepted that a lower level of iron intake will maintain an adult male in iron balance than is necessary for a menstruating female (Moore, 1955; Monsen et al. 1967). In addition, iron deficiency has been shown to occur in certain populations which consume an adequate amount of dietary iron, even in the absence of blood loss (Foy & Kondi, 1956; Apte & Venkatachalam, 1962). The discrepancy between the level of iron intake and the level of absorption in these groups is attributed to the high phytate content of the predominantly cereal diet.

KNOWN DIETARY IRON COMPLEXES

The form in which iron occurs in many food substances is unknown. However, in countries where meat is an important constituent of the diet much of the dietary iron is bound in the porphyrin ring of myoglobin and haemoglobin. Haemoglobin is a conjugated protein with a molecular weight of approximately 60,000. It contains four haem groups which are attached to the protein moieity globin. The latter consists of two pairs of

polypeptide chains known as the alpha (α) and beta (β) chains. According to Xray crystallography of horse haemoglobin, four sub-units are arranged in a tetrahedral form, with the four haem units lying in separate pockets on the surface of the molecule (Perutz, Rossmann, Cullis, Muirhead, Will & North, 1960; Perutz & Muirhead, 1963).

Haem, which constitutes about 4 per cent of the weight of the molecule, is a metal complex consisting of an iron atom in the centre of a porphyrin structure. The porphyrin ring consists of four pyrrole rings united by four methane bridges. The iron content of haemoglobin is 0.3466 per cent of the weight of the molecule. The iron has a coordination valence of six. Of these, four lie in one plane and link the iron to the nitrogen atoms of the pyrrole rings, whereas the remaining two valencies, one on each side of the flat haem molecule, are linked to the globin (Lemberg & Legge, 1949). The linkage form between haem and globin is uncertain (Keilin, 1960; O'Hagan, 1960). Haemoglobin is widely distributed in nature and wherever found has the same fundamental property of reversible oxygenation. The haemoglobins of various plant and animal species do differ widely in other biological, chemical and physical properties. There is no evidence, however, that the differences are related to the haem components (Lemberg & Legge, 1949).

Myoglobin, muscle haemoglobin, is a haem pigment with a

molecular weight of 17,000. It can be identified by paper, starch or acrylamide electrophoresis or by spectrophotometry (Wintrobe, 1968c).

Ferritin and haemosiderin also contribute to the daily food iron intake. Ferritin, originally characterised by Laufberger (1937) has been shown to be both water soluble and capable of crystallisation (Granick, 1942, 1943, 1946; Granick & Michaelis, 1943). Other observations made by these workers indicated that the protein fraction, apoferritin, was constant, whereas the iron fraction was variable and composed as much as 23 per cent of the dry weight of the molecule. The ferritin molecule consists of a nucleus of iron surrounded by a shell of protein approximately spherical in shape (Harrison, 1963). By electron microscopy it appears that the iron is held in six micelles of ferric hydroxide arranged at the corners of a regular octahedron (Granick, 1954).

By contrast, haemosiderin is granular and water insoluble. It is a larger less well defined molecule consisting mainly of iron and is thought to be derived from breakdown of ferritin (Granick, 1946; Harrison, 1964).

Martinez-Torres & Layrisse (1970) have assayed veal muscle labelled with iron^{55} to determine the distribution of the iron compounds. The fractions were monitored for iron content by measuring radioactivity and also by chemical means. More than

90 per cent of the iron was shown to be present as a metallo-porphyrin, most of which was myoglobin.

Other food substances which have been studied include egg yolk in which iron exists in the ferric form strongly complexed to the phosphate of the yolk phosphoproteins (Chodos et alii, 1957), and wine in which the iron occurs predominantly in the ferrous form (McDonald, 1963). The exact nature of iron compounds in wheat has not been determined, but it is known that the iron occurs in the aleurone layer of the grain (Aykroyd & Doughty, 1970; Jenner, 1970).

ABSORPTION OF DIETARY IRON

The determination of absorption of iron from foodstuffs presents formidable difficulties. Chemical balance studies were used initially (McCance & Widdowson, 1937) and are still used (Apte & Venkatachalam, 1962, 1964) due to the difficulties in labelling a variety of foods by isotopic techniques. On the other hand investigations into the absorption of iron salts and haemoglobin iron have utilised radioactive compounds for many years. Iron absorption has been calculated from the amount of radioisotope incorporated into the circulating red blood cells (Hahn, Bale, Lawrence & Whipple, 1939; Saylor & Finch, 1953); the amount of the isotope recovered in faeces; or methods incorporating both faecal loss and red cell iron incorporation

(Dubach, Callender & Moore, 1948; Bothwell et alii, 1958). More recently iron retention has been determined simply and accurately with the use of the whole body liquid scintillation counter (Price, Reizenstein, Cohn, Cronkite & Wasserman, 1961; Van Hoek & Conrad, 1961; Sargent, 1962).

The mean absorption of iron determined chemically from carefully controlled basal diets varied from 11 to 15 per cent (Widdowson & McCance, 1942; Johnston, Frenchman & Burroughs, 1948; Schlaphoff & Johnston, 1949; McMillan & Johnston, 1951). In two of these experiments (McMillan & Johnston, 1951) beef and spinach were added to the basal rations. This made it possible to determine that 21 per cent of iron in beef and 13 per cent of iron in spinach was retained. The mean absorption of iron from a standard meal, to which iron^{59} had been added was 5.3 per cent (Pirzio-Biroli et al. 1958; Jacobs, Rhodes & Eakins, 1967).

With the exception of the few experiments cited above, most of the studies on food iron absorption have been performed on single food items. The results therefore do not give a true indication of iron absorption from a complete diet. As well, different studies of the same foodstuffs have produced various estimates of iron absorption. Thus absorption from unenriched and enriched white bread has ranged from 1 to 12 per cent in normal subjects (Widdowson & McCance, 1942; Steinkamp et al.

1955; Elwood et al. 1968; Callender & Warner, 1968). By contrast, only about 1 per cent of the iron present in brown bread was absorbed. This poor absorption in the face of the much higher total iron content of brown bread was attributed to high phytate content of brown bread (Widdowson & McCance, 1942). Absorption of iron from beef muscle has varied from 20 to 40 per cent in individual series (Johnston et al. 1948; Layrisse et al. 1968; Martinez-Torres & Layrisse, 1970). Egg iron appears to be poorly absorbed in adults (Moore & Dubach, 1956; Chodos et al. 1957), but young children were shown to be able to utilise egg iron very efficiently (Schultz & Smith, 1958). Approximately 10 to 20 per cent of the iron in chicken and sheep liver is retained (Walsh et al. 1955; Moore & Dubach, 1951). This figure is comparable to the mean absorption figure of 14 per cent obtained when chicken muscle was used as the source of iron (Moore & Dubach, 1951). The mean absorption from spinach and lettuce was shown to be approximately 12 per cent by several workers (Johnston et al. 1948; McMillan & Johnston, 1951; Moore & Dubach, 1951; Chodos et al. 1957). The absorption of iron from cereals appears to be much lower than that from animal sources. Accordingly, healthy volunteers absorbed only 2 per cent of the iron in corn (Layrisse et al. 1968), and 3 per cent of wheat iron (Kuhn et al. 1968). Iron in milk is likewise poorly and variably absorbed (Schultz & Smith, 1958). The work of the last group of authors also suggested that food iron

generally was better assimilated by normal children than by adults.

FACTORS AFFECTING FOOD IRON ABSORPTION

Iron deficiency in man produces a reproducible increase in food iron absorption (Chodos et al. 1957; Pirzio-Biroli et al. 1958). However comparative studies have shown that the increase was not as marked as that seen with inorganic iron absorption (Pirzio-Biroli et al. 1958).

Ascorbic acid likewise enhances the absorption of food iron. In the presence of ascorbic acid iron absorption is increased from bread (Callender & Warner, 1968; Höglund & Reizenstein, 1969); from bread enriched by added inorganic iron (Steinkamp et al. 1955); from eggs and rabbit muscle and liver (Moore & Dubach, 1956); and from wheat, corn, and ferritin (Kuhn et al. 1968). Furthermore, absorption of iron from a standard meal, to which a tracer dose of Fe^{59} chloride had been added, was increased in the presence of ascorbic acid (Pirzio-Biroli et al. 1958). However, ascorbic acid has no influence on the absorption of iron from soya beans (Kuhn et al. 1968) and veal muscle (Martinez-Torres & Layrisse, 1970).

Kuhn and his associates (1968) also showed that desferrioxamine, a substance which acts as an iron-chelating agent, decreased the absorption of iron from wheat, corn and ferritin, but not from soya beans. The absorption of iron from veal

muscle was likewise significantly decreased by desferrioxamine (Martinez-Torres & Layrisse, 1970). This reduction was unexpected as these same workers have shown that the iron in veal muscle exists predominantly as a metalloporphyrin. Previous studies (Kuhn et al. 1968; Wheby et al. 1970) have shown that desferrioxamine inhibits iron absorption from certain foods but has no effect on the absorption of the haem-porphyrin.

Soluble sodium phytate as well as the insoluble phytate compounds decreases the absorption of food iron and iron salts (Sharpe et al. 1950; Apte & Venkatachalam, 1962, 1964; Kuhn et al. 1968). It has long been recognised that a large portion of the total phosphorus of cereals and other vegetables is present in the form of phytin, the calcium magnesium salt of inositohexaphosphoric acid. In the more affluent countries only a small percentage of dietary phosphorus is in this form, but in the poorer countries where cereals form the main bulk of the daily food, the daily dietary phytate intake is also very high (McCance & Widdowson, 1935). The prevalence of iron deficiency in the underdeveloped countries can possibly, in part, be attributed to the high level of phytate in the diet. The effects of phytate on iron absorption have been shown to be nullified by high doses of calcium (Apte & Venkatachalam, 1964). The calcium interacts with the phosphate to form an insoluble complex thus leaving the iron free for absorption.

The influence of dietary protein and amino acid content on iron absorption has been studied largely in the rat. In that animal cysteine was shown to enhance inorganic iron absorption (Groen, van den Broek & Veldman, 1947). Later studies suggested that the diet should contain at least 15 to 18 per cent of calories as proteins to maintain adequate dietary iron absorption (Klavins, Kinney & Kaufman, 1962). Further work on individual amino acids showed that glutamic acid, serine, phenylalanine, proline and methionine also increased the absorption of iron (Kroe, Kinney, Kaufman & Klavins, 1963). An enhancing effect similar to that in the intact rat was also shown using isolated proximal small intestine (Kroe, Kaufman, Klavins & Kinney, 1966). Increase in iron transfer was maximal between pH 2.5 and 3.0, and it was postulated that amino acids acted either as buffers or as chelating agents to maintain the iron in solution at the low pH. An alternative theory proposed that the amino acids stimulated a specific or non-specific pathway of intestinal absorption and so facilitated iron absorption.

Corresponding investigations in man are of a limited nature. However, Conrad, Foy, Williams & Knospe (1967a) showed that both starvation and diets deficient in protein produced a decrease in iron absorption in man. Absorption of iron from black beans, which are a major constituent of the diet in South America, was

enhanced two-fold by the addition of fish protein (Layrisse et al. 1968) and a similar increase was obtained by the addition of amino acids in equivalent proportions. The sulphur containing amino acids cysteine and methionine were the active enhancing amino acids whereas basic, aromatic and aliphatic amino acids had no effect in increasing iron absorption (Martinez-Torres & Layrisse, 1970).

The bulk of the meal taken may also affect iron absorption. Sharpe et al. in 1950 added tracer iron to a variety of meals and showed that the proportion of iron absorbed, when the total iron was kept constant, varied inversely with the bulk of the meal. More recent investigations (Brise, 1962a, 1962b; Höglund & Reizenstein, 1969) likewise demonstrated a decrease in inorganic iron absorption when the test substance was taken with a meal.

The role of gastric secretion in modifying absorption of food iron is not yet clearly defined. Ferric chloride labelled with Fe^{59} and baked into bread was absorbed equally well by subjects with intact stomachs as by those who had some form of gastric resection (Choudhury & Williams, 1959). However, when the subjects were anaemic a greater amount of iron was absorbed in those with intact stomachs. No differences in the absorption of Fe^{59}, administered with a standard meal, were demonstrated between haematologically normal subjects with achlorhydria, and

those with normal gastric secretion (Jacobs, Rhodes, Peters, Campbell & Eakins, 1966). These authors also showed that gastric juice of anaemic subjects did not alter the iron absorption in normal and achlorhydric subjects. However, an increase in absorption occurred in the achlorhydric anaemic subjects. Therefore it would appear that acid may play a selective role in food iron absorption.

ABSORPTION OF KNOWN DIETARY IRON COMPLEXES

Ferritin, haemosiderin, haemoglobin and myoglobin constitute a major source of food iron in countries where meat is an important constituent of the diet. The level of absorption of iron from haemosiderin has not yet been determined. With ferritin Turnbull et al. (1962) have shown that approximately 2 per cent is absorbed by the fasting individual and that absorption falls to about half when ferritin is taken with a meal. In a comparative study Hussain, Walker, Layrisse, Clark & Finch (1965) showed that 5 to 10 per cent of iron in ferritin, and 20 per cent of haemoglobin iron was absorbed by the haematologically normal subject. In the presence of anaemia the absorption of the test forms of iron rose appreciably.

Absorption of haemoglobin iron was first demonstrated by Walsh et al. in 1955. Since then it has been increasingly appreciated that haemoglobin forms an important source of dietary

iron. Several further groups have now established that haemoglobin iron is absorbed almost as well as iron salts (Turnbull et al. 1962; Hallberg & Sölvell, 1967) and that the absorption is not decreased when haemoglobin iron is ingested with a meal (Turnbull et al. 1962).

Initially it was assumed that the iron was split from the porphyrin ring in the gut lumen and absorbed in the ionic form. Accordingly, in vitro analyses were developed to measure the amount of ionic iron released from haemoglobin by various degradation procedures. Kaldor in 1957 showed that acid-peptic digestion of untreated and heat denatured haemoglobin released significant amounts of ionic iron from the metalloporphyrin. More ionic iron was released from the denatured haemoglobin, and correlative absorption studies in rats showed that absorption was also higher from the denatured haemoglobin. By contrast, Callender, Mallett & Smith (1957) detected no release of ionic iron from haemoglobin after a variety of digestive procedures. The same workers also demonstrated that haem iron absorption in man was not affected by ascorbic acid. They therefore concluded that haem iron was absorbed by a different pathway than ionic iron. Further evidence for this theory accumulated when later workers (Turnbull et al. 1962; Bannerman, 1965; Hallberg & Sölvell, 1967) confirmed that neither ascorbic acid nor added phytates affected haem iron absorption. Further differences in

absorption of inorganic and haemoglobin iron have since emerged. Thus it has been established that the iron chelating agents EDTA (Conrad, Weintraub, Sears & Crosby, 1966b) and desferrioxamine (Hwang & Brown, 1965; Wheby et al. 1970) have no effect on haemoglobin iron absorption. Phytate likewise did not affect the absorption of iron from haemoglobin (Turnbull et al. 1962).

Intact haem has now been isolated from the intestinal cells (Conrad et al. 1966b; Weintraub, Weinstein, Huser & Rafal, 1968) and it is now accepted that haem iron is absorbed by the intestinal cells complexed to the porphyrin ring. Other recent investigations have suggested that the intestinal mucosa contains a haem splitting enzyme which releases iron from the porphyrin ring before it enters the portal venous system (Conrad et al. 1966b; Conrad, Benjamin, Williams & Foy, 1967b; Weintraub et al. 1968).

Nevertheless it would appear that the regulatory mechanisms for haemoglobin iron absorption are similar to those for ionic iron. Iron deficiency in man and animals produces an increase in haemoglobin iron absorption (Callender et al. 1957; Turnbull et al. 1962; Hallberg & Sölvell, 1967; Wheby et al. 1970). However in all cases the rise was lower than that observed with iron salts. Furthermore iron overloaded guinea pigs absorbed marginally less haemoglobin iron than normal animals (Conrad et al.

1966b). Conrad and his associates also showed an increase in the amount of haemoglobin iron absorption with an increase in the dose; however, as with inorganic iron, the per cent absorption decreased. In addition the administration of non-radioactive ferric chloride together with radioactive haemoglobin decreased the absorption of haemoglobin iron, and likewise the addition of non-radioactive haemoglobin to Fe^{59} chloride decreased the absorption of the latter. Both haemoglobin and ferric chloride given intravenously before oral administration of the organic or inorganic form of iron produced a decrease in the retention of radioactive iron. Similar observations on haemoglobin iron absorption have been made in man. Thus, Turnbull and his co-workers (1962) demonstrated a rise in the haemoglobin iron retained when the dose was increased, with a corresponding fall in the percentage retained. Hallberg & Sölvell (1967) showed that the absorption of haem iron in man was also decreased if the test dose was preceded by inorganic iron, and likewise the absorption of inorganic iron decreased when preceded by haemoglobin administration. The slower rate of absorption of haemoglobin iron (Callender et al. 1957) is thought to be responsible for the lower inhibitory effect of haemoglobin on iron absorption when compared with inorganic iron (Conrad et al. 1966b; Hallberg & Sölvell, 1967).

IN VITRO STUDIES ON FOOD IRON

Various foodstuffs have also been tested to determine the total iron concentration; the amount of ionic iron present, and the release of ionic iron by various digestive processes. Iron present in the ionic form is frequently referred to as the available iron (Lottrup, 1934). Little attempt has been made as yet to correlate the amount of available iron present in foodstuffs with the absorption of iron from that particular item of diet.

In 1934 Sherman and his associates calculated that beef and pork liver and cardiac muscle contained approximately 60 per cent of the total iron as the ionic form; beef skeletal muscle about 50 per cent; and oysters, spinach and blood somewhat less than 25 per cent. Absorption studies in rats with some of these foods indicated that there may be some correlation between available iron present and the haemoglobin regeneration rate. Sandford, in 1960, measured the ionic iron present in silver-beet, sultanas, blood and liver, and demonstrated that the level was increased by acid and acid-peptic digestion. More recently Jacobs and his associates (1969) determined the amount of available iron present in twenty-five common foodstuffs following water & acid-peptic extraction. In the majority of the twenty-five dietary items tested almost half of the iron present was in the ionic form, and once again this was increased by acid-peptic digestion. Cooking, by contrast, decreased the percentage of

ionic iron present.

Certain foods, especially fresh fruits and vegetables, during in vitro gastric digestion caused approximately three-quarters of the iron within the foodstuff or added to it, to be reduced to the ferrous form (Kirch et al. 1947). The reducing properties of the foods were credited to ascorbic acid, proteins, peptides and amino acids. Similar reduction of iron to the ferrous form occurred during intragastric digestion of ingested foods (Bergeim & Kirch, 1948). The conversion of iron to the reduced form has been considered important in increasing absorbability of the iron present (Groen et al. 1947; Kirch et al. 1947; Bergeim & Kirch, 1948). However, adequate correlative absorption studies have not established that reduction of the iron is critical to facilitate absorption.

DIETARY ENRICHMENT

Iron deficiency is so widespread that substantial efforts have been directed towards its prevention on a national level. Thus in Great Britain iron is added to flour at considerable expense (Elwood, 1970). In the United States of America also, many investigators (Finch, 1965; White & White, 1968; U.S. Council on Food & Nutrition, 1968) have recommended a nationwide programme to eradicate iron deficiency. The recommended

preventive measures mainly embrace dietary enrichment of staple foods. The 1970 WHO study group likewise recommended enrichment programmes to be instituted in areas at particular risk of deficiency. Cereals were considered the ideal food for fortification programmes because of their availability to low income groups.

However the effectiveness of iron added to foods in producing an increase in food iron absorption has recently been questioned. Steinkamp et al. (1955) measured the absorption of four iron preparations commonly used in bread and flour enrichment programmes. These were ferrous sulphate, reduced iron powder, ferric orthophosphate and sodium ferric pyrophosphate. Fe^{59} was incorporated into each of the preparations before being baked into bread. All preparations were equally absorbed by the test subjects. Normal subjects absorbed between 1 and 12 per cent of the iron whereas anaemic subjects absorbed from 45 to 64 per cent of the iron. A similar proportion was absorbed from unenriched bread but because the total iron content was lower the absolute amount of iron absorbed was much less. The authors concluded from this study that the iron preparations were altered during baking, so that all were equally effective, and that enrichment of the diet with iron was a valuable procedure. However, more recent studies have shown that some forms of iron added to flour are almost completely unavailable to man. Thus the Panel on Iron in Flour (Ministry

of Health (1968) measured the absorption of radioactive powdered iron, reduced iron and ferric ammonium citrate added to flour and then baked into bread. When given in bread, as part of breakfast, the mean absorptions of the three preparations were 0.86, 3.55 and 10.35 per cent respectively. The mean absorptions, by the same subjects, of radioactive ferrous sulphate from bread was approximately 18, 8 and 13 per cent respectively. The addition of egg to the breakfast menu decreased the absorption of all the forms of supplemental iron. The iron preparations were more readily absorbed by iron deficient subjects; however the increase in absorption of powdered iron was negligible. In addition both powdered iron and reduced iron were very poorly absorbed by subjects with gastric achlorhydria. These investigations indicate that the type of iron compound used in enrichment programmes is critical in determining the availability of the added iron.

The response of the haemoglobin level to iron supplements has been used as a measure of the efficacy of added iron. Natvig, Bjerkedal & Jonassen (1963) studied the effect of 30 mg of supplemental iron given daily to adult men and school children. There was no overall change in the haemoglobin levels and they therefore concluded that dietary iron was adequate in these individuals. Scott & Heller (1964) gave supplements of 6.5 mg of iron per day to the Eskimos, and found a

rise in haemoglobin only in those women who had a haemoglobin level of less than 11.7 g per 100 ml at the start of the study. They concluded that the daily dietary iron intake which had been estimated at 10 to 15 mg was sufficient where there was no abnormal loss of iron. In children, however, iron supplements (5 mg per day) produced no significant change in haemoglobin levels even in those with a pre-treatment level of haemoglobin below 10.0 g per 100 ml (Bradfield, Jensen, Gonzales & Garrayar, 1968). A comparable study indicated that a supplement of 10 mg per day raised the level of haemoglobin in adolescent girls and women, whereas 5 mg had no significant effect (Elwood, Waters & Greene, 1970).

These investigations substantiate the WHO 1970 expert group recommendations that more information is needed regarding both the form and quantity of iron which can be most effectively utilised in the dietary iron enrichment programmes, before these plans can be satisfactorily implemented.

Yet another issue has been raised by Elwood (1970) which he feels should be more fully evaluated before recommendation of dietary supplementation, and this pertains to the assessment of the morbidity and loss of function produced by anaemia. He performed a survey in which 4,000 women were screened and found that 130 of these had haemoglobin levels below 10.5 g per 100 ml.

The women with the low haemoglobin levels were given 90 mg of iron daily, 30 mg of iron daily, or no iron for 12 weeks and the severity of their symptoms was assessed before and after treatment. No change in psychomotor function, cardio-respiratory function or postoperative progress was detected. However, evidence is presented that a fall in function occurs with haemoglobin levels below 8.0 g per 100 ml. As anaemia of this level occurs in less than 1 per cent of the Western population, Elwood queries the advisability of nationwide enrichment programmes except in deprived areas.

IRON ABSORPTION

It is well established that the human subject absorbs iron more readily in the ferrous than in the ferric form (Groen et al. 1947; Brise & Hallberg, 1962a) and that absorption occurs in any part of the gastrointestinal tract distal to the stomach (Hahn et al. 1943; Wack & Wyatt, 1959; Brown, 1963). Maximum absorption has been shown to occur in the duodenum, and to diminish distally with very small amounts being absorbed in the large bowel (Endicott, Gillman, Brecker, Ness, Clarke & Adamik, 1949; Brown & Justus, 1958; Hemmati, 1968).

It is also recognised that iron deficiency produces an increase in the absorption of food and inorganic iron, and conversely that excessive iron stores diminish iron absorption

(Bothwell et al. 1958; Pirzio-Biroli & Finch, 1960; van Hoek & Conrad, 1961; Conrad & Crosby, 1963; Hallberg & Sölvell, 1967). However, the mechanisms regulating iron absorption have not been clearly elucidated, and theories concerning this can be divided into two groups. The first maintains that the intestinal cells regulate the absorption whereas the second holds that luminal factors are critical and act to make the iron more or less available.

MUCOSAL FACTORS IN IRON ABSORPTION

A theory of mucosal regulation or "block" was first proposed by Hahn and his associates in 1943. These investigators observed that when a dog received a large dose of iron followed by a dose of radioiron, less radioactivity appeared in the erythrocytes than might have been expected. They concluded that the first dose blocked the absorption of the second labelled dose. The mucosal block theory was subsequently expanded by Granick (1946, 1949, 1951). He initially demonstrated that guinea pigs which were fed huge quantities of iron accumulated ferritin crystals in the intestinal mucosa. He then postulated that the ferrous ions entering the mucosal cell stimulated the production of an acceptor protein, apoferritin. After entry into the cell, the divalent ions were oxidized to the ferric state under the redox conditions at the luminal border and the trivalent ions combined with apoferritin to form ferritin. At

the vascular border the ferritin was reduced by a different redox environment with consequent release of ferrous ions which passed from the cell into the blood stream and after reoxidation were bound to transferrin. When the body's requirements were met further absorption was curtailed by accumulation of mucosal ferritin. An additional pathway was included for conditions of exceptional iron need when redox potentials of the cell allowed ferrous iron to traverse the cell directly.

Recent studies have discredited the mucosal block theory in its original concept and attention has now been focused on the iron content of the absorptive intestinal cells as the important factor in regulating the cell's capacity to absorb iron. The studies of Conrad & Crosby (1963), Weintraub, Conrad & Crosby (1964) and Conrad, Weintraub & Crosby (1964) demonstrated that changes in iron absorption were inversely proportional to the iron content of the intestinal tissues. They postulated that the absorptive capacity of the intestinal cells was determined by the amount of non-haem iron incorporated into the cells. Furthermore, they showed that the amount of iron present in the cell was dependent on the rate of plasma iron clearance. Thus when plasma iron clearance was rapid, indicating rapid iron utilisation, lesser quantities of iron were available to the mucosal cell and accordingly the cell was able to accept increased quantities of dietary iron. The proposed regulating mechanism

consisted of three steps: the uptake of the iron into the absorptive cell from the lumen, mucosal transfer of the iron to the plasma and storage of unwanted iron in the cell. The first two steps were rapid and energy dependent while any iron incorporated into the storage form was turned over very slowly and the bulk lost when the cell was sloughed off at the villous tip. The life span of these cells has been calculated to be between two and five days.

LUMINAL FACTORS IN IRON ABSORPTION

(a) Gastric factors. The role of the stomach in iron absorption remains uncertain. Iron deficiency occurs commonly after gastric surgery (Vaughan, 1932; Wallensten, 1954) and although haemorrhage is probably an important aetiological factor (Kimber, Patterson & Weintraub, 1967) poor absorption may also occur. Although absorption of inorganic salts and food iron was not found to be consistently reduced following gastric surgery (Smith & Mallett, 1957; Baird & Wilson, 1959; Choudhury & Williams, 1959), iron absorption in response to anaemia was impaired. Hallberg, Sölvell & Zeberfeldt (1966), using a more sophisticated technique involving double isotope studies, were able to show that defects existed in the absorption of inorganic and haemoglobin iron in subjects with incomplete stomachs, and that this impairment was greater in the presence of anaemia. Murray & Stein (1967) also demonstrated impaired absorption

in gastrectomised rats following haemorrhage or haemolysis. This defect in absorption following gastric resection was largely reversed by added hydrochloric acid (Moeschlin, Schmid & Schneider, 1965). It was therefore postulated that decreased acid secretion after gastric resection may be the important factor in inducing impaired iron absorption.

Achlorhydria has been recognised as an associated feature of iron deficiency for many years (Witts, 1930). There has, however, been continuing disagreement about the role of gastric acid in iron absorption. In early studies iron absorption was found to be facilitated in the presence of normal gastric acidity (Minot & Heath, 1932) while later reports (Grace, Doig & Wolff, 1954; Pirzio-Biroli et al. 1958) found no evidence that gastric secretion altered iron uptake. More recently a series of papers (Goldberg, Lochhead & Dagg, 1964; Cook, Brown & Vallberg, 1964; Jacobs, Bothwell & Charlton, 1964) have documented increased absorption in subjects with normal gastric secretion when compared with control subjects with histamine-fast achlorhydria. Gastric acid enhanced the absorption of ferric salts and food iron, but had little effect on ferrous salts and haemoglobin iron (Choudhury & Williams, 1959; Baird & Wilson, 1959; Biggs et al. 1961; Jacobs et al. 1964). Acid production seems to be a more important factor in regulating iron absorption in anaemic subjects than in subjects with a

normal level of haemoglobin (Jacobs et al. 1966) in whom the effect of added acid is marginal. The enhancing effect of acid in inorganic iron absorption is attributed to the fact that ferric iron is maintained in solution at acid pH and therefore may form soluble complexes with endogenous and exogenous agents (Schade, Cohen & Conrad,1968). These complexes are then available for absorption.

It is thought that the mechanism of action of acid gastric juice in increasing food iron absorption is related to the release of ionic iron during acid and acid-peptic digestion (Bergeim & Kirch, 1948; Callender et al. 1957; Jacobs et al. 1969).

Latterly reports have been made suggesting that gastric juice may affect iron absorption in a manner unrelated to acid production. Beutler, Fairbanks & Fahey (1963) discovered a stabilizing factor in human gastric juice which prevented the precipitation of iron at an alkaline pH. However, gastric juice taken from iron deficient patients had no greater stabilizing capacity than pooled gastric juice from normal subjects. They therefore concluded that variations in the amount of such stabilizing substances were unlikely to play an important physiological role in the regulation of iron absorption.

Koepke & Stewart (1964) found that gastric juice of anaemic dogs enhanced the absorption of inorganic iron by normal dogs.

This enhancing effect was not confirmed by Mignon, Russell, Semb, Morgan, Finch & Nyhus (1965), however, in either normal or anaemic animals.

In rats, inorganic iron absorption was increased by added gastric juice from human subjects with iron deficiency and haemochromatosis (Murray & Stein, 1968a, 1968b). The increase was slight in normal animals and more striking in anaemic gastrectomised animals. This work has not been confirmed by subsequent investigations (Smith & Williams, 1968; Smith, Studley & Williams, 1969).

In humans, gastric juice from patients with iron deficiency anaemia has been shown to increase iron absorption (Turnberg, 1968). Gastric juice from normal subjects also increased inorganic iron absorption in patients with gastric atrophy or achlorhydria (Jacobs, Rhodes & Eakins, 1967; Jacobs, 1968; Jacobs & Owen, 1969).

In addition to the effects of whole gastric juice, crude and purified hog intrinsic factor concentrates increased the absorption of haemoglobin iron two-fold in patients with pernicious anaemia and iron deficiency (Waxman, Pratt & Herbert, 1968). These workers also showed that neutralized gastric juice had a similar effect in these patients.

An iron binding protein has been described in human gastric juice which was postulated to inhibit iron absorption (Davis, Luke & Deller, 1966; Luke, Davis & Deller, 1967). The basis of this claim was the reported finding of low levels of this protein in iron deficiency anaemia and in haemochromatosis. The presence of an iron binding protein in gastric juice was confirmed by other workers, but they could not substantiate the alterations in levels in disease states (Wynter & Williams, 1968); Smith, 1968; Jacobs & Miles, 1968; Powell & Wilson, 1970). Furthermore, Rudzki (1970) showed that the iron protein complex is particulate and does not represent a stoichiometrically pure compound, and that a purified preparation of this protein did not alter inorganic iron absorption in rats.

It is therefore evident that much confusion exists regarding the role of human gastric juice components, other than acid, in iron absorption.

(b) Pancreatic secretions. The role of the pancreatic exocrine secretions in iron absorption remains confused. It has been shown that the uptake of radioiron is increased in the presence of chronic pancreatic disease (Davis & Badenoch, 1962); that the elevated uptake is inhibited by administration of pancreatic extract (Biggs & Davis, 1963); and that the increased iron absorption in haemochromatosis is depressed by oral pancreatic extract (Davis, 1964). Subsequent work by these

authors showed that the commercial pancreatic extract contained a water soluble, heat labile fraction which could depress iron absorption in isolated rat jejunum (Davis & Biggs, 1965). The relevance of these findings to the in vivo human situation is seriously open to question. Indeed more recent work has failed to confirm the association of increased iron uptake with loss of pancreatic function (Balcerzak, Peternel & Heinle, 1967; Kavin, Charlton, Jacobs, Green, Torrance & Bothwell, 1967).

Benjamin, Cortell & Conrad (1967) examined the effects of added bicarbonate, the main anion in pancreatic secretions, on iron absorption. Iron absorption was decreased in normal rats but not in iron deficient or iron overloaded animals. It was found that added bicarbonate produced precipitation and macro-molecular aggregation of iron particles and that these large complexes were poorly absorbed. The authors concluded that the secretion of bicarbonate by the pancreas may have an effect on the interplay of iron and its various binding agents in the gut, thereby playing some role in iron absorption.

Nevertheless, Murray & Stein (1966) and Crosby (1968) after a thorough review of the literature, concluded that the pancreas plays little or no role in the physiological regulation of iron absorption.

(c) Bile. Bile contains ascorbic acid (Bockus, 1965).

Ascorbic acid taken orally enhances the absorption of inorganic and food iron, the effect being most striking with doses in excess of 200 mg (Pirzio-Biroli et al. 1958; Brise & Hallberg, 1962b; Kuhn et al. 1968). Bile could therefore be expected to enhance iron absorption.

In vitro addition of bile to ferric chloride decreased the precipitation of iron by sodium hydroxide (Conrad & Schade, 1968). They also demonstrated that ligation of the bile duct in normal rats reduced the absorption of ferrous and ferric salts instilled into the duodenum, but no change was detected when the test dose of iron was injected into the stomach. In addition no alteration in absorption of ferrous salts by anaemic rats was found, but impaired absorption of ferric salts by these animals occurred.

Sorensen (1967) found that bile decreased absorption of iron in rats and had no effect on absorption in man when the iron was given with a meal. Bile salts did not alter absorption in man (Brise, 1962a, 1962b). Therefore the role of bile in the absorption of iron in man remains uncertain.

A comprehensive theory of the role of luminal factors in iron absorption has been proposed by Saltman and his co-workers. These investigators demonstrated that sugars were able to form stable chelate complexes with iron (Charley, Sarkar, Stitt and Saltman, 1963) and that such complexes facilitated iron absorption from an isolated segment of rabbit gut (Charley, Stitt, Shore & Saltman, 1963). It was similarly demonstrated that low molecular weight chelates increased the uptake of iron by rat liver slices incubated in physiological medium (Saltman, Fiskin & Bellinger, 1956; Saltman, Frisch, Fiskin & Alex, 1956).

On the basis of these findings Saltman (1965) postulated that regulation and control of iron metabolism depended on the ability of iron to be chelated by low molecular weight ligands, the chemical nature of the ligand determining the rapidity with which the iron would move across the membrane. The transport mechanism was postulated to be a passive one as no metabolic energy was required for its operation.

Recently several endogenous components with iron binding ability have been demonstrated in gastric juice (Beutler et al. 1963; Davis et al. 1966; Wynter & Williams, 1968; Rudzki, 1970) as mentioned earlier in this chapter. However, the physiological importance in regulation of iron absorption of these substances is uncertain.

CHAPTER III

INTRODUCTION

The total iron content (McCance & Widdowson, 1960) and the overall iron absorption from basic foodstuffs has been quantitated by both chemical (Widdowson & McCance, 1942) and radioisotopic techniques (Pirzio-Biroli et al. 1958; Martinez-Torres & Layrisse 1970). However the physical and chemical nature of dietary iron complexes remains poorly understood and little information is currently available about the changes induced by cooking or enzymatic digestion of various foodstuffs. These latter changes may cause iron to be more or less available for absorption, and may be critical to an understanding of deficiency states, and to a closer understanding of the factors which modify physiological iron absorption.

Accordingly this section of the study was designed to quantitate the iron in a particular food substance; to determine its chemical nature; and to observe any changes induced by cooking, by enzymic digestion or both, on the iron complexes present.

Sheep, calf or chicken liver is a rich source of dietary iron (Moore & Dubach, 1951; Walsh et al. 1955) containing about 13 mg of iron per 100 g of tissue (McCance & Widdowson, 1960). The iron content of guinea pig liver is comparable to that of larger animals (personal observation) and livers from these small

animals were selected as the initial model because of their availability and because the liver iron in this species could be easily and rapidly labelled using moderate doses of high specific activity radioactive iron.

It was apparent at the outset that a major problem of technique, particularly in the later stages of this thesis, would be the accurate determination of the small quantities of iron present in the various food fractions. Isotopic labelling of food iron was thus selected to allow efficient and accurate monitoring of the iron fractions, where the levels of iron were such that they could not be accurately detected by other means.

MATERIALS AND METHODS

All the glassware and polythene apparatus used was washed in Pyroneg detergent, rinsed in deionised water and then soaked overnight in concentrated hydrochloric acid. The apparatus was rinsed in deionised water several times prior to use to remove surface iron contamination. All the chemicals used were of analytical grade.

Stock ferric chloride solution

A standard method was used to prepare a solution of 0.05 M ferric chloride. Rust free iron wire (A.R.) was dissolved in

approximately 50 ml of 5 M hydrochloric acid. Oxidation of the ferrous ions to ferric ions was produced by the addition of 30 ml of hydrogen peroxide. The solution was then evaporated to dryness under an infra red lamp, and the residue transferred quantitatively to a one litre volumetric flask and made up to volume with 0.01 M hydrochloric acid.

Collection of guinea pig liver

The guinea pigs were housed in metabolic cages and fed a standard diet of rabbit pellets (William Charlick Ltd. Adelaide, South Australia) containing 222.5 μg iron per g. The animals were sacrificed by ether anaesthesia, and the liver was immediately removed through a transverse abdominal incision. The liver was then thoroughly washed with distilled water and patted dry with filter paper. It was stored at $0^{o}C$ until required.

Estimation of total iron content

1. Wet ashing. This method was modified from that described by Gübler, Lahey, Ashenbrucker, Cartwright & Wintrobe (1952). The liver tissue was wet ashed in 200 ml digest flasks. The acid mixture used comprised concentrated nitric acid, concentrated sulphuric acid and concentrated perchloric acid in the volume ratio of 3:1:1. Approximately 5 ml of the acid mixture was added to each 1 g of liver tissue. The resultant solution was heated very slowly until the brown fumes of nitrous oxide were given off,

and after this heating was allowed to continue more briskly until a clear colourless solution remained in the digest flask. The solution was cooled and made up to either 25 ml or 50 ml with deionised water for iron estimation. For each wet ashing procedure a control was performed without added liver to allow for unpredicted iron contamination.

2. Measurement of iron.

Atomic absorption spectrophotometry. The iron concentration was determined using a Unicam SP 90A atomic absorption spectrophotometer. Iron was estimated at a wavelength of 248.3 mμ and a slit width of 0.1 mm. The burner height was 1.5 cm and the gas flow 1 litre per minute for acetylene and 5 litres per minute for air. Standard solutions in the range of 1 to 30 μg Fe per ml were made by the necessary dilution of the stock solution of 0.05 M ferric chloride with 0.01 M hydrochloric acid. The test solutions within this range were then read by reference to the standard line.

Fractionation of the liver iron.

1. Preparation of the homogenate. A known weight, normally between 1 and 2 g of liver was placed in a 50 ml polythene centrifuge tube with 5 ml of deionised water. The mixture was homogenised for 30 seconds (Ultra-Turrax homogeniser, Janke & Hunkel, K.L. Staufen, G.Br.). The homogeniser was washed twice in deionised water in another 50 ml polythene tube, and the

washing water was pooled with the initial homogenate. The homogenate was then transferred quantitatively to a calibrated 50 ml pyrex test tube. The final volume of the homogenate was adjusted with deionised water so that there was 1 g of liver in 10 ml. This concentration was constant throughout unless specifically otherwise stated.

2. *Non-haem iron extraction.* This method was modified from that described by Bruckmann & Zondek in 1940. Five ml aliquots of the liver homogenate were transferred to 15 ml centrifuge tubes, and 4 ml of an equal mixture of 10 per cent trichloroacetic acid and 4 per cent sodium pyrophosphate was added. The resultant mixture was heated for 20 min in a boiling water bath to allow precipitation of protein and release of the iron not bound to the porphyrin ring. Then it was cooled and centrifuged at 2,000 g for 20 min. The supernatant, containing the non-haem iron fraction was carefully decanted into graduated glass tubes. The precipitate was subjected to a repetition of the extraction process, but on the second occasion 2.5 ml of an equal mixture of trichloroacetic acid and sodium pyrophosphate was used. Separate tubes with the same amounts of 10 per cent trichloroacetic acid and 4 per cent sodium pyrophosphate were heated along with the samples for the blank and standards, and were transferred accordingly. The purpose of this was to keep the volume of the extracting solution the same, and thereby

insure that the blank, standard and samples had the same reacting substances in the final solution. The standards ranged from 1 to 30 μg Fe per ml.

3. *Haem iron extraction and estimation.* Haem was isolated by the method of Labbe and Nishida (1957). The extraction solvent was composed of one part of 2 per cent strontium chloride hexahydrate in glacial acetic acid and 3 parts of acetone. This was prepared immediately before use to avoid crystallisation of the strontium chloride. Ten ml of liver homogenate was added, with stirring, to 120 ml of the extraction solvent. The resultant solution was allowed to stand for 30 min to allow release of the haem and precipitation of the protein. The mixture was then filtered and the residue washed twice with 10 ml of the extraction solvent. The combined filtrate containing the haem was heated to 100°C and concentration was allowed to proceed slowly. Crystallisation of the haem began as the solution became concentrated and was completed when the solution was cooled to room temperature. The haem was centrifuged and washed twice with 50 per cent acetic acid and water. The crystals were redissolved in 0.1 M sodium hydroxide and the volume of the solution adjusted to 25 ml with deionised water. The iron content was determined directly by atomic absorption spectrophotometry.

4. Ferritin iron determination. Ferritin was extracted from the liver homogenate by the method of Granick (1942). The heat supernatant obtained was titrated with horse anti ferritin (whole rabbit serum, Calbiochem) to the point of maximum iron precipitation (Linder-Horowitz, Ruettinger & Munro, 1970). In this method ferritin was precipitated by incubating the ferritin extract with between 0.1 and 0.5 ml of antiserum made up to 2.5 ml with isotonic saline. The mixture was incubated for 18 hours at 4°C. After incubation the precipitate was spun down by centrifugation at 2000 g and was washed twice with deionised water. The precipitate was then dissolved in several drops of 3:1:1 nitric, sulphuric and perchloric acid digest mixture, adjusted to 3 ml with deionised water, and the iron content determined by atomic absorption spectrophotometry. This volume was used to calculate the ferritin iron content per gram of tissue.

Enzyme Digestions

Enzyme digestions were performed in a thermostatically controlled water bath at 37°C ± 0.1^{o}C. A mechanical shaker within the water bath ensured constant agitation of the digest. The following enzymes were examined.

Pepsin. Ten ml of the homogenate was incubated for 2 hr at pH 1.6 with pepsin (Sigma/400-525 units/mg) prepared in distilled water. The quantity of pepsin was 1% w/w of the

substrate. The pH was checked routinely at the end of the incubation and was always below pH 2. The enzyme was inactivated by heating the incubation mixture to 100°C.

Trypsin. Liver homogenate, 1 g in 8 ml of deionised water, was prepared as described. To this was added 2 ml of 0.1 M tris buffer pH 7.8, an aqueous solution of crude pancreatic extract containing 10 mg trypsin (type I Sigma) per ml, the total enzyme added constituting 1% of the substrate w/w. Finally 0.5 ml of 0.02 M calcium nitrate was added. The final pH was 7.6 to 7.8. The digestion period was 2 hours. The enzyme was inactivated by heating the digest mixture at 100°C for 30 seconds.

Digests were always carried out in triplicate. Controls without added enzyme were also performed with each experiment. The activity of the enzyme in each case was tested by submitting egg albumin to digestion concurrently with the test homogenates.

Cooking. The liver homogenate was heated at 100°C for 10 minutes, with continuous stirring.

RESULTS

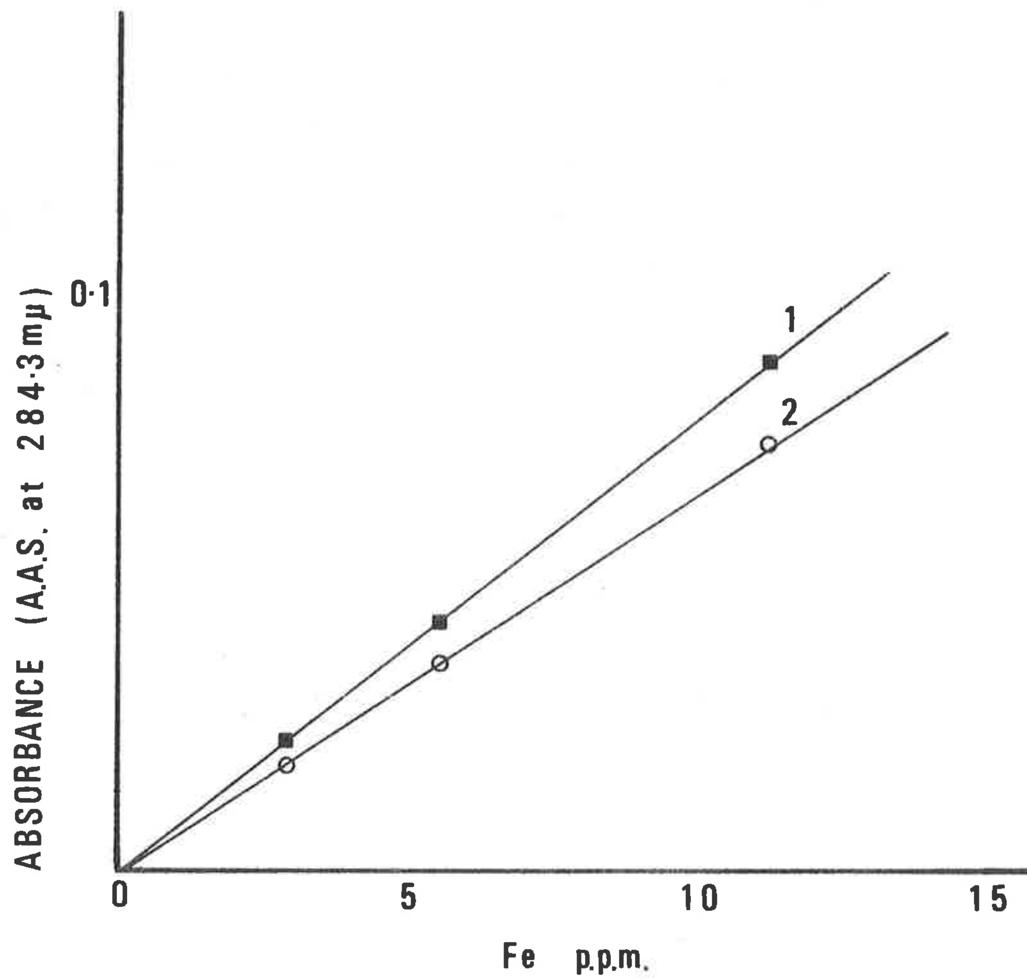
The absorbance of the standard iron solutions plotted against the iron content (ppm) always produced a straight line which passed through the origin. The standards used for the

non-haem iron estimations, made up with 10 per cent trichloroacetic acid and 4 per cent sodium pyrophosphate, always produced higher readings; however, the standard reference line did on all occasions pass through the origin (fig. 1).

The total guinea pig liver iron was determined in ten animals. In each animal, 1 g samples of tissue were taken from three separate sites. The mean values for the iron content ranged from 136.3 ± 3 (mean ± 1 S.D.) μg Fe per g to 188.3 ± 2.4 μg (mean ± 1 S.D.) Fe per g of liver wet weight. The iron appeared to be distributed uniformly throughout the liver as there was minimal variation of the iron content of the tissue samples in individual guinea pigs. Table 1 is representative of the separate values obtained for five animals. No iron was detected in the controls without added liver.

Of the total iron, approximately 60 per cent was present as non-haem iron, mostly as ferritin, and the rest as haem iron. These figures were derived from duplicate extractions of non-haem iron, haem iron and ferritin iron from two separate animals. In the first animal the total liver iron content was 180 μg Fe per g liver, 38 per cent was haem iron, 62 per cent was non-haem iron and 58 per cent of the total iron was present as ferritin. In the second animal, with a liver iron content also of 180 μg Fe per g, 41 per cent occurred as haem iron, 59 per cent as non-haem iron and 55 per cent as ferritin.

FIGURE 1



1. Reference line obtained for standard iron solutions made up in equal volumes of 10% T.C.A. and 4% $Na_2P_4O_7$.
2. Reference line obtained for standard iron solutions made up in 0·01M HCl.

TABLE 1. Values for iron content of guinea pig liver

(μg Fe per g wet tissue)

		Mean	± 1 S.D.
Guinea pig A	185 190 190	188.3	2.4
Guinea pig B	145 145 142	144.0	1.4
Guinea pig C	135 138 136	136.3	1.3
Guinea pig D	148 145 140	144.3	3.3
Guinea pig E	165 165 172	167.3	3.3

Cooking and enzymic digestion did not alter the ratio of haem and non-haem iron in the guinea pig liver (tables 2 and 3). The effects of cooking and enzymic digestion on the haem and non-haem iron fractions of guinea pig liver were studied in duplicate on liver samples from two animals. In the first series of experiments (table 2) the non-haem iron was extracted from the homogenate before and after cooking and enzymic digestion, whereas the haem iron content was obtained by subtraction of the non-haem iron from the total iron content. In the second series (table 3) the haem iron was extracted from the liver homogenate and the non-haem iron content determined by calculation. The lack of sufficiently sensitive methods for the detection of iron prohibited the extraction of haem and non-haem iron from aliquots of the same sample.

COMMENT

Such elements as magnesium, calcium, phosphorus and sodium produce gross interference with iron detection by flame emission methods (Allan, 1959). However, Allan demonstrated no interference by 100 fold excess of sodium and 50 fold excess of phosphorus when iron was detected by atomic absorption spectrophotometry. Furthermore, 1 per cent trichloracetic acid has been shown to produce no interference with iron measurement by atomic absorption (Zettner, Sylvia & Capacho-Delgado, 1966). The concentration of sodium and phosphorus in the non-haem iron extraction solution was approximately 750 ppm. Thus the ratio

TABLE 2. Liver non-haem iron expressed as μg Fe per g of liver, and as percentage of total iron. (Haem iron determined by subtraction.)

Sample	Non-haem Fe		Haem Fe	
	μg Fe	%	μg Fe	%
Raw liver	110	61	70	39
Cooked liver	110	61	70	39
Peptic hydrolysed (2 hr) liver	112	62	68	38
Cooked and peptic hydrolysed (2 hr) liver	108	60	72	40
Tryptic digested liver	105	59	75	41

(Total iron of liver used was 180 μg per g liver)

TABLE 3. Haem iron in liver, μg per g of liver, and per cent of total Fe. (Non-haem Fe determined by subtraction.)

Sample	Haem Fe		Non-haem Fe	
	μg Fe/g	%	μg Fe/g	%
Raw liver	48	34	92	66
Cooked liver	50	36	90	64
Peptic hydrolysed (2 hr) liver	50	36	90	64
Cooked and peptic hydrolysed (2 hr) liver	52	37	88	63
Tryptic digested liver	49	35	91	65

(Total iron content of liver used was 140 μg/g liver)

of the iron concentration in the standard solutions to the sodium and phosphorus concentration was about 1:100. Therefore it would be unlikely that sodium produced the interference (fig. 1). However the phosphorus concentration may have been responsible. In addition the trichloroacetic acid concentration was higher than that tested by Zettner and his associates and therefore this may have also contributed to the interference. The interference detected did not affect the iron estimation since the standard reference line was a straight line passing through the origin, and the preparation of the non-haem iron extract and the iron standards ensured that the concentration of any interfering substance was identical.

The mean total liver iron of guinea pigs used in this study was comparable to that of sheep, pig and beef cattle liver studied by McCance & Widdowson (1960). For this reason the observations on guinea pig liver are considered a reflection of the handling of dietary liver ion iron. Furthermore, Van Wyck Linder-Horowitz & Munro (1971) separated rat liver iron into haem and non-haem iron fractions. Using liver which had been perfused with saline, the levels of non-haem iron were of a similar order to those reported in this thesis, and this was also shown to be mostly ferritin. However, the haem iron values were considerably lower, and this is attributed to the saline perfusion of the liver by Van Wyck and his associates.

These findings add further support to the postulate that species differences with regard to the liver iron make up are not great and so the guinea pig liver was accepted as a working model.

The lack of change produced in the haem, non-haem iron partition by cooking and enzymatic digestion is not in complete agreement with earlier reports. Kaldor (1957) reported that 12 to 13 per cent of the total haemoglobin iron was liberated by acid-peptic digestion, and was detectable as ionic iron. Sandford (1960) however, found minimal release of iron from haemoglobin by acid-peptic digestion alone, although approximately 10 per cent of the haem iron was released by a combination of cooking and peptic hydrolysis. Since it has been realised that the haem moiety is absorbed intact by the mucosal cells (Conrad et al. 1966b) interest has been diverted to an examination of the release of haem from haemoglobin within the intestine, and to its subsequent transport through the mucosal cell, rather than the release of iron from the haem moiety.

The degradation of haemoglobin to haem by commercial enzyme preparations and intestinal secretions has been extensively studied by Conrad et al. (1966a). Factors affecting absorption of haemoglobin iron have also received widespread attention (Turnbull et al. 1962; Conrad et al. 1966a, 1966b; Conrad, Benjamin, Williams & Foy, 1967b). More recently, Weintraub et

al. (1968) focused interest on a haem splitting enzyme within the mucosal cells. Earlier experiments (Hallberg & Sölvell, 1967) had indicated that following mucosal uptake of haem, iron is split from the porphyrin ring before transport to the plasma. These studies are of great significance towards the understanding of dietary iron metabolism since haem in the form of haemoglobin and myoglobin is a major source of food iron in many countries (Wheby et al. 1970).

Ferritin iron constituted most of the non-haem guinea pig liver iron. This was anticipated from the knowledge of iron distribution in the human body. The bulk of the iron in man exists as haemoglobin, myoglobin and storage iron in the forms of ferritin and haemosiderin (Moore & Dubach, 1956). It is also known that the liver, along with the spleen and bone marrow, is one of the main iron storage organs (Granick, 1946; Ramsay, 1957; Wintrobe, 1968b). Fractionation studies of liver iron in man (Morgan & Walters, 1963) and animals (Gabrio, Shoden & Finch, 1953; Van Wyck et al. 1971) have demonstrated that ferritin is the main form of liver storage iron. The difference between the total liver non-haem iron and the ferritin iron was approximately 10 per cent and this is assumed to consist of haemosiderin, free serum iron, transferrin bound iron and iron in the various enzyme systems.

Acid-peptic and tryptic digestion of ferritin has been shown to release small amounts of iron from the molecule (Mazur & Shorr, 1950) and it was also demonstrated that the iron released is increased markedly when the ferritin is denatured by heat prior to the enzymic digestion. Thus degradation of the ferritin molecule with the liberation of iron undoubtedly occurred in the experiments reported in this chapter. However at this stage of the study interest was directed at the behaviour of the haem and non-haem iron fractions collectively and not to the changes within individual components of these fractions.

CHAPTER IV

PHYSICAL CHARACTERIZATION OF LIVER IRON COMPOUNDS

INTRODUCTION

The first part of the study comprised measurement of liver iron stores and a determination of the broad distribution of iron as haem, ferritin and other non-haem iron complexes. The next phase of the study was to assess the physical size of the iron compounds and to monitor any change in complex size produced by cooking and enzymatic digestion. The most sensitive colorimetric methods currently available (Ramsay, 1957; Fischer & Price, 1964) did not allow accurate detection of iron following the various fractionation procedures, all of which produced a considerable dilutional effect. Consequently a rapid method was devised for the isotopic labelling of the guinea pig liver iron stores. The introduction of the Fe^{59} radioisotope of iron into the system made it possible to monitor the iron complexes with precision.

MATERIALS AND METHODS

In vivo labelling of guinea pig liver iron stores with Fe^{59}

Guinea pigs were lightly anaesthetised with ether (anaesthetic B.P.) and bled by cardiac puncture. A volume of 20 ml of blood was removed over a period of two weeks by repeated bleedings. This comprised approximately 60 per cent of their blood volume (Courtice, 1943; Berlin, Hyde, Parsons & Lawrence, 1949). A further two weeks elapsed to allow the

available liver iron stores to be mobilized for erythropoiesis (Wintrobe, 1968a). At the end of this time 2 ml of 0.05 M ferric chloride (5.6 mg elemental iron) labelled with 50 μCi Fe^{59} chloride (specific activity 10 μCi/ μg) was injected intraperitoneally in a divided dose, on two consecutive days. After a further two weeks, during which the injected iron was incorporated into the liver iron pool (personal observation), the animal was killed and the liver removed for use.

$Fe^{59}Cl_3$ stock solution

Fe^{59} was obtained from the Commonwealth X-Ray and Radium Laboratories as ferric citrate. Complete oxidation of the citrate was achieved by adding a mixture of concentrated nitric and perchloric acids in equal volumes, and evaporating the solution to dryness under an infra-red lamp. The residue was redissolved in 0.01 M hydrochloric acid to the required concentration.

Measurement of radioactivity

The Fe^{59} gamma radiation was measured in two ways. When the sample was less than 10 ml, activity was recorded using an automatic well type sodium iodide crystal scintillation counter (Philips type PW 4003). With this system the counting efficiency was greater than 25 per cent and the background approximated 3 counts per second. In samples exceeding 10 ml, Fe^{59} activity was recorded using a large volume gamma counter (Nuclear Data large volume gamma counter A.E.I.). All samples were counted in

duplicate for 100 seconds with adjustments such that the background counts were less than 0.5 per cent of the standard. The counting efficiency exceeded 20 per cent.

Cooking and digestion

The cooking and enzymatic digestion methods employed have been described in Chapter III.

Acid digestion

The homogenate, (1 g in 10 ml) the pH having been adjusted to pH 1.6 with 2 M hydrochloric acid, was incubated at 37°C for 2 hours. The incubation was performed in a thermostatically controlled water bath. A mechanical shaker within the water bath ensured constant agitation of the digest.

Centrifugation

Homogenates of guinea pig liver were adjusted to 10 ml volume with deionised water and centrifuged at 10,000 g for one hour. This time and centrifugal force were chosen after a pilot study had shown that precipitation was complete. In the pilot study centrifugation of the homogenates was carried out at 10,000 g for time intervals ranging from ½ an hour to 2 hours. In another series of experiments the homogenates were centrifuged for a fixed time of one hour and the centrifugal force was varied from 5,000 g to 20,000 g. After centrifugation the supernatant was decanted, the volume adjusted to 10 ml with deionised water and the iron content determined by atomic

absorption spectrophotometry. The precipitate was wet ashed using the concentrated nitric, sulphuric and perchloric acid mixture, as described in Chapter III, prior to iron estimation.

The estimates of soluble and precipitated iron were repeated after cooking, acid-peptic digestion, acid digestion, tryptic digestion and cooking in combination with peptic hydrolysis. All estimations were performed in triplicate.

Dialysis

Samples of liver homogenate were dialysed against both deionised water and dilute hydrochloric acid to estimate the amount of iron which was present in the low molecular weight fraction. Raw liver, cooked liver and liver previously digested by acid-pepsin, trypsin and acid alone were all treated in a similar manner. Fe^{59} labelled guinea pig liver made up in 10 ml of deionised water was placed within the cellulose dialysis tubing (Visking). The total iron content of the homogenate was determined in triplicate and ranged from 140 μg Fe to 168 μg Fe giving a final concentration between 0.00025 M and 0.0003 M. The initial activity of the homogenate was determined by duplicate counting (Nuclear Data A.E.I.). Dialysis was performed, in triplicate, against 300 ml glass distilled water for 24 hours at 4°C and against an identical volume of 0.16 M hydrochloric acid. The dialysable iron present was determined by

duplicate counting (Nuclear Data A.E.I.) of the homogenate after dialysis. The initial count represented the total iron present, and the dialysable iron was calculated by difference from the activity remaining in the dialysis bag after 24 hours.

Nature of iron in dialysate

The dialysate was concentrated by rotary evaporation to approximately 20 ml. Haem estimation was performed on aliquots of the dialysate by the benzidine method of Crosby & Furth (1956). Non-haem iron was determined using the modified method of Bruckmann & Zondek (1940).

Dialysis of ferritin iron

Radioactive ferritin was prepared from liver according to the method of Granick (1942) referred to in chapter III. Two grams of Fe^{59} labelled liver was used as the substrate. The heat filtrate, containing the ferritin, was made up to a volume of 10 ml with deionised water. The ferritin iron concentration of this solution ranged from 14.0 to 16.0 mg per ml. The ferritin solution was dialysed in its 'raw' state, and also after cooking and digestion. Dialysis of this solution was carried out against 300 ml deionised water, and 300 ml 0.16 M hydrochloric acid for 24 hours at $4^{o}C$. The dialysable fraction was determined by the difference between the initial and final radioactivity in the dialysis bag. All these

experiments were performed in triplicate.

Sephadex gel filtration

The technique of molecular sieving (Porath, 1955; Andrews, 1965) was used for further separation of the liver iron compounds.

Fractionation of liver homogenate

(1) Liver preparation for fractionation. Homogenate was prepared from Fe^{59} labelled guinea pig liver (1 g/10 ml) as described previously in chapter III. The homogenate in the raw, cooked and digested state was then subjected to centrifugation at 10,000 g for 1 hour. The supernatant was collected and 4 ml aliquots were applied on Sephadex columns.

(2) Sephadex gel filtration. Gel filtration was carried out on Sephadex G200 (Pharmacia) with a fractionation range of M.W. 1,000-200,000. The sephadex G200 was packed in a 45 cm column (type K25/45) and eluted by upward flow. The eluting buffer was 0.9% NaCl with 0.01 M tris adjusted to pH 8. The effluent from the columns was collected as 5 ml fractions in a refrigerated fraction collector.

The supernatant of peptic hydrolysed liver was fractionated on sephadex G200 and G50 (fine), the latter having a fractionation range of M.W. 500-10,000. The sephadex G50 was packed in a 90 cm type 15K/90 column. The conditions for filtration were the same as those used for sephadex G200.

(3) Analysis of the eluted fractions.

Estimation of protein. The eluted fractions were monitored for their relative protein content by estimating the ultra-violet light absorption at 280 mμ by the aromatic amino-acids (Layne, 1957).

Estimation of carbohydrate. The carbohydrate content of the fractions was monitored by the phenol sulphuric method of Dubois, Gilles, Hamilton, Debers & Smith (1956).

Estimation of iron. The iron content of the fractions was monitored by counting (Philips type PW 4003) the radioactivity of the Fe^{59}.

Estimation of haem. The haem content of the fractions was estimated by reading the chromogen directly at 540 mμ.

Detection of ferritin. Fe^{59} containing eluted fractions in the high molecular weight area were pooled and concentrated to 2 ml with lyphogel (Gelman). The presence of ferritin was determined by the Ouchterlony (1949, 1958) immunodiffusion technique. This antigen-antibody precipitation reaction was performed on agar gel plates (Hyland) with 10 μl of horse anti-ferritin (whole rabbit serum, Calbiochem) being applied in the centre well and 10 and 20 μl of the test solution in the peripheral wells. The plates were stored in a humidity chamber for 36 to 72 hours, after which period the absence or presence of precipitin bands was assessed. The results were recorded by photographing

the agar gel slides.

Ferritin fractionation

(1) Ferritin preparation for fractionation. Radioactive ferritin was prepared from 2 g of Fe^{59} labelled liver. Three ml. of deionised water was used for the extraction procedure (Granick, 1942). The resultant heat filtrate was adjusted to 4 ml with deionised water. The iron content of the ferritin solution ranged from 139 to 145 µg. Raw, acid-peptic digested, acid digested and cooked plus acid-peptic digested aqueous ferritin solution (4 ml) was layered on the sephadex G200 column.

The presence of ferritin in the raw, cooked and digested aqueous ferritin extract was determined by the Ouchterlony immuno-diffusion technique. The method was similar to that described on the previous page.

(2) Sephadex gel filtration. The methods and materials used were identical to those used for the fractionation of the liver homogenate.

(3) Analysis of the eluted fractions. The 5 ml eluted fractions were monitored for iron and protein content. The iron content was monitored by counting the radioactivity of the Fe^{59} in the fractions. The protein content was monitored by the method of Layne (1957) and referred to previously in this chapter.

Fractionation of rabbit haemoglobin

(1) Preparation of labelled rabbit haemoglobin. Radio-active haemoglobin was prepared by lysis of washed red cells

from a rabbit which had received 50 μCi Fe^{59} citrate intraperitoneally two weeks prior to phlebotomy. A solution containing 140 gm per ml radioactive haemoglobin was prepared. Aliquots of this were further diluted 1:5, and 2 ml of the resultant solution was applied on the sephadex G200 column.

(2) Sephadex gel filtration. The procedures employed for fractionation by sephadex G200 were the same as those described earlier in this chapter.

(3) Analysis of eluted fractions. The fractions were monitored for haemoglobin content. The elution pattern was obtained by estimating the radioactivity of the Fe^{59} labelled haemoglobin.

RESULTS

Centrifugation

Approximately two-thirds of the total iron in the liver homogenate was present in the supernatant after centrifugation at 10,000 g for one hour. This fraction was designated as the soluble iron fraction (Jacobs et al. 1969). One-third of the iron remained in the precipitate. No significant increase was achieved by increasing the centrifugation time to 2 hours (table 4) or the centrifugal force to 20,000 g (table 5). For this reason a centrifugation time of one hour and force of 10,000 g was selected and used throughout the study. The results

TABLE 4. Centrifugation of liver homogenate at 10,000 g with varying time

	Fe in supernatant expressed as % of total iron	Fe in precipitate expressed as % of total iron
½ hour	69	31
1 hour	65	35
2 hours	62	38

TABLE 5. Centrifugation of liver homogenate for 1 hour at varying centrifugal force

	Fe in supernatant expressed as % of total iron	Fe in precipitate expressed as % of total iron
5,000 g	68	32
10,000 g	70	30
15,000 g	76	24
20,000 g	66	34

presented were obtained from duplicate estimations for all samples.

The amount of soluble iron in the supernatant did not increase following acid-peptic digestion, acid digestion, or cooking followed by acid-peptic digestion (table 6). In all these studies the distribution of soluble and precipitated iron was similar to that in the raw liver homogenate with about two-thirds of the total iron in the supernatant and one-third in the precipitate. However, both cooking alone and tryptic digestion produced a reversal of the distribution. When liver homogenate was so treated, only approximately 30 per cent of the total iron was recovered from the soluble supernatant fraction, and about 70 per cent remained in the precipitated material.

Dialysis

Dialysis of raw liver against deionised water resulted in a loss of 2 per cent (range 1.0 to 2.6%) of the radioactive iron (table 7). The dialysable fraction increased to 8 per cent (range 6.6 to 9.0%) when dialysis was performed against 0.16 M hydrochloric acid.

After cooking there was no detectable loss of radioactivity either against water or dilute hydrochloric acid.

Acid-peptic digestion for 2 hours increased the dialysable iron to 30 per cent (range 28.2 to 33.0%) against deionised

TABLE 6. Distribution of liver iron following centrifugation for 1 hour at 10,000 g

	Fe in supernatant expressed as % of total iron	Fe in precipitate expressed as % of total iron
Raw liver homogenate	65	35
Liver homogenate acid-peptic digested for 2 hours	65	35
Liver homogenate acid digested for 2 hours	60	40
Cooked liver homogenate	30	70
Liver homogenate cooked and acid-peptic digested for 2 hours	60	40
Liver homogenate tryptic digested for 2 hours	30	70

TABLE 7. Dialysable liver Fe expressed as percentage of total iron, after dialysis against H_2O, and 0.16 M HCl

(Figures represent mean of 3 estimations)

	H_2O	HCl
Liver homogenate	2%	8%
Liver homogenate cooked	nil	nil
Liver homogenate acid-peptic digested for 2 hours	30%	35%
Liver homogenate acid digested for 2 hours	30%	32%
Liver homogenate cooked and acid-peptic digested for 2 hours	40%	45%
Liver homogenate tryptic digested for 2 hours	20%	25%
Liver homogenate acid-peptic digested for 2 hours and then cooked	63%	67%

water, and 35 per cent (range 31 to 38.2%) against 0.16 M hydrochloric acid. In a separate experiment it was shown that the initial contact of raw liver homogenate with the acid-peptic digestion mixture caused a loss of 29 per cent of the radioactive iron from the dialysis tubing. The dialysable fraction did not increase with periods of digestion of up to 2 hours (table 8).

Acid digestion produced similar results, with a loss of 30 per cent (range 26.8 to 34%) of the radioactivity when dialysed against deionised water, and 32 per cent (range 28 to 35.6%) against 0.16 M hydrochloric acid (table 7).

Following tryptic digestion approximately 20 per cent (range 17.2 to 23.7%) of the iron was dialysable either against water or dilute hydrochloric acid.

Cooking followed by acid-peptic digestion further increased the dialysable iron to 40 per cent (range 37.0 to 44%) when dialysed against deionised water, and 45 per cent (range 40.3 to 48.5%) against hydrochloric acid.

The greatest increase in dialysable iron was achieved by peptic hydrolysis followed by heating at 100°C for 10 minutes. Following such treatment (table 7) the dialysable iron fraction both against water and 0.16 M hydrochloric acid exceeded 60 per cent.

TABLE 8. Effect of duration of acid-peptic digestion on dialysable liver iron

Time (mins)	Dialysable Fe as percentage of total iron
0	29
15	30
30	31
60	29
90	30
120	29

Haem recovery from the dialysate is recorded in table 9. For all digestion mixtures recovery ranged from 0.05 to 0.18 μg haem per g of liver when dialysis was carried out against deionised water, and from 0.01 to 0.17 μg haem iron per g of liver against 0.16 M hydrochloric acid. The calculated percentages of total iron recovered as haem iron are also recorded (table 9). These range from a minimum of 0.01 per cent to a maximum of 0.13 per cent.

None of the radioactive ferritin contained in aqueous solution was removed after dialysis against deionised water or 0.16 M HCl (table 10). However, prior acid-peptic digestion, cooking plus peptic hydrolysis and tryptic digestion resulted in a loss of more than 20 per cent of the ferritin iron during dialysis (table 10).

Sephadex gel fractionation

The supernatant of raw liver homogenate separated into three distinct protein peaks (fig. 2). The major protein fraction was a low molecular weight peak. Radioactivity was associated only with the high molecular weight and intermediate molecular weight protein fractions. The intermediate peak was shown to contain haemoglobin by coincidence of the O.D. 540 mμ peak, and also by fractionation of labelled rabbit haemoglobin (fig. 3) which showed that haemoglobin was eluted in the same fractions as the intermediate radioactive protein peak. The

TABLE 9. Dialysable haem iron expressed in μg haem Fe per gram of liver, and as a percentage of the total iron after dialysis against H_2O and .16 M HCl.

(The figures represent a mean of 2 estimations)

	H_2O		HCl	
	μg haem Fe	% total Fe	μg haem Fe	% total Fe
Liver homogenate acid digested for 2 hours	.02	.01	.01	.01
Liver homogenate acid-peptic digested for 2 hours	.05	.04	.04	.03
Liver homogenate cooked and acid-peptic digested for 2 hours	.16	.11	.10	.07
Liver homogenate acid-peptic digested for 2 hours, and then cooked	.18	.13	.17	.12
Liver homogenate tryptic digested for 2 hours	.05	.04	.02	.01

TABLE 10. Dialysable ferritin iron expressed as percentage of total ferritin iron. Dialysed against distilled water and .16 M hydrochloric acid.

(Figures represent the mean of 2 estimations)

	H_2O	HCl
Ferritin	Nil	Nil
Ferritin, after acid-peptic digestion for 2 hours	23%	25%
Ferritin, after cooking and acid-peptic digestion for 2 hours	28%	30%
Ferritin, tryptic digested	22.5%	28%

Fig. 2: Gel filtration of raw liver iron components by sephadex G200. The sample was 4 ml of supernatant obtained by centrifugation of raw liver homogenate for 1 hour at 10,000 g. The eluant was 0.9% NaCl-0.01 M tris pH 8. For simplicity not all measured values have been included.

▽------▽ radioactivity

●———● absorbance 280 mμ

□———□ absorbance 540 mμ

FIGURE 2

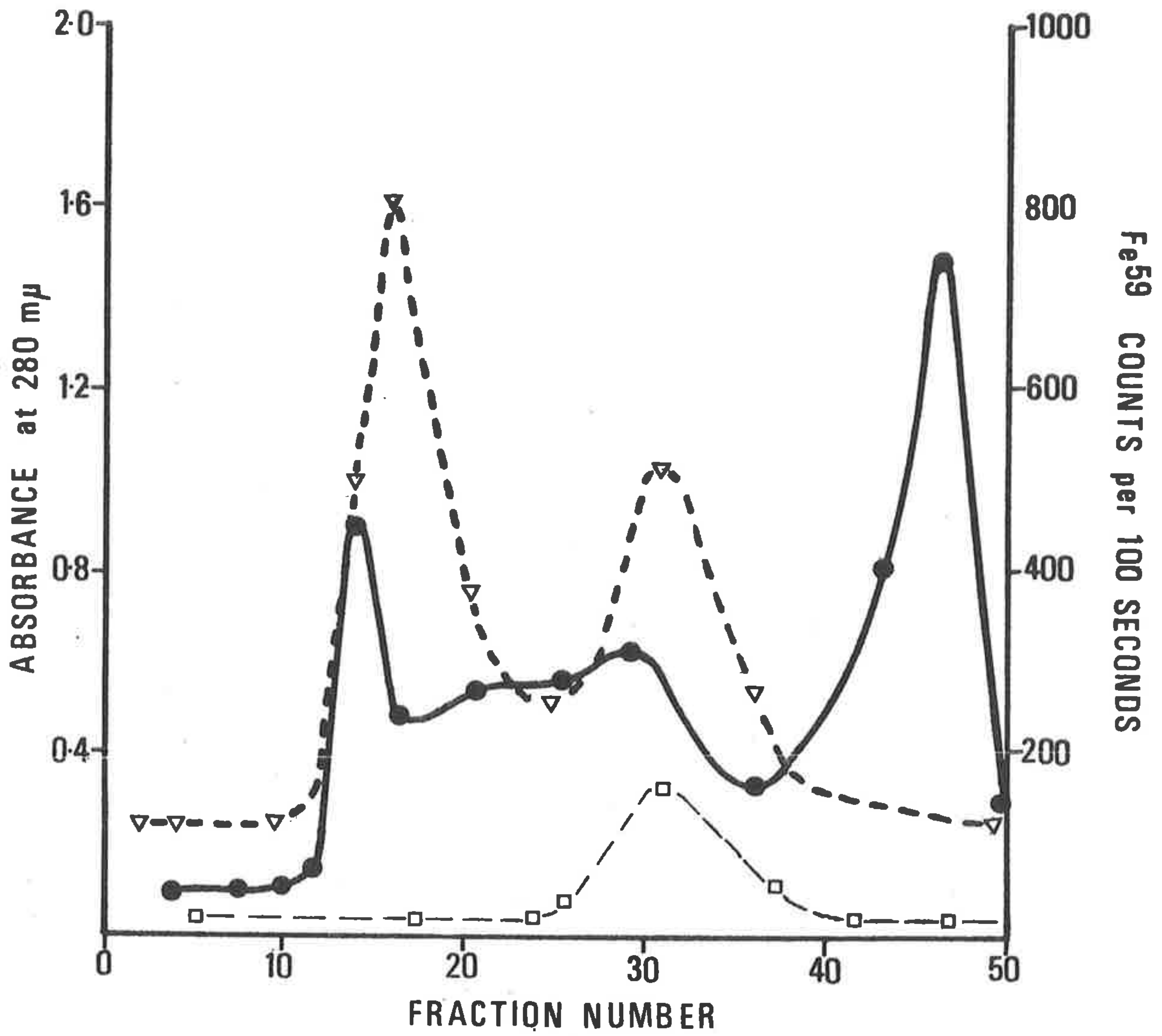


Fig. 3: Gel filtration by sephadex G200.

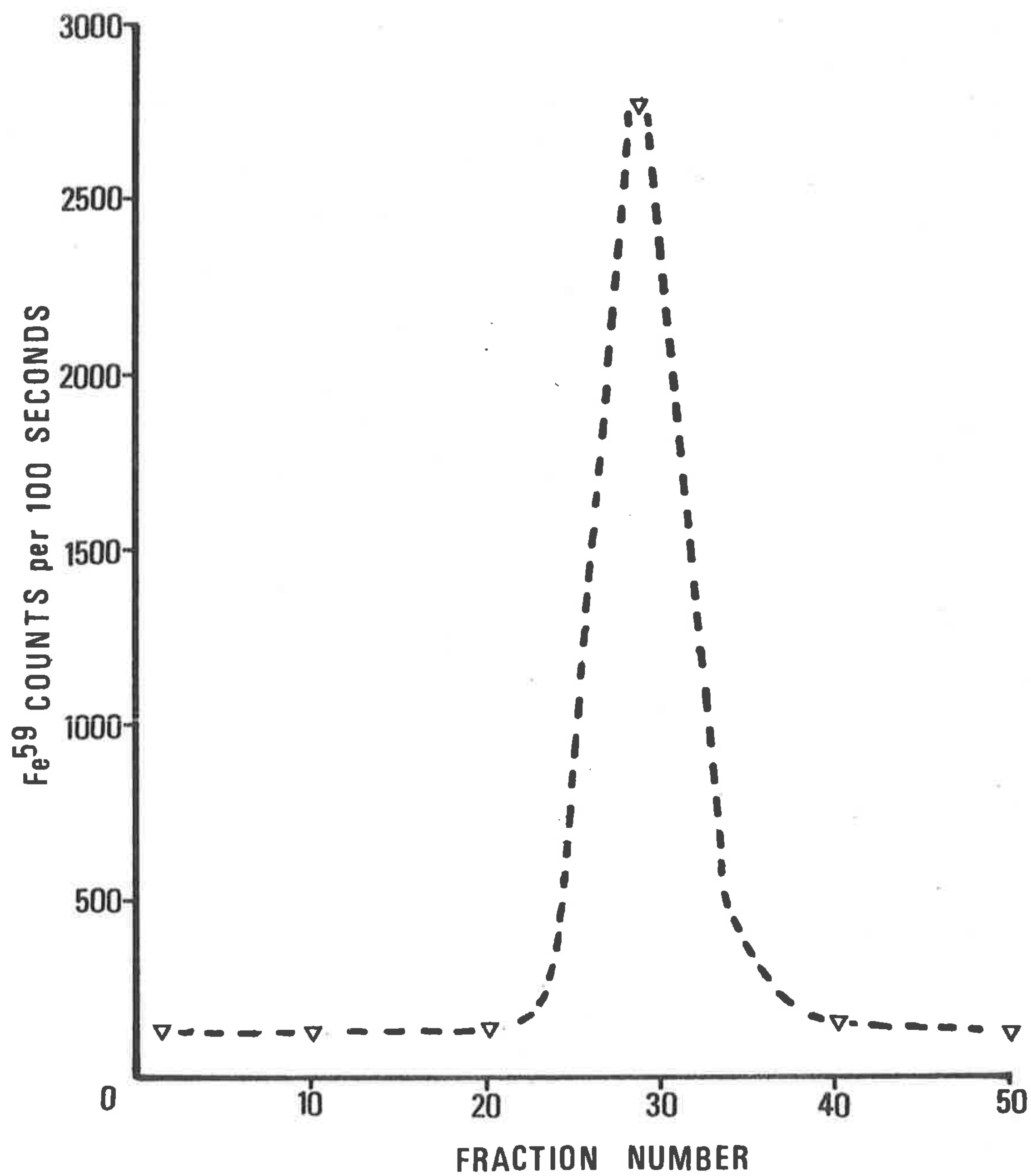
The sample was 2 ml of Fe^{59} labelled rabbit haemoglobin (28 mg Hb/ml).

The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all measured values have been included.

∇------∇ radioactivity

FIGURE 3



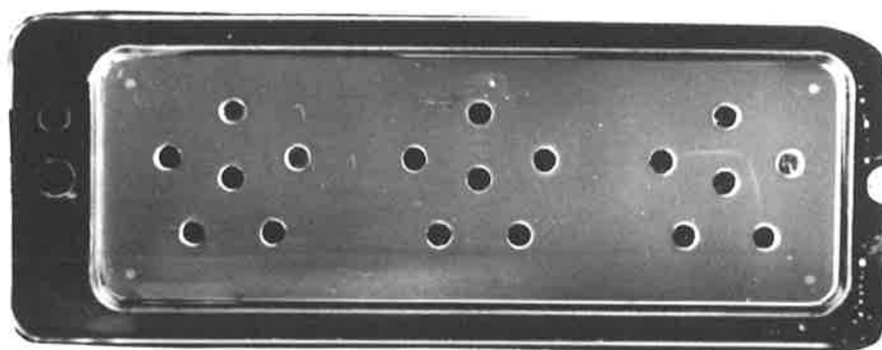
high molecular weight peak corresponded with the excluded fraction (M.W. > 200,000). Precipitin tests by immunodiffusion on agar gel showed that the concentrate of the pooled fractions under the large molecular weight radioactive protein peak, contained ferritin (photograph 1). The phenol sulphuric colour reaction for the detection of carbohydrate did not coincide with the radioactivity peaks.

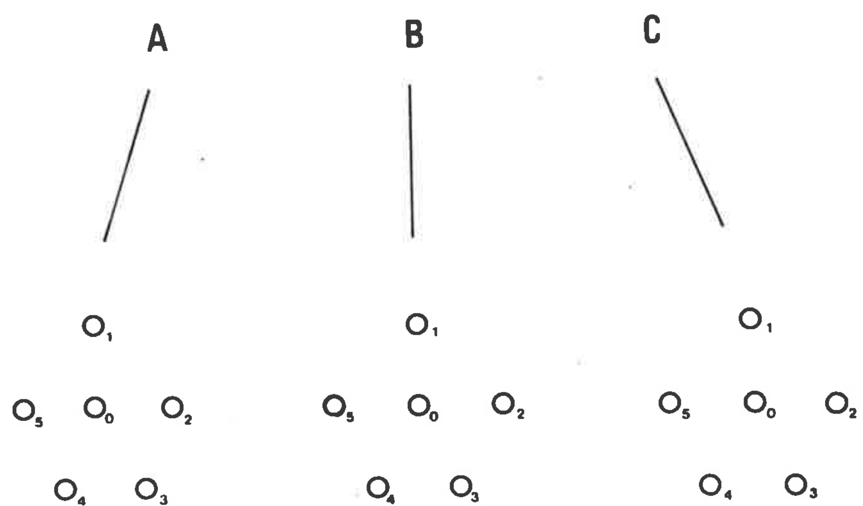
Following cooking (fig. 4) only two protein peaks were apparent. The majority of protein was present as low molecular weight components, but there was a small high molecular weight fraction which contained all the radioactivity. Ferritin was no longer detectable in the remaining high molecular weight fraction.

Liver homogenates which had been previously digested with acid-pepsin contained only small molecular weight protein complexes (fig. 5). The Fe^{59} activity was likewise localised to the single low molecular weight fraction (fig. 5). Further separation of the low molecular weight components released by acid-peptic digestion was then undertaken using sephadex G50 (fig. 6). There were two species of iron complexes present. The first corresponded with the excluded fraction (M.W. > 10,000) and the second was of a lower molecular size.

When liver was cooked and then digested with acid-pepsin

PHOTOGRAPH 1





B_0 – 5 µl antiferritin

B_1, B_2 – 5 µl pooled fractions

C_0 – 10 µl antiferritin

C_1, C_2 – 10 µl pooled fractions

Immunodiffusion of pooled, concentrated high molecular weight Sephadex gel fractions against horse antiferritin.

Fig. 4: Gel filtration of cooked liver iron components sephadex G200. The sample was 4 ml of supernatant obtained by centrifugation of cooked liver homogenate for 1 hour at 10,000 g.

The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all measured values have been included.

∇------∇ radioactivity

●———● absorbance 280 mμ

FIGURE 4

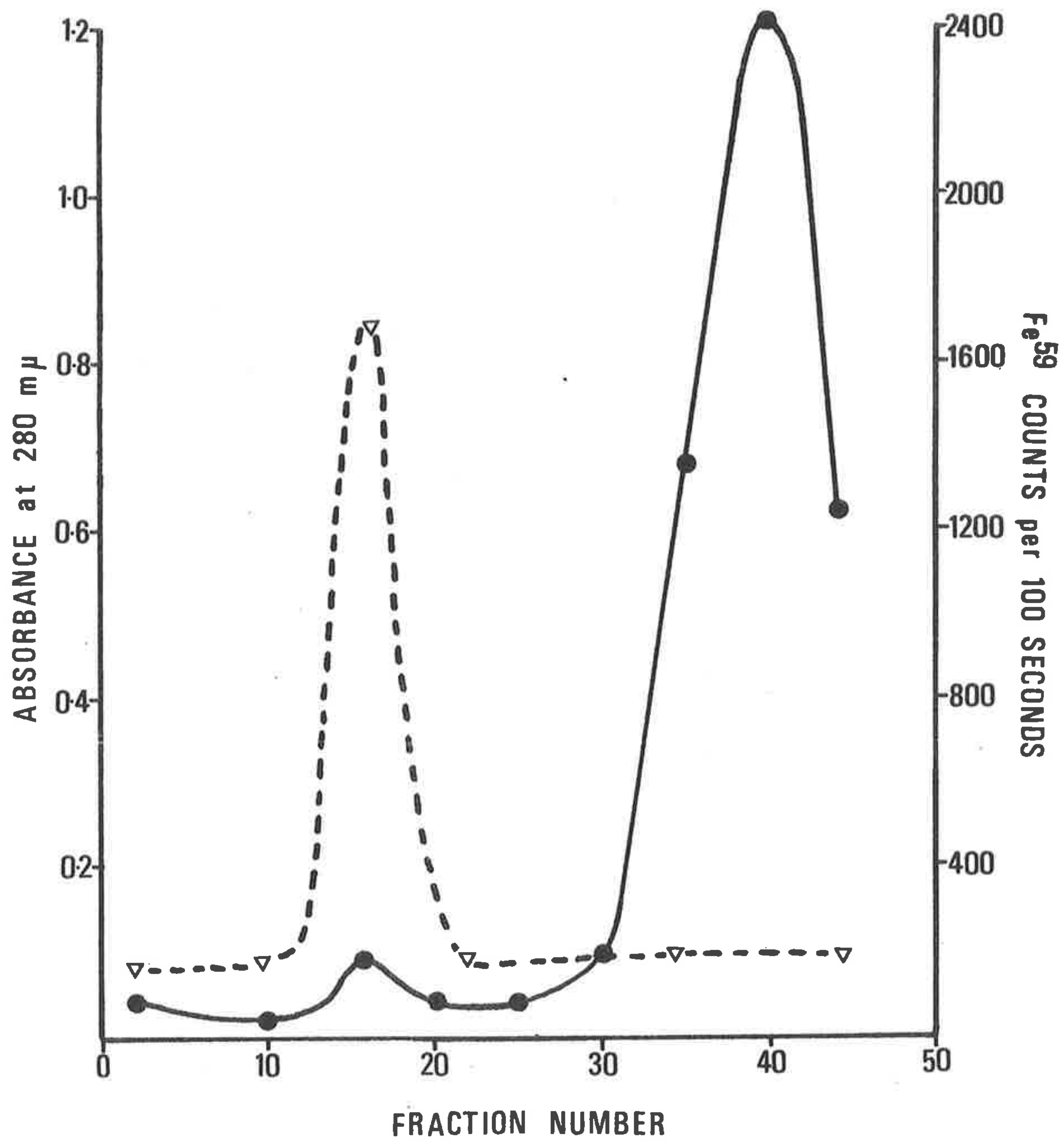


Fig. 5: Gel filtration of acid-peptic digested liver iron components by sephadex G200.

The sample was 4 ml of supernatant obtained by centrifugation of acid-peptic digested liver homogenate for 1 hour at 10,000 g.

The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all measured values have been included.

▽------▽ radioactivity

●———● absorbance 280 mμ

FIGURE 5

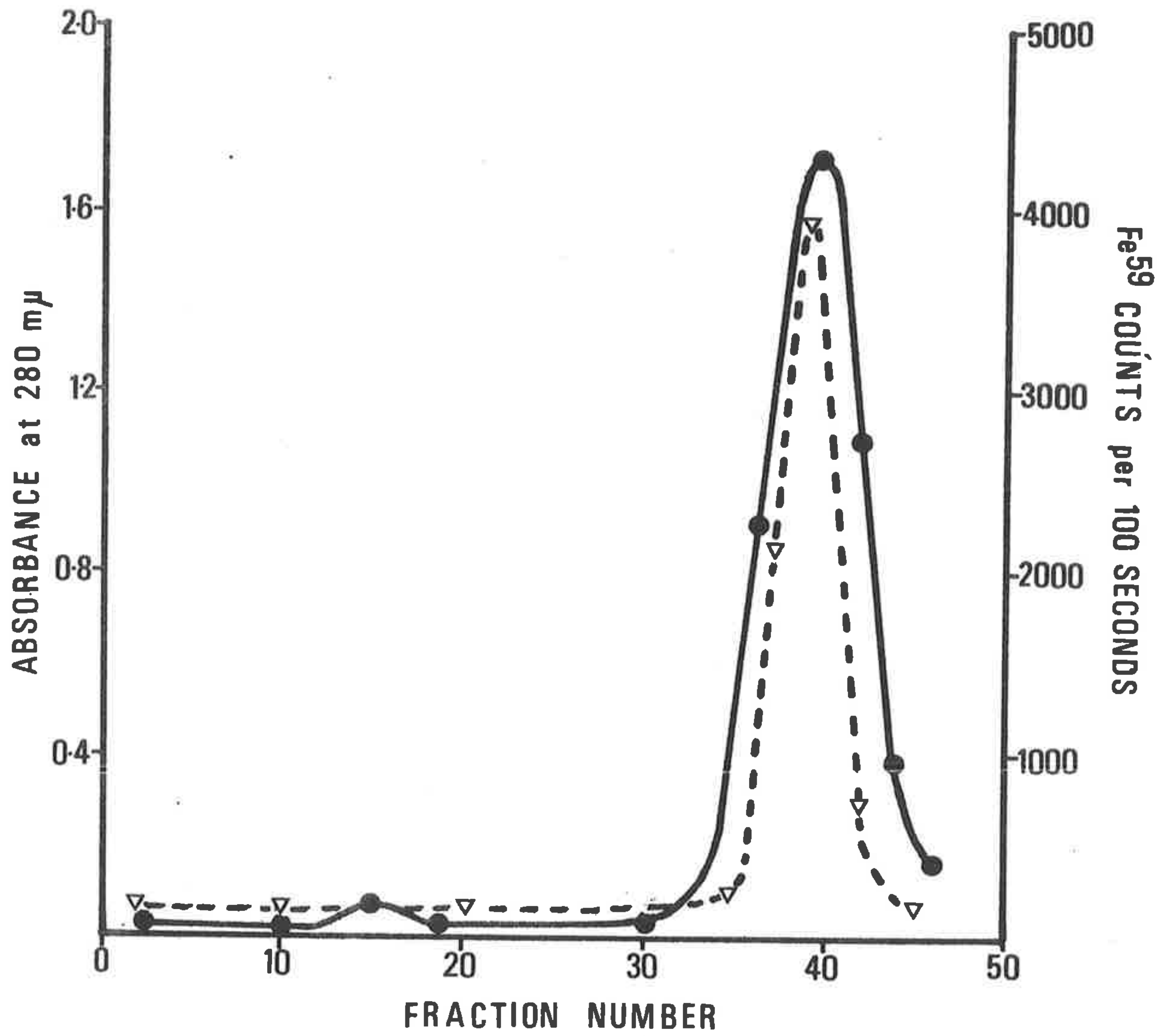


Fig. 6: Gel filtration of acid-peptic digested liver iron components by Sephadex G50.

The sample was 4 ml of supernatant obtained by centrifugation of acid peptic digested liver homogenate for 1 hour at 10,000 g.

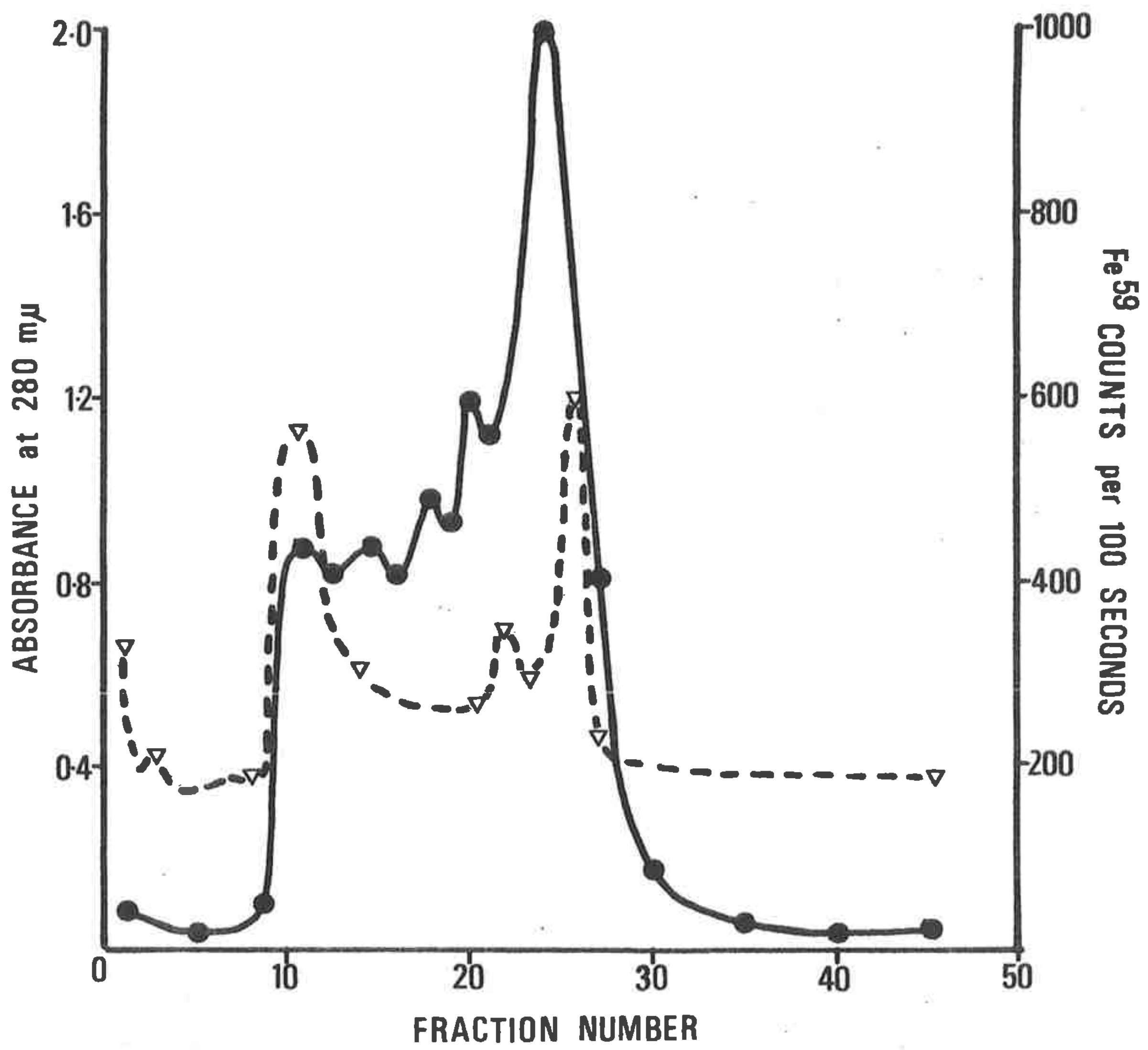
The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all measured values have been included.

∇------∇ radioactivity

●———● absorbance 280 mμ

FIGURE 6



there was no further change in the physical size of iron complexes. As with liver-acid-pepsin digests there was a single low molecular weight protein peak which corresponded to the Fe^{59} activity (fig. 7). The lower radioactivity peak was due to the lower activity of the guinea pig liver used.

Acid digested liver was resolved into two protein fractions (fig. 8). The Fe^{59} peak was coincident with the larger protein fraction in the low molecular weight range.

Following tryptic digestion the supernatant of the liver homogenate was separated into three protein fractions as was the raw liver (fig. 9). By comparison with untreated liver, the high molecular weight fraction was reduced and the major protein fraction comprised material of low molecular size. Radioactivity, however, remained localised to the high and intermediate molecular size protein fractions (fig. 9).

The aqueous ferritin extract, the presence of ferritin having been confirmed by immunodiffusion (photograph 2), was separated into high and low molecular weight protein fractions (fig. 10). The radioactivity was associated only with the high molecular weight peak.

Fractionation of ferritin following acid-peptic digestion, cooking plus acid-peptic digestion, and acid digestion alone,

Fig. 7: Gel filtration of cooked and acid-peptic digested liver iron components by sephadex G200.

The sample was 4 ml of supernatant obtained by centrifugation of cooked and acid-digested liver homogenate for 1 hour at 10,000 g.

The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all measured values have been included.

▽------▽ radioactivity

●———● absorbance 280 mμ

FIGURE 7

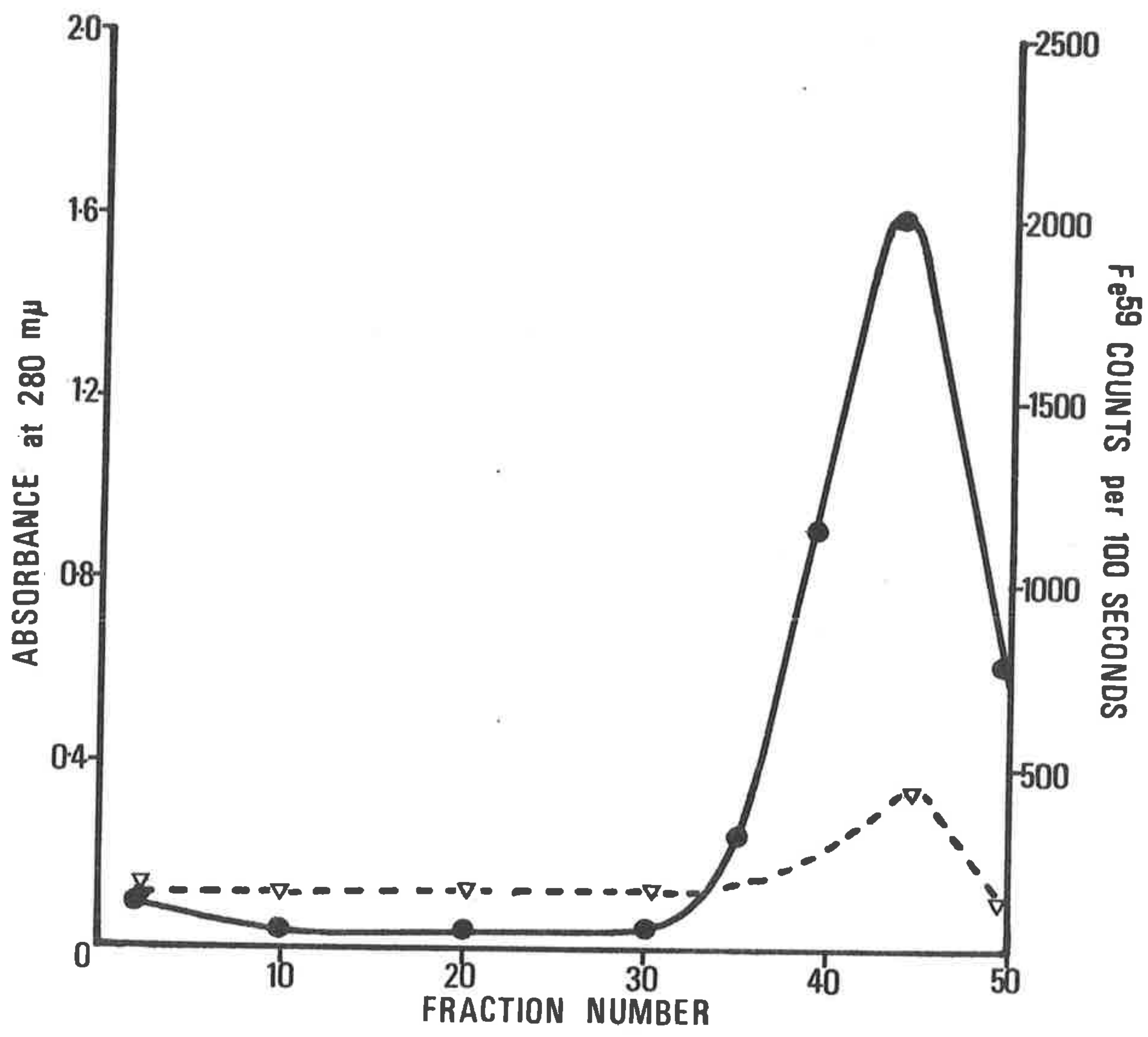


Fig. 8: Gel filtration of acid digested liver iron components by sephadex G200.

The sample was 4 ml of supernatant obtained by centrifugation of acid digested liver homogenate for 1 hour at 10,000 g.

The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all values measured have been included.

∇------∇ radioactivity

●———● absorbance 280 mμ

FIGURE 8

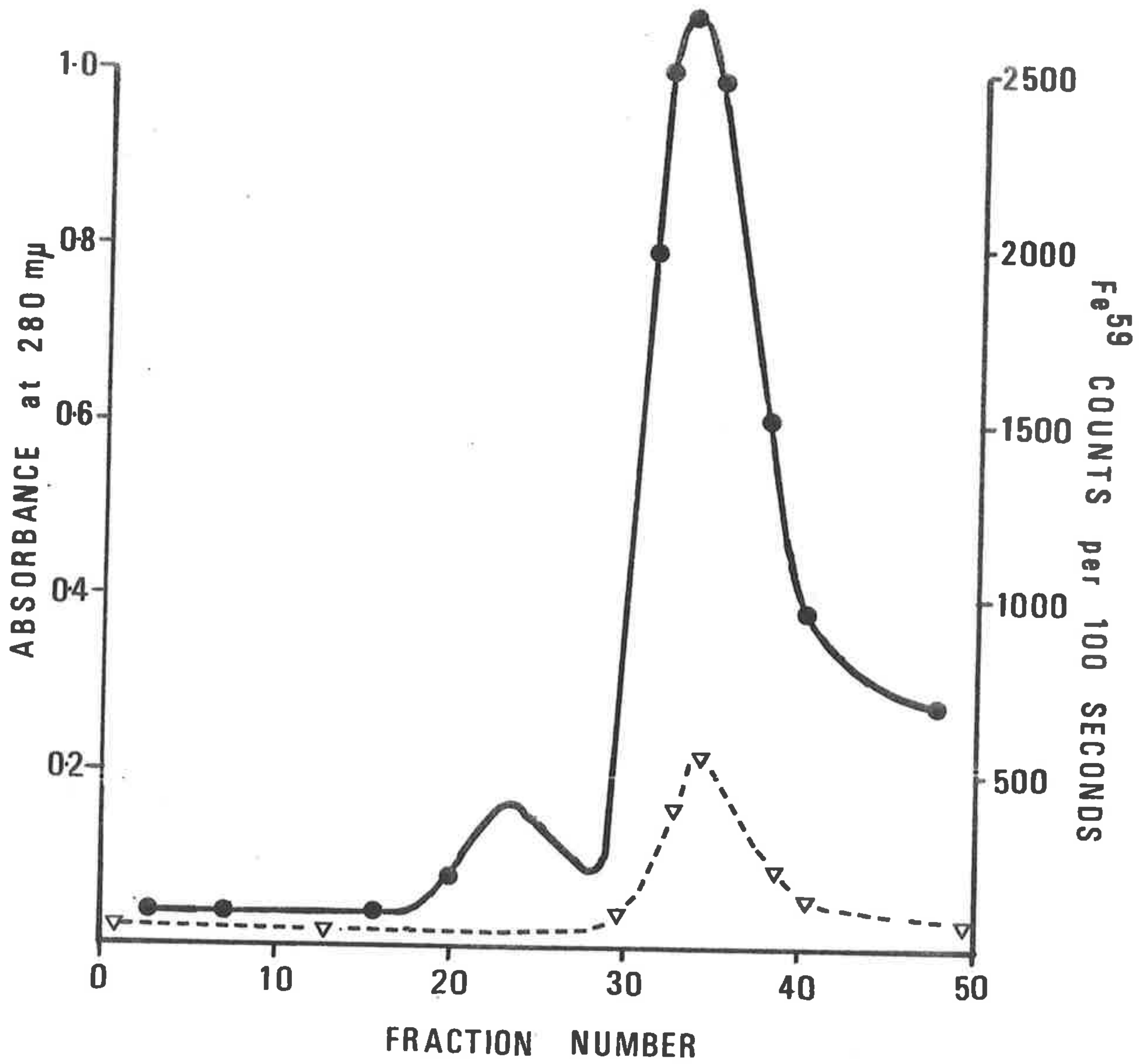


Fig. 9: Gel filtration of tryptic digested liver iron components by sephadex G200.

The sample was 4 ml of supernatant obtained by centrifugation of tryptic digested liver homogenate for 1 hour at 10,000 g.

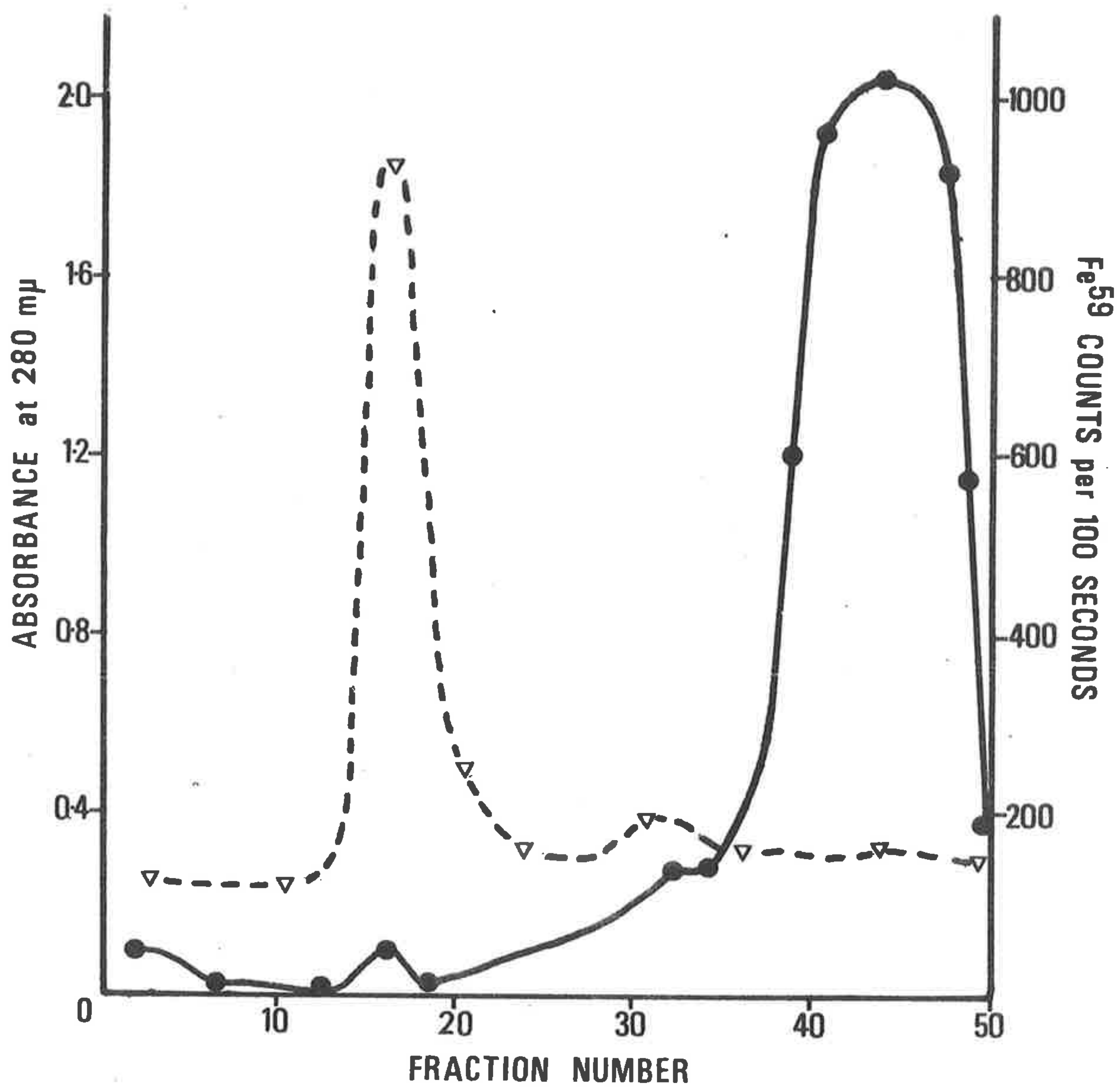
The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all values measured have been included.

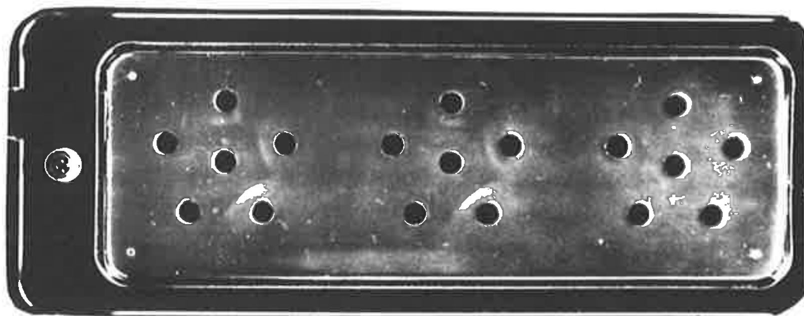
∇------∇ radioactivity

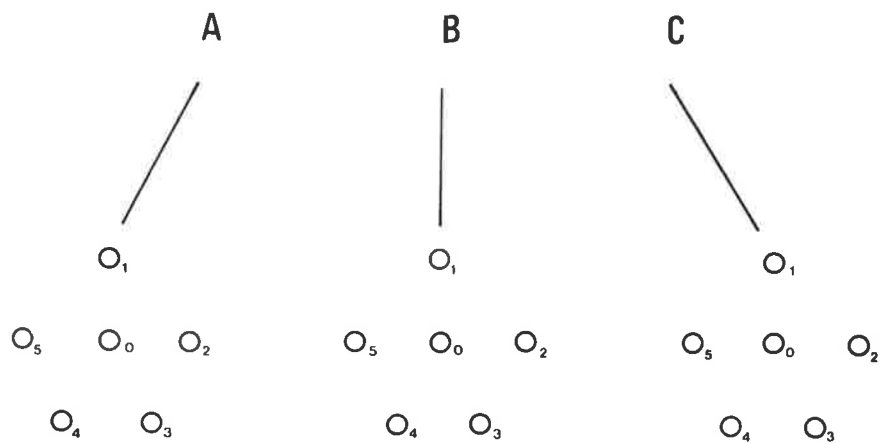
●———● absorbance 280 mμ

FIGURE 9



PHOTOGRAPH 2





A_0 – 5 µl antiferritin

A_2 – 5 µl ferritin

A_3 – 10 µl ferritin

B_0 – 10 µl antiferritin

B_2 – 5 µl ferritin

B_3 – 10 µl ferritin

Immunodiffusion of guinea pig liver ferritin against horse antiferritin.

Fig. 10: Gel filtration by sephadex G200.

The sample was 4 ml of aqueous ferritin solution.

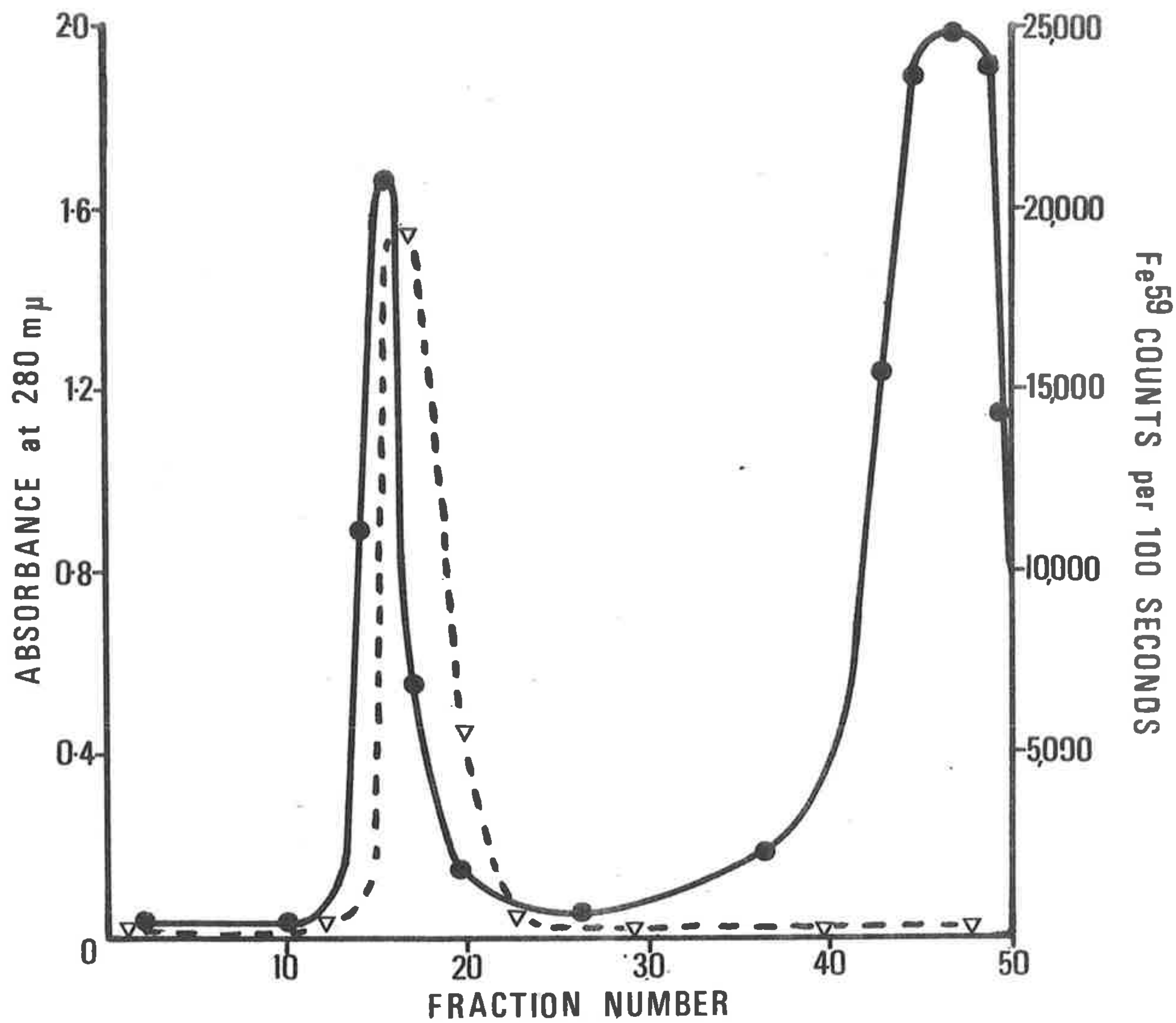
The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all values measured have been included.

∇------∇ radioactivity

●———● absorbance 280 mμ

FIGURE 10



produced a similar elution pattern in all cases (fig. 11, 12, 13). The ferritin solution in each case was resolved into high and low molecular weight protein fractions. However, all digestion procedures produced reduction of the high molecular weight peak. The radioactivity was associated with both of the protein fractions, but the greatest proportion occurred with the high molecular weight protein fraction (fig. 11, 12, 13). Immunologically active ferritin was not detected following these three procedures. Photograph 3 is specific for acid-peptic digested ferritin, but the other digestive procedures produced similar results. Precipitin bands were visible in agar gel within 36 hours when the undigested ferritin solution was diffused against the horse antiferritin. However, following the digestive procedures, immunodiffusion of the resultant ferritin solution produced no precipitin bands even after a time interval of 72 hours.

Tryptic digestion of ferritin resulted in a minimal change in the elution pattern when compared with that of undigested ferritin (fig. 14). The main difference was a small Fe^{59} peak, coincident with the low molecular weight protein fraction. No diminution in the large molecular weight protein fraction was evident, but in spite of this the presence of ferritin was once again not detected by immunodiffusion following tryptic digestion of the ferritin solution.

Fig. 11: Gel filtration by sephadex G200.

The sample was 4 ml of acid-peptic digested ferritin solution.

The eluant was 0.9% NaCl-0.01M tris pH 8.

For simplicity not all values measured have been included.

▽------▽ radioactivty

●———● absorbance 280 mμ

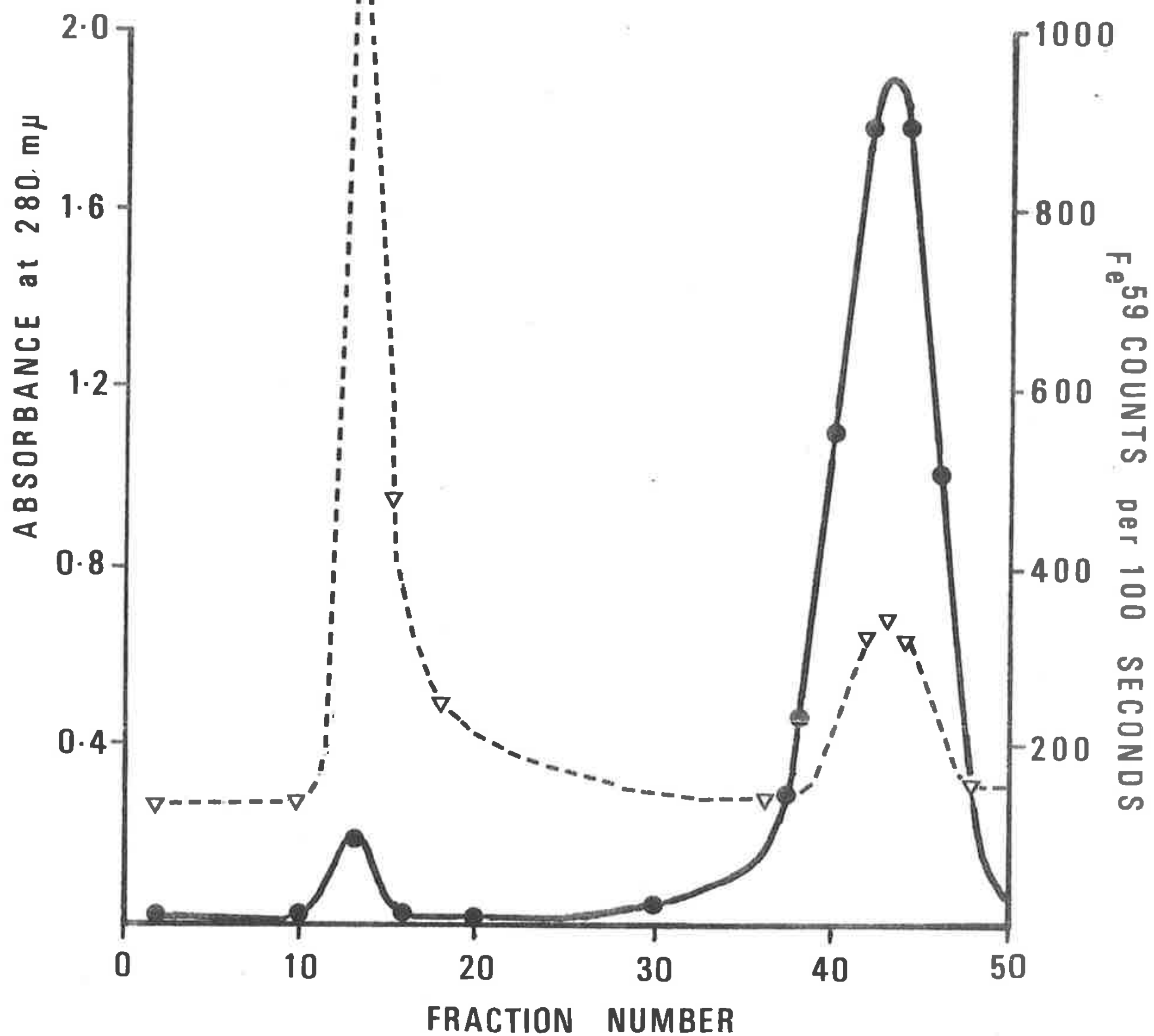


FIGURE 11
ABSORBANCE at 280 mμ
2·0
1·6
1·2
0·8
0·4
0
10
20
30
40
50
FRACTION NUMBER
1000
800
600
400
200
Fe59 COUNTS per 100 SECONDS

Fig. 12: Gel filtration by sephadex G200.

The sample was 4 ml of cooked and acid-peptic digested ferritin solution.

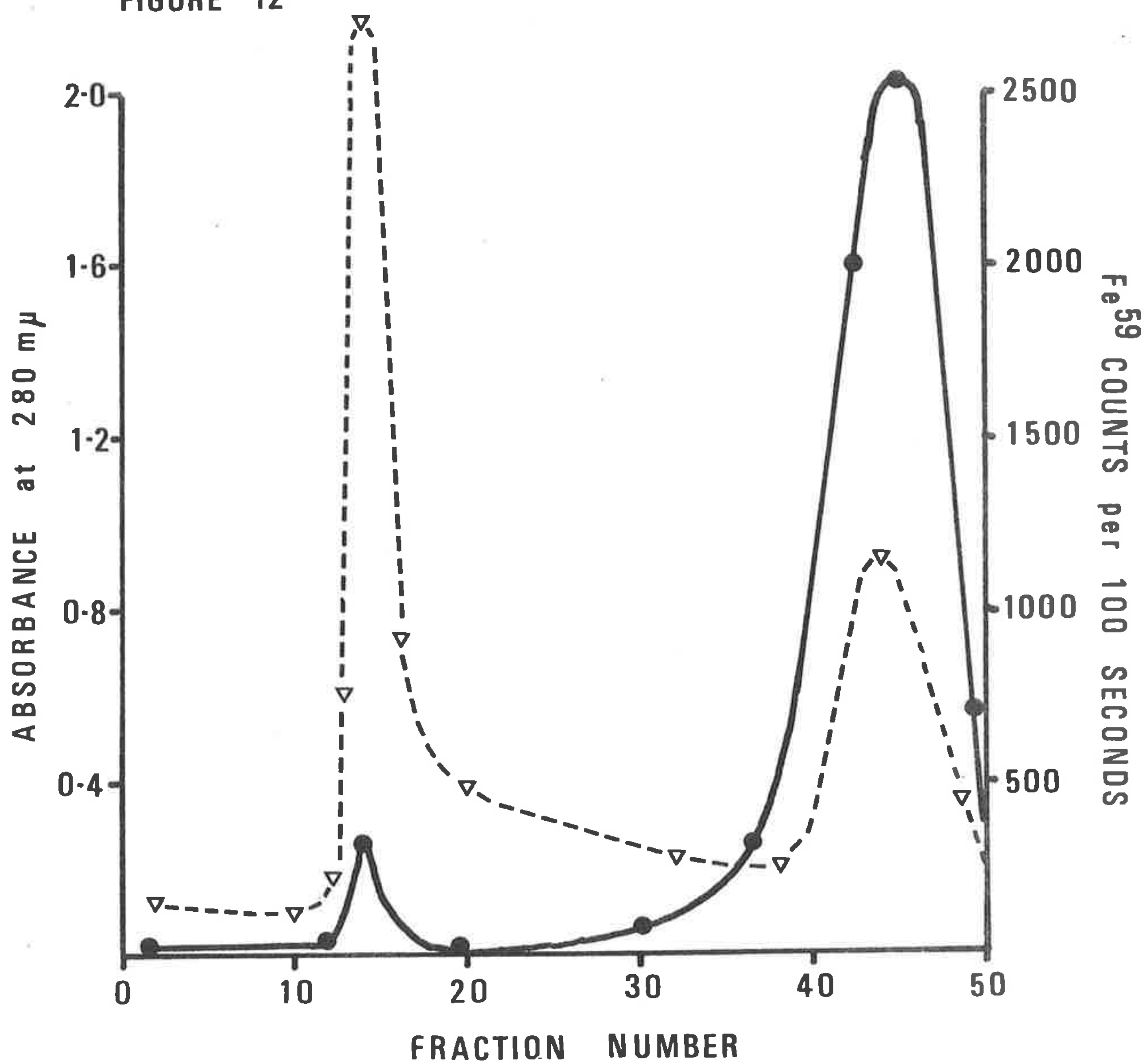
The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all values measured have been included.

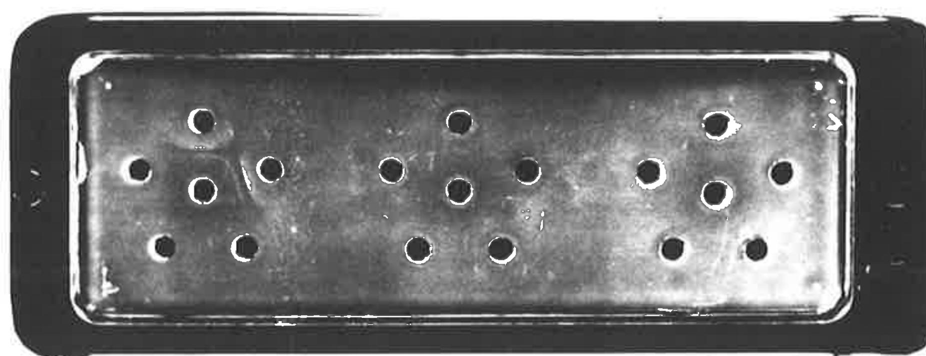
∇------∇ radioactivity

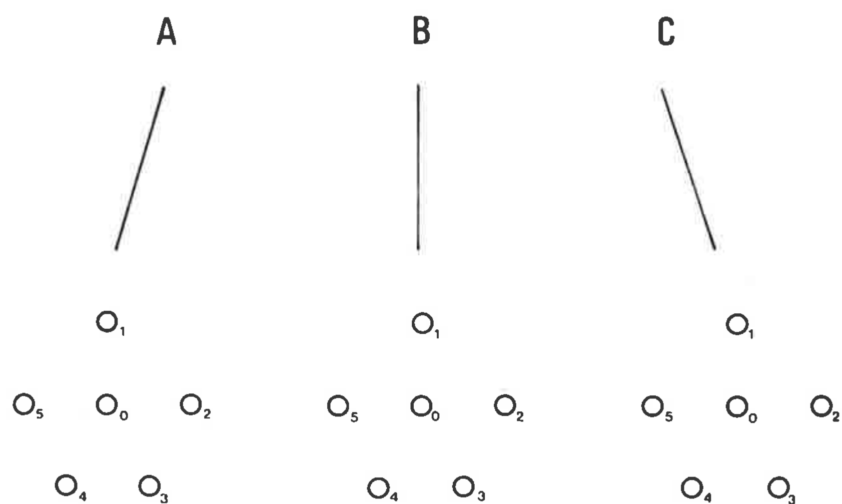
o———o absorbance 280 mμ

FIGURE 12



PHOTOGRAPH 3





A_0 – 10 µl antiferritin

A_1, A_2 – 10 µl ferritin

B_0 – 10 µl antiferritin

B_1, B_2 – 10 µl ferritin

Immunodiffusion of guinea pig liver ferritin, before and after acid-peptic digestion, against horse antiferritin.

Fig. 14: Gel filtration by sephadex G200.

The sample was 4 ml of tryptic digested ferritin solution.

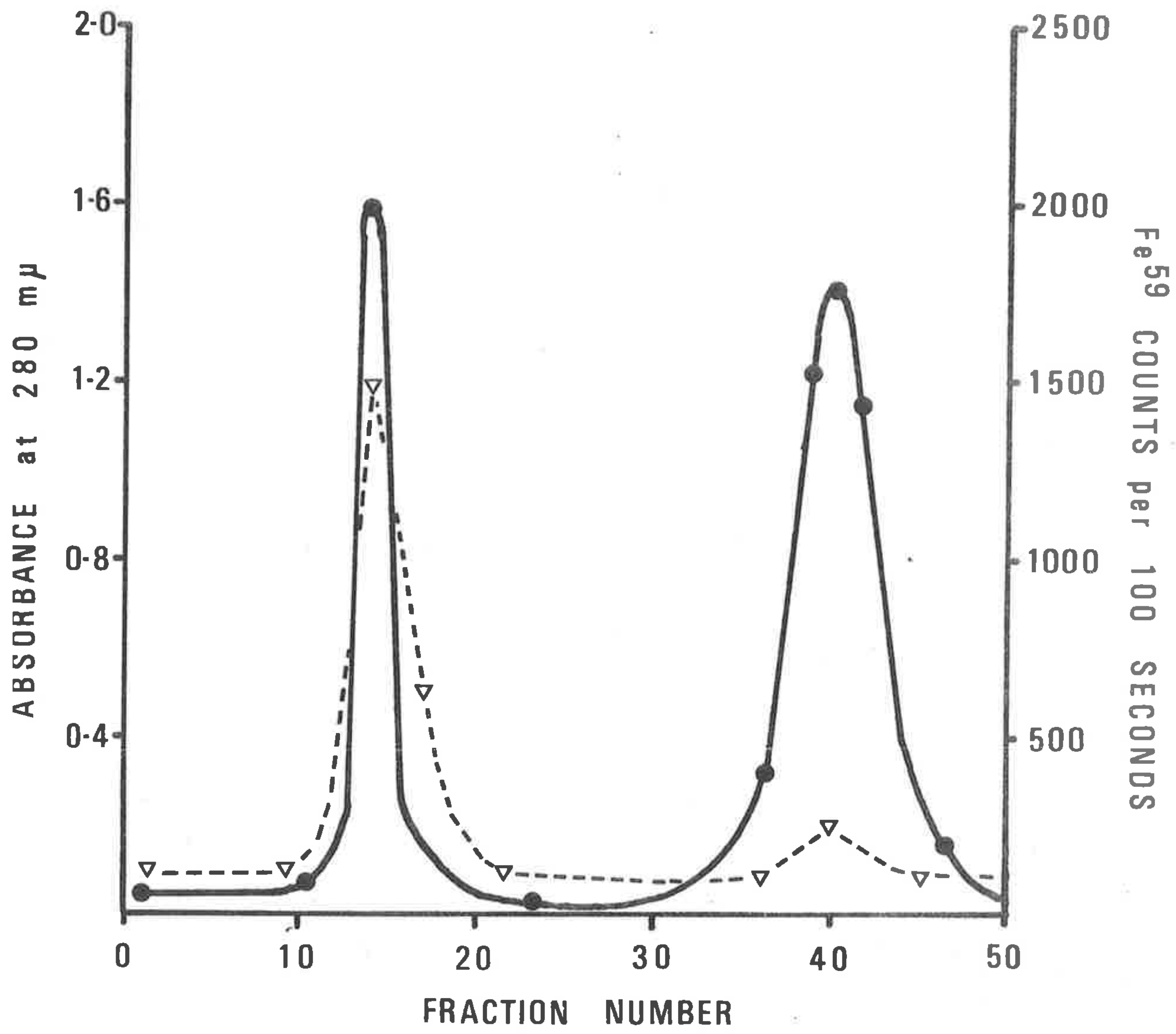
The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all values measured have been included.

▽------▽ radioactivity

●———● absorbance 280 mμ

FIGURE 14



COMMENT

The results indicate that a reduction in the molecular size of the iron complexes in guinea pig liver is produced by acid and enzymatic digestion, and that the most striking changes occurred following peptic hydrolysis of the liver homogenate in conjunction with cooking. The changes appear to reflect changes in the ferritin component of liver iron, whereas the haem iron component is resistant, and once the haem is split from haemoglobin, remains unaltered by digestive processes.

In this study there was no increase in soluble iron after acid digestion, acid-peptic digestion or cooking in association with acid-peptic digestion. The failure of such digestive procedures to release soluble iron is at variance with earlier reports (Shackleton & McCance, 1936; Kirch et al. 1947; Sandford, 1960). Although the first studies mainly involved estimates of ionic iron rather than soluble iron components, and may thus not be directly comparable, Jacobs et al. (1969) have recently estimated the soluble and ionisable iron in extracts of 25 common foods. Overall, they found that acid-peptic digestion and cooking prior to acid-peptic digestion produced a 20 to 40 per cent increase in the soluble iron content of these foods. Ox, pig, and lamb livers were included in their study and in these foods acid-peptic

digestion caused a two-fold increase in soluble iron. A much greater increase in soluble iron was produced by acid-peptic digestion of raw liver than of liver which had been previously cooked.

Although failure to release soluble iron from the guinea pig liver by peptic hydrolysis may merely reflect a species difference, differences in technique also exist. In the study of Jacobs et al. cited above, the concentration of liver homogenate was much greater than that in the present study. It is possible that the differences in the total iron concentration of the homogenate are responsible for the differences in iron partition, particularly as all the values for soluble iron reported by Jacobs and his co-workers are lower than those obtained for guinea pig liver.

A marked reduction of the soluble iron occurred after cooking and also following tryptic digestion. Jacobs et al. (1969) similarly showed that cooking alone, without subsequent peptic hydrolysis, resulted in a 30 per cent reduction in the soluble iron of pig and lamb liver homogenates, but found no change in the case of ox liver homogenate. The effects of cooking, during which the liver homogenate was maintained at $100^{o}C$ for 10 minutes, are probably attributable to denaturation and aggregation of protein at this temperature (Granick, 1946). It is known too, (Granick, 1942) that ferritin is irreverisbly

denatured, undergoing macromolecular aggregation, when subjected to temperatures in excess of 90°C.

Ferritin has been shown to be relatively resistant to tryptic digestion (Granick, 1942, 1946) and only a small amount of iron is released during contact with this enzyme. However, Granick also demonstrated that when ferritin is denatured by 1 N alkali, the iron micelles are rapidly removed from the protein fraction. Thus the excess precipitation of iron which occurred during tryptic digestion may be related predominantly to the alkaline conditions of the digest rather than to specific tryptic activity. Any iron released from the ferritin molecule would precipitate as insoluble ferric hydroxide at the alkaline pH of the tryptic digests. Furthermore, Conrad et al. (1966a) have also shown that about one-third of haemoglobin is degraded to haem by tryptic activity, and that the haem monomers polymerise in an alkaline environment. Therefore the combined effect of trypsin and the alkaline medium would be to decrease the soluble iron component of the liver.

Even though peptic digestion produced no increase in the soluble iron fraction, very significant physical and chemical changes occurred during the digestion period. These were reflected by alterations in the dialysable iron component and also by changes in physical sizing on sephadex gels. Virtually no dialysable iron was detected in raw liver homogenate, but this

increased markedly following the enzymatic digestions. The quantity of dialysable iron was approximately 30 per cent of the total iron after both acid and acid-peptic digestion. This suggested that acid was the important factor in altering the iron complexes. The importance of acidity rather than the peptic component was also indicated by the failure of prolonged peptic digestion to increase the dialysable material. Release of small molecular weight iron complexes occurred immediately on contact with acid, with no further increase during the total digest period. This contrasts with the known effect of pepsin, and other enzymes which produce initially a linear increase in the degradation of the substrate (Dixon & Webb, 1966).

Tryptic digestion likewise increased the proportion of iron which was removed by dialysis. Such small molecular weight components were not detected in the soluble iron fraction probably due to precipitation at alkaline pH. However they indicate that protease digestion is potentially important in altering the physical and chemical characteristics and thus the availability of dietary iron.

The dialysate contained negligible amounts of haem iron. Thus it appeared likely that the dialysable iron was released from the ferric hydroxide micelles of the ferritin molecule. This was confirmed by ferritin dialysis. There was no dialysable iron detected in the aqueous ferritin solution. By contrast,

approximately 13 per cent of the total ferritin iron passed through a dialysis membrane after peptic hydrolysis, and 28 per cent after cooking in conjunction with peptic hydrolysis. The changes in the ferritin molecule may likewise be important in increasing the amount of iron, present in this form, which can be absorbed.

In the present study haem was not detected in the dialysate of either raw or digested liver. It is known (Conrad et al. 1966a, 1967b; Wheby et al. 1970) that gastric and intestinal enzymes do split haem from haemoglobin, both in vivo and in vitro, and that the haem component so released is readily absorbed. The failure to detect haem after enzymatic digestion in this study probably reflected the unfavourable conditions under which dialysis was performed rather than a lack of haemoglobin degradation. Conrad et al. (1966b) separated haem from incubated mixtures of haemoglobin and intestinal enzymes by placing the mixtures in cellulose dialysis bags and immersing these in solutions buffered to pH 8, at which pH haem was lost from the dialysis bag. Globin degradation products, on the other hand, were removed by dialysis against a 0.1 M KCl-HCl buffered solution pH 4; the haem was precipitated and remained in the bag, until subsequently the dialysis bag was transferred to an alkaline environment to separate the haem from haemoglobin. Therefore it is apparent that the conditions under which dialysis

was performed in this study would cause precipitation of the haem within the dialysis bag.

Fractionation of the liver iron components using sephadex gels confirmed that the size distribution of the iron complexes changed following cooking and enzymatic digestion. In raw liver iron was associated with the large and intermediate molecular weight protein peaks. The former was shown to contain ferritin and the latter haemoglobin. Acid, acid-peptic digestion, and cooking plus peptic digestion all resulted in a single low molecular weight protein peak and the Fe^{59} radio-activity was associated with this, thus indicating that both the major iron components of liver were broken down by these procedures. Further separation on sephadex G50 showed that two species of low molecular weight iron complexes were contained within this single iron containing protein peak. The first iron fraction corresponded with the excluded fraction (M.W. $>$ 10,000), and as filtration was performed using a buffer at pH 8, it probably represented polymers of haem (Conrad et al. 1966a). The second iron fraction was of a lower molecular weight, being within the fractionation range of sephadex G50 (M.W. $<$ 10,000 and $>$ 500), and probably represented aggregates of ferric hydroxide released from the ferritin iron micelles.

With cooking, the high molecular weight protein fraction was

partially denatured with release of low molecular weight components. However there was no release of iron from the large complexes. This finding is in accord with the results of dialysis of cooked liver, in which no release of iron was detected in the dialysate, either against deionised water or dilute hydrochloric acid.

A similar pattern was observed following tryptic digestion in that the high molecular weight protein peak was partially denatured with release of low molecular weight complexes. However tryptic digestion also produced a slight release of iron from the large molecular weight complexes. This finding is also in accord with the results of dialysis of tryptic digested liver, in which some iron was released into the dialysate both against deionised water and 0.16 M hydrochloric acid.

The liver ferritin extract separated into two major protein components on sephadex G200. All the radioactivity was associated with the high molecular weight complexes (M.W. > 200,000) due to the molecular weight of ferritin, M.W. 460,000 (Granick, 1946). The low molecular weight protein fraction has been shown to consist of contaminant proteins (Lindner-Horowitz et al. 1970). Monitoring of the radioactivity in the eluted fractions following all digestive procedures showed that a variable portion of the radioactivity was now present in the low molecular size region. This confirmed that iron is released

from the ferritin molecule, and substantiated the dialysis findings. In addition, the loss of ferritin antigenicity following acid and enzymatic digestion indicated that ferritin had been sufficiently degraded during these procedures to alter the antigen/antibody binding sites.

It is therefore apparent that cooking and digestion modify liver iron, the main effect being towards reduction in size of the iron complexes. It is proposed that the reduction in size is aimed at facilitating the absorption of the liver iron compounds. Cooking alone did not reduce the size of the complexes and so it can be argued that this common preparative procedure plays no role in dietary iron absorption. However, when cooking is considered in the light of the complete process of food preparation, ingestion, and digestion, and when it is recalled that cooking in combination with digestion induced the greatest changes in liver iron, then it appears that cooking may influence liver iron absorption.

CHAPTER V

LIVER IRON ABSORPTION STUDIES IN RATS

INTRODUCTION

The absorption of iron from a variety of dietary sources has been investigated by numerous workers over a long period of time (Widdowson & McCance, 1942; Chodos et al. 1957; Callender & Warner, 1968; Martinez-Torres & Layrisse, 1970). During the same period other groups (Bergeim & Kirch, 1948; Sandford, 1960; Jacobs et al. 1969) have recorded the effects of cooking and enzymatic digestion on iron contained in a number of food-stuffs. However, up to the present time no attempt has been made to correlate changes induced in iron complexes by cooking and enzymatic digestion with their subsequent absorption. It has been shown (chapter IV) that the greatest reduction in the size of the liver iron complexes occurred when liver was cooked and then exposed to acid-peptic digestion. As these in vitro conditions broadly mimic the handling of dietary iron complexes it was postulated that the processes of cooking and digestion combined to enhance dietary iron absorption. The next phase of the investigation was undertaken to test this hypothesis by studying the absorption of radioactive iron from in vivo labelled guinea pig liver in the raw, cooked and digested states. In addition absorption from the main liver iron components was also measured.

The rat was chosen as the experimental animal for the initial absorption studies due to difficulties in procuring sufficiently large quantities of in vivo labelled guinea pig liver to enable a complete survey to be performed in man. This necessarily imposed a restriction on the interpretation of the results and extrapolation to the state in man. In view of this limitation, absorption of Fe^{59} from selected liver preparations was subsequently tested in human volunteers.

METHODS AND MATERIALS

Experimental animals. Iron absorption studies were performed on female albino rats of the Lister strain. The weights ranged from 100 to 150 g with an average weight of 131 g, and the age, at the onset of the study, varied between 4 and 5 weeks. The rats were housed in metabolic cages and fed a standard diet of M & V rat cubes (William Charlick Ltd. Adelaide). All test animals were fasted for 12 hours before administration of the test dose. The rats were lightly anaesthetised with ether, and a fine polythene tube (approximately 14 gauge) was passed into the stomach. The test substance was administered through this tube. All animals received the test substance in a volume of 1.25 ml, followed by 0.25 ml deionised water. The test substances were:

(1) Ferric chloride. Ferric chloride solution was made by diluting 0.25 ml (687.5 µg elemental iron) of the stock

0.05 M ferric chloride solution with 0.01 M hydrochloric acid. Ten μCu Fe^{59} chloride (10 μCi/μg) were added to the solution and the final volume adjusted so that the iron concentration was 20 μg per 1.25 ml.

(2) Liver preparations. Homogenates were prepared from Fe^{59} labelled guinea pig liver (1 g/10 ml) as previously described in chapter III. The iron content of the homogenates was determined in triplicate and ranged from 15.0 μg per ml to 16.0 μg per ml. Absorption of iron from raw, cooked and digested homogenates was measured in separate groups of rats. The test doses each contained 20 μg elemental iron, and the volume of each was 1.25 ml.

(3) Major liver iron components.

Ferritin. Radioactive ferritin was prepared from 6 g of Fe^{59} labelled guinea pig liver by the method of Granick (1942) previously referred to in chapter III. The volume of the ferritin containing heat filtrate was 8 ml and the ferritin iron content was 23 μg per ml. The necessary dilution of the solution was performed with deionised water, giving a final ferritin iron concentration of 20 μg per 1.25 ml. Raw ferritin was administered to one group of rats. Another group of animals received cooked plus acid-peptic digested ferritin. The aqueous ferritin solution was prepared as outlined above, and

then cooked and acid-peptic digested for 2 hours before administration to the experimental animals.

Haem

Haemoglobin haemolysate. Haemoglobin haemolysate was prepared by acid-peptic digestion of in vivo labelled (Fe^{59}) rabbit haemoglobin. The preparation of isotopically labelled rabbit haemoglobin has been described in Chapter IV. 3.9 g of radioactive haemoglobin in 100 ml of deionised water was acid-peptic digested for 12 hours. The enzymatic digestion methods have been detailed in chapter III. The iron content of the digest being 30 μg per ml, further dilution to 20 μg Fe per 1.25 ml was performed with deionised water.

Chemically purified haem. Fe^{59} labelled haem was extracted from 3 g of radioactive guinea pig liver by the method of Labbe & Nishida (1957). The crystalline haem was redissolved in 5 ml of 0.1 N sodium hydroxide; the iron content of the resultant solution was 24.4 μg per ml. The required iron concentration (20 μg per 1.25 ml) was achieved by dilution with deionised water.

(4) Dialysable and non-dialysable liver iron fractions

Three 5 g aliquots of radioactive guinea pig liver were homogenised in the usual manner, and the final volume of each homogenate was adjusted to 50 ml with deionised water. The homogenates were cooked, and then subjected to peptic hydrolysis

in the manner previously described in chapter III. The resultant digests were dialysed against 0.16 M hydrochloric acid for 24 hours at 4°C. At the end of this period the low molecular weight dialysable iron complexes in the dialysate were concentrated by rotary evaporation. The volume of the dialysate was reduced to 10 ml, and the iron content was then determined by atomic absorption spectrophotometry. The final volume of the dialysate was adjusted with deionised water so that the iron content was 20 μg per 1.25 ml.

The non-dialysable liver iron preparation was obtained by pooling the contents of the dialysis bags at the end of the 24 hour period. The volume of the resultant solution was adjusted to 150 ml with deionised water. The content of iron remaining in the dialysis bag was determined by atomic absorption spectrophotometry following wet ashing of three 20 ml aliquots of the pooled dialysis bag contents. The iron content was 8.9 μg per ml, and so the pooled solution was concentrated with lyphogel (Gelman) to produce a final iron concentration of 20 μg per 1.25 ml.

The concentration of iron was always adjusted so that each test solution contained 20 μg Fe in 1.25 ml of the solution, with the exception of two groups of animals, both of which received a test solution of 1.25 ml containing 40 μg Fe. In one of these

groups of animals the effect of liver on the absorption of inorganic iron was tested. These animals were given 20 μg of Fe^{59} labelled ferric chloride and unlabelled liver homogenate (133 mg of liver) containing 20 μg Fe. The other group was the control, and these animals were given 40 μg of Fe^{59} labelled ferric chloride.

Ferric chloride was included as a reference control so that the absorption of the various liver fractions could be compared with that of inorganic iron. The number of animals in each group varied from 5 to 13.

"Gastrectomised" rats

In a further group of animals the effect of by-passing the stage of in vivo gastric digestion on liver iron absorption was assessed. In this group of rats the test dose was injected directly into the duodenum following pyloric ligation.

In this technique rats were anaesthetised with 0.5 to 0.6 ml (6 mg/ml) sodium pentobarbitone given by intraperitoneal injection. The abdomen was then opened through a left paramedian incision and the stomach was isolated. The pylorus was exposed and gently tied with black silk without impairment to the blood supply. The test dose was injected into the duodenum with a 19 gauge hypodermic syringe. The abdomen was closed with Michel clips. The rats were counted in a large volume

gamma counter (Nuclear Data) within 2 hours of receiving the dose. Under a light ether anaesthetic, 6 hours later, the abdomen was re-opened and the pyloric ligature removed. The abdomen was then re-closed using black silk through and through sutures.

The control group of rats for the above experiment were subjected to a sham gastrectomy. The control rats were treated in an identical manner to that previously described with the exception that the pyloric ligature was released within 2 minutes, and the liver preparation was injected into the stomach. After 6 hours the abdomen was re-opened, the pylorus again exposed, and then the abdomen was resutured.

Measurement of Fe^{59} *retained*

Whole body radioactivity was measured in the large volume scintillation detector (Nuclear Data) previously described. The rats were placed in a well ventilated plastic container approximately 5 cm x 10 cm in which they were virtually immobilised, and the container was placed between the two 3 inch crystals. All animals were counted in duplicate. Standards were prepared for each group by measuring the test doses into 250 ml water filled plastic bottles. These were also counted in duplicate. The initial count was made within 2 hours of administration of the dose. The final count was performed under

identical conditions on the 8th test day.

The calculation of the iron retained was by the method of Forrester, Conrad & Crosby (1962). The initial counts represented the 100 per cent value and the activity eight days later represented the iron retained. The percentage of Fe^{59} retained after eight days was calculated from the formula below.

$$\frac{r_8/S_8}{r_0/S_0} \times \frac{100}{1} = \text{per cent } Fe^{59} \text{ retained}$$

where r_0 = initial whole body count

S_0 = initial standard count

r_8 = whole body count after 8 days

S_8 = standard count after 8 days

RESULTS

The results of the absorption experiments in rats are detailed in tables 11-16 and figs. 15-17. In the first group of animals absorption of ferric chloride was compared with iron absorption from raw, cooked and digested liver (table 11, fig. 15). The retention of ferric chloride was 34.7 ± 3.1 per cent; this was significantly better ($p < 0.001$) absorbed than iron from raw liver, acid and enzymatically digested liver, and liver which had been cooked alone. However when liver was cooked and

TABLE 11. Comparison of iron absorption from ferric chloride and raw, cooked and digested liver

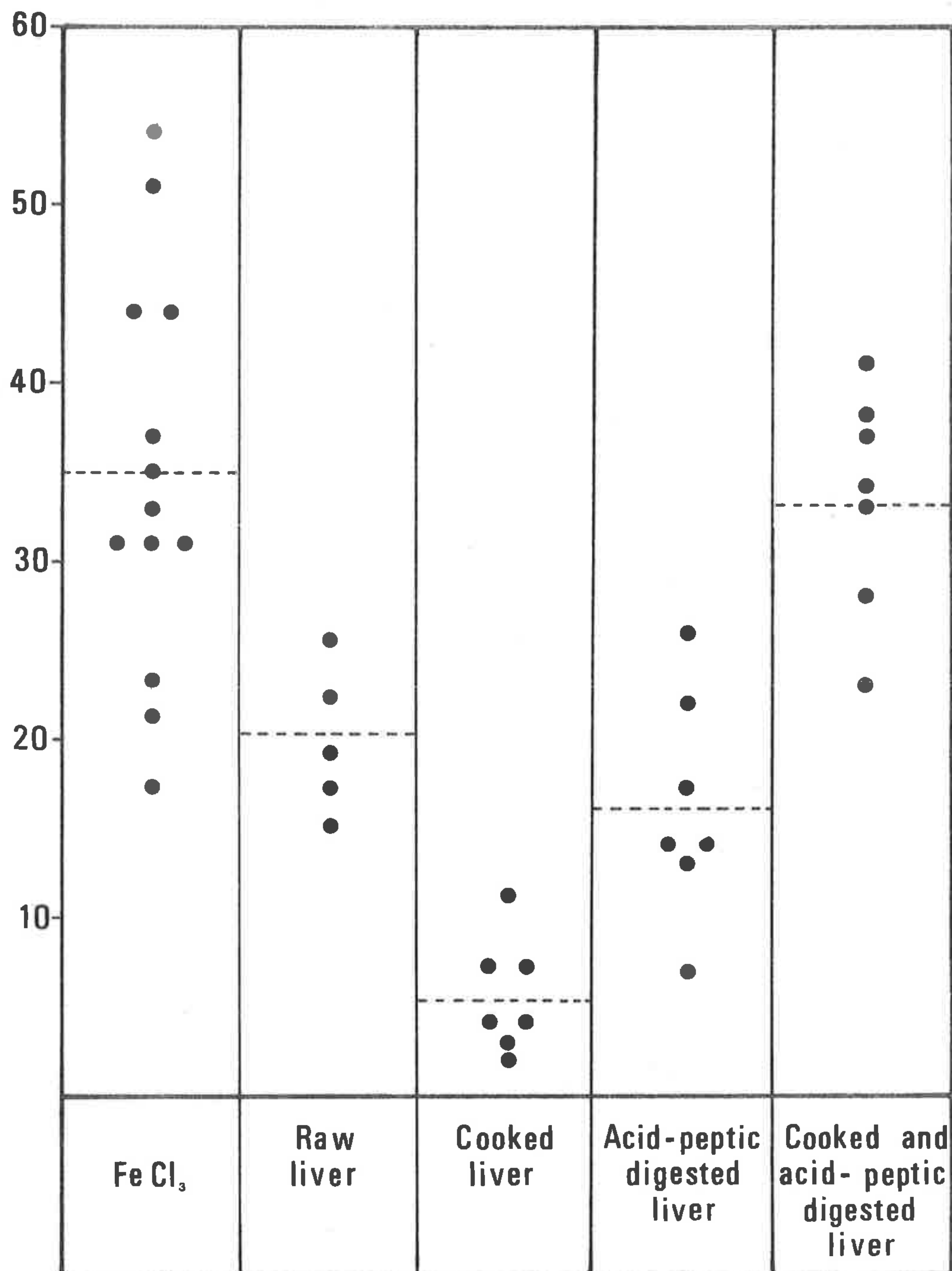
Test substance		% Fe^{59} retained Mean	± S.E.	**Significance level (compared to $Fe^{59}Cl_3$)
Ferric^{59} chloride	*(13)	34.7	3.1	–
Raw liver	(5)	19.9	1.8	$p < .001$
Cooked liver	(7)	5.3	1.6	$p < .001$
Cooked, and peptic hydrolysed (2 hrs) liver	(7)	33.3	1.2	$p > .1$
Peptic hydrolysed (2 hrs) liver	(5)	12.8	1.9	$p < .001$
Peptic hydrolysed (24 hrs) liver	(7)	15.9	2.4	$p < .001$
Acid digested liver	(11)	14.1	1.8	$p < .001$
Peptic hydrolysed (2 hrs) then cooked liver	(6)	40.2	3.3	$p > .1$
Tryptic digested liver	(10)	11.8	1.5	$p < .001$

*Numbers of animals used

**The statistical methods used are given in appendix I

FIGURE 15

Absorption of Liver Fe following Cooking and Acid-peptic Digestion (20 μg Fe)



then acid-peptic digested for 2 hours absorption was 33.3 ± 1.2 per cent, and when initial peptic digestion was followed by cooking for 10 minutes, iron retention was 40.2 ± 3.3 per cent. Thus, following combined cooking and peptic digestion, absorption of liver iron was comparable with that from ferric chloride.

Table 12 and fig. 15 compare the absorption of iron from raw liver, and liver previously cooked, digested or both cooked and digested. The retention of Fe^{59} from raw liver homogenate was 19.9 ± 1.8 per cent. Cooking the liver homogenate markedly decreased liver iron retention by the rat. Mean retention of cooked liver homogenate was 5.3 ± 1.6 per cent, which was significantly less than iron absorption from raw liver ($p < .0001$). Neither peptic digestion for 2 or 24 hours, acid digestion alone or tryptic digestion significantly altered the mean iron retention when compared with the raw liver substrate. However when liver was cooked and acid peptic digested the iron retention was significantly greater ($p < .002$) than from raw liver. Similarly iron absorption from acid-peptic digested and then cooked liver was significantly increased ($p < .001$) when compared to raw liver homogenate.

In the third test group iron absorption was compared between total raw liver iron, haemoglobin haemolysate, crystalline haem and the ferritin component (table 13, fig. 16). Iron

TABLE 12. Comparison of iron absorption from raw liver and cooked and enzymatically digested liver

Test substance		% Fe^{59} retained Mean	± S.E.	**Significance level (compared to raw liver)
Raw liver	*(5)	19.9	1.8	-
Cooked liver	(7)	5.3	1.6	$p < .001$
Cooked and peptic hydrolysed (2 hrs) liver	(7)	33.3	1.2	$p < .002$
Peptic hydrolysed (2 hrs) liver	(5)	12.8	1.9	$p > .05$
Peptic hydrolysed (24 hrs) liver	(7)	15.9	2.4	$p > .1$
Acid digested liver	(11)	14.1	1.8	$p > .05$
Peptic hydrolysed (2 hrs) then cooked liver	(6)	40.2	3.3	$p < .001$
Tryptic digested liver	(10)	11.8	1.5	$p < .02$

*Numbers of animals used

**The statistical methods used are given in appendix 1.

TABLE 13. Comparison of iron absorption from total liver iron and the liver iron components

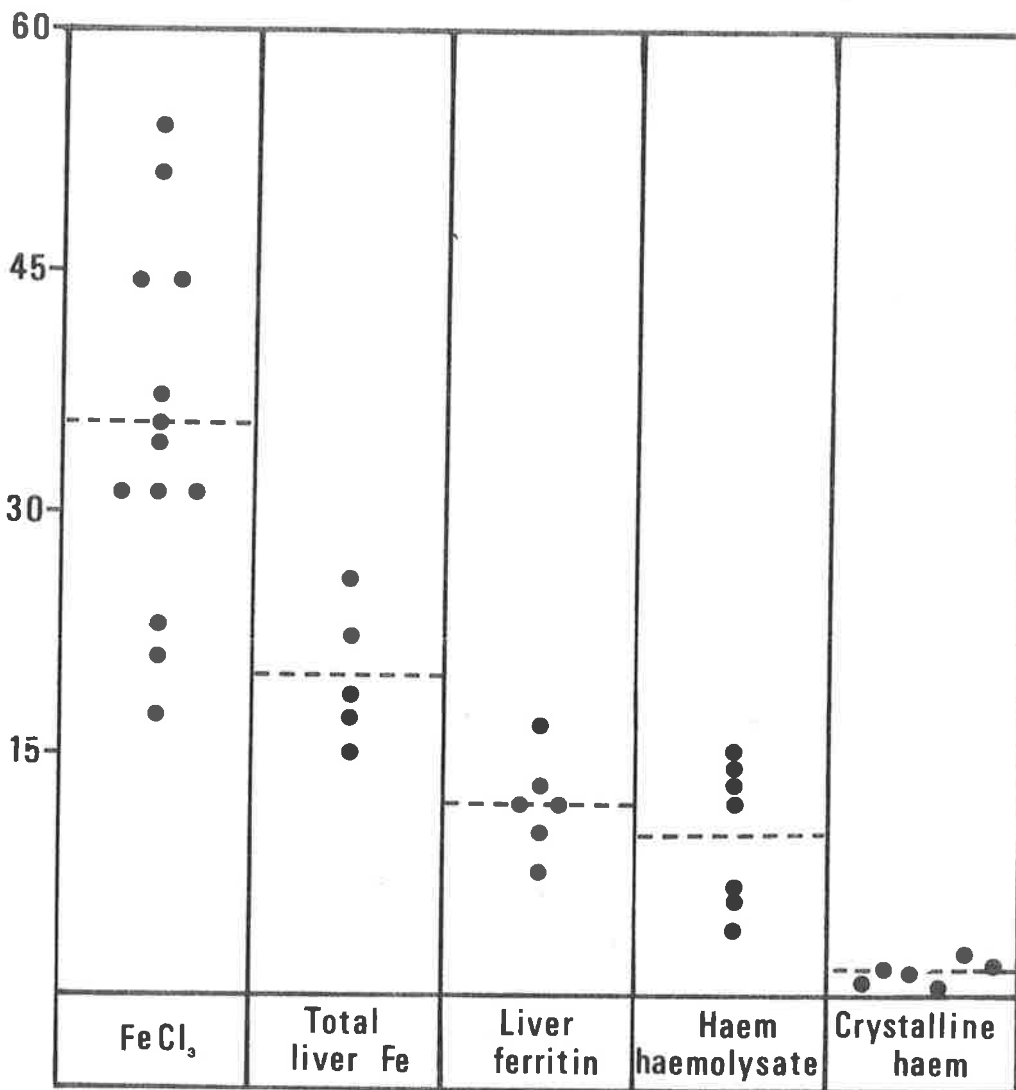
Test substance		% Fe^{59} retained Mean	± S.E.	**Significance level (compared to raw liver)
Raw liver	*(5)	19.9	1.8	-
Haemoglobin haemolysate	(7)	10.2	1.7	$p < .01$
Chemically prepared haem	(5)	2.2	0.4	$p < .001$
Ferritin	(6)	11.9	1.3	$p < .01$

*Numbers of animals used.

**The statistical methods used are given in appendix 1.

FIGURE 16

Absorption of Fe from Liver, and from Major Liver Fe Components (20 μg Fe)



retention in the four groups was 19.9 ± 1.8, 10.2 ± 1.7, 2.2 ± 0.4 and 11.9 ± 1.3 per cent respectively. Although the mean levels of iron absorption from ferritin and haemoglobin haemolysate were lower than from raw liver extract, the differences were statistically only of probable significance ($p < 0.01$). By contrast, chemically prepared haem was significantly ($p < .001$) less well absorbed than all the other liver components.

As with raw liver, cooking and acid peptic digestion of the ferritin extract greatly increased the amount of iron which was retained by the test animals. The mean retention of treated ferritin, by a group of 10 rats, 25.2 ± 2.3 per cent, was significantly greater than that from the untreated preparation (11.9 ± 1.3 per cent, $p < .001$).

The levels of iron absorption from small molecular weight components prepared from the dialysate of cooked and acid-peptic digested liver and from larger molecular weight components retained by the cellulose dialysis tubing are represented in table 14 and fig. 17. 38.5 ± 4.3 per cent of the iron was absorbed from the dialysable iron complexes compared with 12.2 ± 1.4 per cent from the larger molecular weight non-dialysable complexes. This difference was highly significant ($p < .001$). When compared to raw liver (table 14, fig. 17)

TABLE 14. Comparison of iron absorption from the dialysable, non-dialysable and total liver iron components.

Test substance		% Fe^{59} retained Mean	± S.E.	**Significance level
Dialysable Fe, from cooked and peptic hydrolysed liver	*(19)	38.5	4.3	-
Non-dialysable Fe, from cooked and peptic hydrolysed liver	(9)	12.2	1.4	$p < .001$
Raw liver	(5)	19.9	1.8	-
Dialysable Fe, from cooked and peptic hydrolysed liver	(19)	38.5	4.3	$p < .001$
Non-dialysable Fe, from cooked and peptic hydrolysed liver	(9)	12.2	1.4	$p < .01$

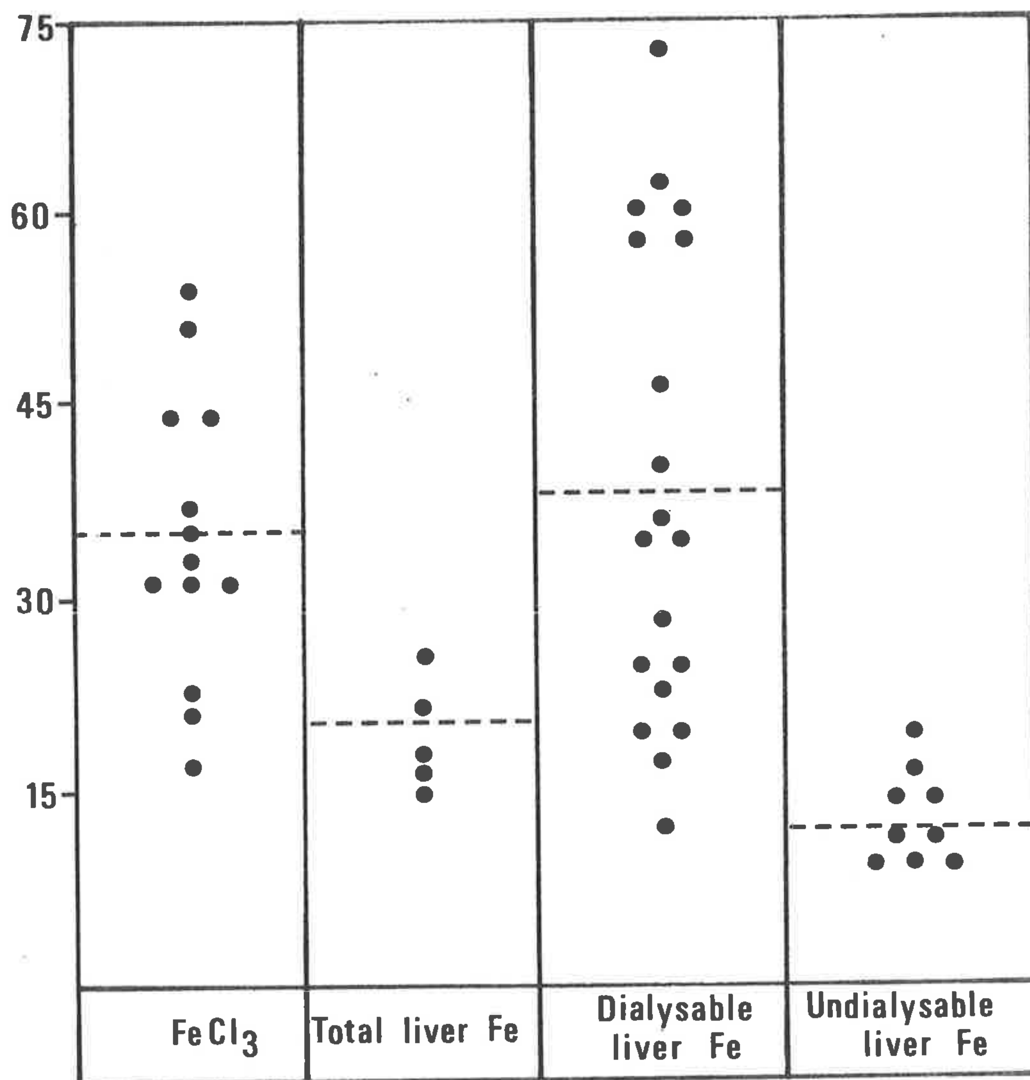
*Number of animals used.

**Statistical methods used are given in appendix 1.

FIGURE 17

Absorption of Dialysable and Non-dialysable Liver Fe Components

(20 µg Fe)



the absorption of the smaller fragments was significantly increased ($p < .001$) and that of the larger molecular weight fractions probably significantly decreased ($p < .01$). Furthermore, the iron absorption from smaller fragments was comparable with that from ferric chloride (fig. 17).

The effect of in vitro digestion on the absorption of liver iron (table 15) was tested by measuring the absorption by normal rats, in which the raw liver homogenate was instilled in the stomach, and by rats which had undergone pyloric ligation. In the latter group the test dose was injected directly into the duodenum. Mean retention of liver iron by normal rats was 19.9 ± 1.8 per cent, and this did not differ significantly from the 17.3 ± 2.8 per cent retained by the "gastrectomised" rats. The absorption of liver iron by the control group of animals which were sham "gastrectomised" was also comparable to that from raw liver substrate (table 15).

In the final test group, the effect of liver homogenate on iron absorption was studied (table 16). Rats given 20 μg ferric ^{59} chloride alone, retained 34.7 ± 3.1 per cent, whereas rats given 20 μg ferric ^{59} chloride and a quantity of liver containing 20 μg Fe retained 19.9 ± 3.7 per cent of the Fe^{59}. This difference is probably statistically significant ($p < .01$). In addition test animals receiving 40 μg of $ferric^{59}$ chloride

TABLE 15. Comparison of raw liver iron absorption by different groups of rats

Experimental animals		% Fe^{59} retained Mean	± S.E.	**Significance level
Normal rats	*(5)	19.9	1.8	-
"Gastrectomised" rats	(11)	17.3	2.8	$p > .1$
Sham "gastrectomised" rats	(7)	13.0	1.8	$p > .02$

*Number of animals used.

**The statistical methods used are given in appendix 1.

TABLE 16. Comparison of iron absorption from different test doses of iron.

Test substance		% Fe^{59} retained Mean	± S.E	**Significance level
$Fe^{59}Cl_3$ 20 µg	*(13)	34.7	3.1	-
$Fe^{59}Cl_3$ 20 µg + 20 µg raw liver Fe	(8)	19.9	3.7	$p < .01$
$Fe^{59}Cl_3$ 40 µg	(9)	22.3	3.3	$p < .01$
$Fe^{59}Cl_3$ 20 µg + 20 µg raw liver Fe	(8)	19.9	3.7	-
$Fe^{59}Cl_3$ 40 µg	(9)	22.3	3.3	$p > .1$

*Number of animals used.

**The statistical methods used are given in appendix 1.

retained 22.3 ± 3.3 per cent, which was also less than the percentage retained from the lower dose of inorganic iron. However, there was no significant difference ($p > 0.1$) in the absorption of iron from test doses with equivalent amounts of iron, regardless of the nature of the iron (table 16). Thus the absorption from 40 µg of ferric^{59} chloride was 22.3 ± 3.3 per cent and from a mixture of 20 µg ferric^{59} chloride and 20 µg raw liver iron was 19.9 ± 3.7 per cent.

COMMENT

The present study has shown that iron from guinea pig liver is well absorbed by normal rats, and that combined cooking and acid-peptic digestion of liver increase the iron retention to a level similar to that found with inorganic ferric chloride. This increase in liver iron absorption appears to be related to a reduction in the size of the liver iron components, and in particular to a release of iron from the micelles of the ferritin molecule. This is substantiated by the increased absorption of the dialysable liver iron fraction, which was shown to be predominantly non-haem iron, when compared with the non-dialysable iron fraction and total raw liver iron. The increase in the absorption of the dialysable liver iron component was such that it was comparable to that from ferric chloride. In addition the retention of Fe^{59} from radioactive

ferritin was increased twofold by subjecting the ferritin solution to cooking and peptic hydrolysis before administration to the test animals.

Many investigators (Steinkamp et al. 1955; Pirzio-Biroli et al. 1958; Callender & Warner, 1968) have reported that in man inorganic iron salts are more efficiently absorbed than food iron. In the guinea pig it is known too (Conrad et al. 1966b) that ferric chloride is absorbed more readily than haemoglobin iron. In our studies in rats, essentially the same pattern has emerged in that absorption of ferric chloride is significantly higher than absorption of raw liver iron. Peptic hydrolysis alone failed to increase iron retention. However, cooking plus acid-peptic digestion of the liver prior to administration achieved an appreciable increase in iron absorption such that retention approached the high levels found with ferric chloride.

The observations on the absorption of iron from liver in the raw, cooked and enzymatically digested states did not correlate absolutely with the physical and chemical changes induced by these procedures in the iron complexes. Acid digestion, and acid-peptic digestion did not increase iron absorption when compared with raw liver despite a great increase in the dialysable small molecular weight components and a degradation of the high

and intermediate molecular weight iron fractions to a single low molecular weight iron fraction as assessed by sephadex G200 fractionation. It was considered possible that the liver homogenate was digested sufficiently in vivo during the gastric phase to mimic the effect, on the size of the complexes, of in vitro hydrolysis. However raw liver was absorbed readily even when the stomach was by-passed and liver iron injected directly into the duodenum following pyloric ligation. Murray & Stein (1967) have reported likewise that the stomach, in rats, does not appear critical in normal iron absorption. Tryptic digestion within the duodenum remains a possible source of low molecular weight iron complexes although cooking and tryptic digestion in vitro both produced a reduction in liver iron absorption.

Both these procedures reduced the soluble liver iron component and resulted in no increase in the low molecular weight iron complexes as assessed by sephadex G200 filtration. However tryptic digestion increased dialysable liver iron whereas cooking had no such effect. It would appear that in spite of the reduction of the soluble iron following tryptic digestion some iron is released in a readily available form. The sum of these two effects may well be that the iron in tryptic digested liver homogenate is absorbed equally well as that from preparations with a higher level of soluble and

dialysable iron.

The results following cooking were contrary to expectations. It was anticipated that the iron compounds would be denatured by heat and so be made more susceptible to digestion by the rats' intestinal secretions. Certainly the in vitro peptic hydrolysis of cooked liver homogenate resulted in a dramatic increase in the retention of Fe^{59}. The extent of the increase in absorption was such that it was equivalent to the absorption of iron from a similar test dose of ferric chloride. Therefore it is obvious that in vitro cooking and peptic hydrolysis produces enhancement of the absorption of iron by the rat. However, the role of the intestinal secretions of the rat in iron absorption remains dubious.

Iron from raw homogenised liver was better absorbed than iron from purified extracts of haemoglobin and ferritin. This suggested that the liver may contain a specific substance or a number of substances which enhance absorption of the individual food-iron components. Such an effect may be due to liver amino acids as it has been previously demonstrated (Kroe et al. 1966; Layrisse et al. 1968) that certain amino acids enhance iron absorption. Furthermore, Conrad and his associates (1967b) have shown conclusively that haem iron is better absorbed in the presence of globin degradation products than as purified haem. The poor absorption of crystalline haem in our

study further confirms this. No such enhancing effect of liver amino acids was shown when liver homogenate was administered with $Fe^{59}Cl_3$. Indeed percentage absorption of the total iron fell appreciably. This fall was attributed to the higher iron content of the combined test dose, rather than any liver component, since there was no significant difference in absorption when compared with an equivalent dose of ferric chloride.

The exact mechanism by which cooking and digestive procedures influence liver iron absorption is not clearly elucidated. The observation that the dialysable iron was more readily absorbed than the non-dialysable and total liver iron tends to indicate that the size of the iron complexes is an important factor in determining the degree of absorption. Furthermore, cooking and acid-peptic digestion of ferritin released iron from the molecule, as demonstrated by dialysis, and produced a two-fold increase in ferritin iron absorption, thus providing more evidence to support the theory that the smaller sized iron species are more readily absorbed.

At the same time it is impossible to exclude the action of some other factors which may be involved. Apart from the release of iron and the degradation of the iron compounds into smaller fractions, a breakdown of the liver proteins to peptides

and amino acids would be expected to occur following cooking and enzymatic digestion. It has already been stated that the breakdown products of proteins affect the absorption of both dietary and inorganic iron. Therefore the role of cooking and enzymatic digestion may be directed towards the release of these products and enhance the iron absorption in this way, even though such an effect was not demonstrated in this study. However the most feasible role would be that of an effect on the iron compounds in addition to the breakdown of the liver proteins.

CHAPTER VI

LIVER IRON ABSORPTION STUDIES IN HUMAN SUBJECTS

INTRODUCTION

The initial tests of the absorption of food iron were carried out in rats. These preliminary studies suggested that cooking alone, and cooking plus enzymic digestion affected iron absorption. However it is difficult always to extrapolate the results of animal experiments to the situation which exists in human subjects. For this reason certain critical absorption studies were repeated in man utilising the facilities of the whole body monitor.

The substances selected for the absorption studies were cooked liver and liver which had been cooked and then subjected to in vitro peptic hydrolysis. These preparations were selected for two reasons. Firstly, it was considered that such preliminary processing broadly resembled the normal situation, in which meat foodstuffs were cooked and then digested by exposure to intestinal secretions. Secondly, the preliminary rat experiments had demonstrated that the lowest value for iron absorption was found from cooked liver and the highest value from liver which had been cooked and peptic hydrolysed. In addition the investigations into the physical nature of the iron complexes in these two liver preparations revealed the greatest differences between the iron species present.

MATERIALS AND METHODS

Test Subjects

Radioiron absorption tests were performed on six haematologically normal male adults. Their ages ranged from 19 to 30 years. All were of normal body build and weighed from 68.2 to 87.3 kg. The haematological status of the subjects was assessed by the Haematology Division of the Institute of Medical and Veterinary Science, Adelaide. The haemoglobin level was measured by estimating cyan-methaemoglobin with the Coulter S counter (Pinkerton, Spence, Ogilvie, Ronald, Marchant & Ray, 1970). Serum iron concentration, total iron binding capacity and latent iron binding capacity were determined by a modified method of Schade, Omara, Reinhart & Miller (1954).

Liver Preparation

Guinea pig liver iron stores were labelled with Fe^{59} as previously described in chapter IV. The animals were killed by placing them in a desiccator containing 20 ml of anaesthetic ether (BP). This method met the requirements of the local health authorities. The liver was removed and washed with deionised water, until the water remained clear. Samples of the livers used were examined by light microscopy and by culture to exclude contamination by pathogenic organisms. Culture media used included blood agar, Salmonella-shigella-

agar and selenite F mannitol enriched medium. The liver was declared fit for human consumption provided that the cultures were negative for Salmonella and Pasteurella pestis and light microscopy of sections after Ziehl-Neelsen's staining was negative for Mycobacteria. The bacteriological scrutiny of the guinea pig liver slices was performed by the Division of Bacteriology, Institute of Medical and Veterinary Science, Adelaide.

Test Dose

An equal mixture of liver from three guinea pigs was used. The iron content of each liver was determined in triplicate by atomic absorption spectrophotometry following wet ashing of the samples as described in chapter III. The iron concentration of each of the three livers was found to be 190 μg per gram of liver. Each test dose contained 1 mg of iron, this being equivalent to 5.3 g of liver. 5.3 g aliquots of the radioactive liver were homogenised for 30 seconds, and then either cooked alone or both cooked and acid-peptic digested for 2 hours, as described in chapter III. The final volume of each administered test preparation was made up to 20 ml with deionised water.

All subjects were fasted for 12 hours prior to receiving the test substance and for a further 2 hours following ingestion. The test dose was administered orally, and it

was followed by 70 ml of deionised water. Each subject received both the cooked liver homogenate and the cooked plus acid-peptic digested homogenate. The administration of the two test preparations was randomised.

Whole Body Counting

Absorption of Fe^{59} from the test doses of liver was measured in a whole body liquid scintillation detector. Whole body radioactivity was measured before, 5 minutes after, and 14 days after ingestion of the liver preparations. At the end of this period all unabsorbed radioactive iron has been excreted (Price, Cohn, Wasserman, Reizenstein & Cronkite, 1962). This includes the iron retained by mucosal cells, but not absorbed into the body (Powell & Wilson, 1970) and which is lost by exfoliation of mucosal cells.

During the counting procedure the subject lay firmly supported on a couch, around which were four sodium iodide (thallium activated) crystals measuring 11.25 cm in diameter and 5 cm in depth. These scintillation detectors were motor driven to scan the full length of the subject with end pauses. The crystal geometry, scanning speed and end pauses were designed to make counting efficiency independent of the source geometry, so that the "100 per cent value" could be determined immediately after ingestion

of the radioactive substance. The total counting time was 15 minutes. Background radioactivity was minimised by housing the assembly in a 6 inch thick steel castle. Gamma emission of the radioactive iron^{59} was measured with a single channel pulse height analyser set to admit gamma rays of energy 0.8 to 1.7 MeV. The reference standard was prepared for each experiment by measuring an aliquot of the test dose into a 500 ml plastic bottle filled with water. This was counted before and after counting the subjects at each visit(S). This enabled correction for daily variations in overall counting efficiency and the decay of Fe^{59}.

On the first visit the subject was counted prior to administration of the labelled liver. This first counting was taken as the measure of the natural body radioactivity (C_0). After oral administration of the radioisotope the whole body activity was measured again (C_1) to establish the 100% value. Fourteen days later the subject was recounted to determine the percentage Fe^{59} retained (C_{14}). The percentage Fe^{59} retained was obtained by the formula expressed below

$$\% \ Fe^{59} \text{ retained} = \frac{C_{14} - C_0}{C_1 - C_0} \times \frac{S_0}{S_{14}} \times 100$$

After the measure of (C_{14}), the subject was given the second test dose of radioactive liver preparation and counted again (C_{14a}). The retention of radioiron from the second test dose was determined by recounting 14 days later again (C_{28}). The formula for Fe^{59} retained in the second study is

$$\% \ Fe^{59} \text{ retained} = \frac{C_{28} - C_{14}}{C_{14a} - C_{14}} \times \frac{S_{14}}{S_{28}} \times 100$$

where C = total body count, the numeral indicating the day of counting

S = standard count, the numeral indicating the day of counting

RESULTS

All of the subjects had a normal haemoglobin value and normal levels of iron in the serum (table 17).

The results of the absorption studies are detailed in table 18 and fig. 18. The mean absorption of Fe^{59} from cooked liver was 5.9 per cent, and 11.4 per cent from liver both cooked and peptic hydrolysed. Statistically there was no significant difference in iron absorption from the two liver preparations. However all of the subjects with the exception of B.M., absorbed more iron from liver which had been cooked and peptic hydrolysed than from cooked liver. Two subjects, G.P. and K.C., retained more than twice the

TABLE 17. Haematological status of the male volunteers used in the absorption study

	Hb g/100 ml	Serum iron µg/100 ml	L.I.B.C. µg /100 ml	T.I.B.C. µg/100 ml	% saturation
A.C.	14.6	185	95	180	66
B.M.	15.5	130	120	300	43
G.P.	13.4	185	135	320	58
K.C.	15.3	120	185	305	39
G.W.	15.3	145	200	345	42
J.W.	16.2	115	165	380	41

TABLE 18. Comparison of iron absorption from cooked liver, and cooked and peptic hydrolysed liver

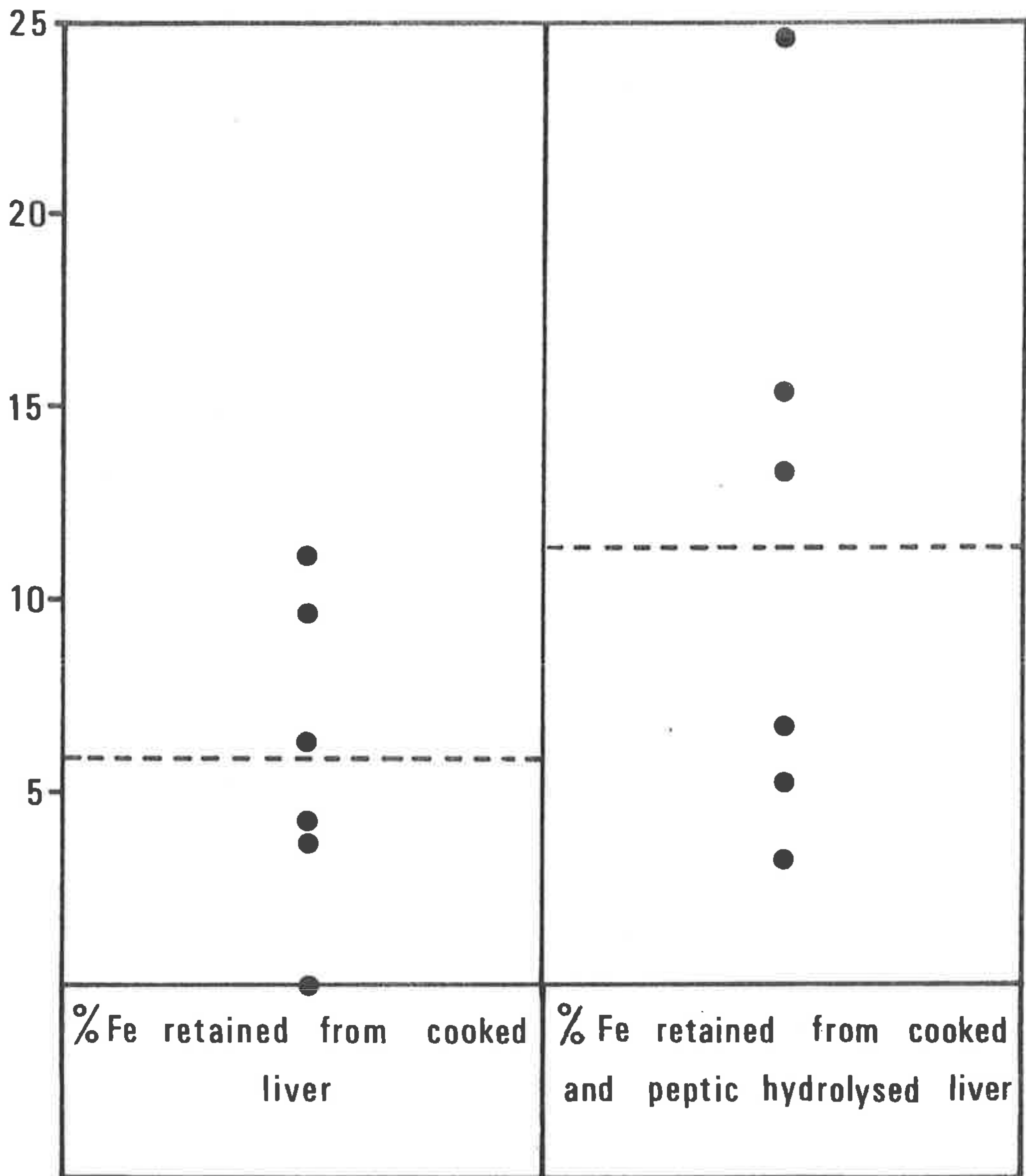
Subject	% Fe retained from cooked liver	% Fe retained from cooked and peptic hydrolysed liver
A.C.	4.2 (2)	6.9 (1)
B.M.	9.8 (1)	3.2 (2)
G.P.	6.3 (1)	24.7 (2)
K.C.	0 (2)	13.3 (1)
G.W.	3.9 (2)	5.1 (1)
J.W.	11.1 (1)	15.4 (2)
Mean ± SE	5.9 ± 2.4	11.4 ± 3.0

(1) First test dose ingested

(2) Second test dose ingested

FIGURE 18

Absorption of Liver Fe by Human Subjects (1·0 mg Fe)



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amount of Fe^{59} from cooked and peptic hydrolysed liver when compared to the Fe^{59} retention from liver cooked alone.

COMMENT

In the group of human subjects studied there was no significant difference between the mean absorption of iron from cooked liver and from liver which had been cooked and then peptic digested. This was in striking contrast to the experimental situation in the rat when a six-fold difference was found (chapter V). This inconsistency highlights the difficulty of extrapolating results from small laboratory animals to the human situation. However, with the exception of one subject (B.M.), paired studies did show higher percentage absorption of cooked and peptic hydrolysed liver when compared with a control of cooked liver. Furthermore, in two subjects (G.P. and K.C.), there was a very marked increase in absorption from peptic hydrolysed liver. Thus the trend is similar both in human subjects and in rats.

A dose of 1 mg of liver iron was chosen because of difficulties in obtaining sufficiently large quantities of isotopically labelled guinea pig liver. The 1 mg dose is somewhat less than that used in other investigations of dietary iron absorption, where iron doses have ranged from 3 to 17 mg (Moore & Dubach, 1951; Steinkamp et al. 1955;

Kuhn et al. 1968). However, a test dose of 1 mg of dietary iron has been used for absorption studies in man by other workers (Moore & Dubach, 1951; Layrisse et al. 1968).

The mean absorption of cooked guinea pig liver approached 6 per cent, which was similar to that in previous studies. However a direct comparison between series is not possible since in all cases different quantities of iron were used and the total numbers are small. Moore & Dubach (1951), using labelled chicken liver baked into patties prior to administration, calculated that 4.2 per cent of a 16.6 mg test dose was absorbed; 5.9 per cent of 6.2 mg; and 9.4 per cent of 7.2 mg. Each of these figures was derived from only one observation. Walsh et al. (1955) measured radioactive iron retention from in vivo labelled sheep's liver both in a healthy male blood donor and in a male with haemochromatosis. The normal male retained 8.0 per cent from a test dose of 51.0 mg of sheep liver iron; the haemochromatotic subject retained 20.8 per cent from a test dose of 40.0 mg.

The absorption of iron from cooked liver was in general lower than that from liver both cooked and peptic hydrolysed in vitro. However these differences were not statistically significant. It is possible that the cooked liver was sufficiently altered by peptic digestion in vivo, following

ingestion, so that the final form of the test substances presented at the intestinal absorptive site was very similar. Thus it can be postulated that the differences between the two test substances were masked by in vivo gastric digestion. If this is so then it must also be postulated that the human digestive processes have an effect different from that of the rat, since absorption of iron from cooked liver, by the rat, remained low despite exposure to gastric contents during digestion.

Absorption of iron from raw liver by man has not been determined. Therefore the effect of cooking and digestion, whether it occurs in vivo or in vitro, cannot be definitely stated. However in view of the increase in low molecular weight iron complexes produced by these procedures in vitro (chapter IV) it is reasonable to postulate that cooking plus peptic digestion act to release iron from high molecular weight conjugates thus making it more readily available for absorption.

The possible effects of other factors, particularly protein breakdown products, on the absorption of iron has not been excluded. The role of peptides and amino acids in iron absorption has been discussed in the previous chapter.

CHAPTER VII

STUDIES ON WHEAT IRON

INTRODUCTION

Grain products contribute approximately one-fifth of the dietary iron intake in the western world (Food and Nutrition Board, 1970) and a much higher proportion in the under-developed countries (Patwardhan, 1962). In South America black beans constitute the bulk of the dietary food intake in the lower socio-economic groups (Martinez-Torres & Layrisse, 1970). A recent study of nutritional anaemia in this area (Cook, Alvarado, Gutnisky, Jamra, Labardini, Layrisse, Linares, Loria, Maspes, Restrepo, Reynafarje, Sanchez-Medal, Velez & Viteri, (1971) showed that iron deficiency was the main causative factor. The authors postulated that the iron deficiency probably arose from malabsorption of dietary iron, and that low dietary protein intake, reflected by low serum albumin levels, was probably a further contributing factor.

The most widely consumed cereal product is rice; however, wheat and its products form a staple part of the diet in many parts of the world. Furthermore, flour is frequently used as the vehicle for dietary iron enrichment programmes (U.S. Council on Food & Nutrition, 1968; Steinkamp et al. 1955). In order to further examine the nutritive value of a cereal product it was decided to document the total iron content of wheat, to determine the size

distribution of the iron complexes and to study the effects of cooking and enzymatic digestion on the wheat grain iron.

MATERIALS AND METHODS

Wheat

Gabo variety wheat was used for all the experiments. The strain used was obtained from the fields of the Waite Agricultural Research Institute (University of Adelaide). Stalks with growing ears of grain were cut 28 days after flowering and immediately placed in water. At this stage of ripening, starch formation occurs at the rate of 1 mg of starch per wheat grain per day (Jenner, 1970). This process does not depend on, nor require, sunlight (Jenner, 1970). In the laboratory the stalks were cut diagonally under water, and were then transferred to 2 per cent sucrose solution (pH 6). This ensured that the ears of grain continued to mature. The stalks were divided into bundles of 5, and each bundle was kept in a 1 inch diameter test tube containing approximately 30 ml of the 2 per cent sucrose solution. On alternate days the stalks were cut diagonally under water; the sucrose solution was discarded and replaced with fresh solution. Maturation was allowed to proceed for 10 days, at which time uptake of the sucrose solution by the stalks had ceased.

On cessation of maturation of the wheat, the ears of grain were dried in an oven at 80°C for one hour. Following drying, the ears were threshed, by rubbing between two sheets of filter paper, to remove the husks. The threshed grain was then further dried in an oven at 60°C overnight.

Total Iron Estimation

The dried wheat was weighed in five separate 1 g portions and these were wet ashed using a mixture of concentrated nitric, perchloric and sulphuric acids in the volume ratio 3:1:1. The wet ashing procedure has been detailed in chapter III. The iron concentration was then estimated directly using the atomic absorption spectrophotometer (Unicam SP90A). The technique of atomic absorption spectroscopy was identical to that described in chapter III. A mean value of iron concentration was then determined from the five random specimens.

Incorporation of Radioactive Iron (Fe^{59}) into the wheat grain

Ten μCi of radioactive ferric citrate (S.A. 10 μCi/μg Fe) were added to the first 2% sucrose solution. A further 5 μCi of ferric citrate were added when the sucrose solution was replaced on alternate days. A total of 30 μCi of Fe^{59} was added to each test tube. Incorporation was allowed to proceed for 10 days, at which time maturation of the ears of grain had ceased.

Distribution of Radioactive Iron within the Wheat Grain

Three pairs of whole unthreshed wheat grains were counted in the Philips gamma counter. Following the initial counting, the pairs of grain were threshed and the husks separated from the kernels. The radioactivity of the husks and kernels was then measured separately. The initial count represented the total radioactivity within the unthreshed grain and the distribution of the radioactivity in the husks and kernels was expressed as a percentage of this. The Fe^{59} gamma radiation was measured using an automatic well-type sodium iodide crystal scintillation counter (Philips type PW 4003). The counting efficiency was greater than 25 per cent and the background approximated 3 counts per second. The whole grains, husks, and kernels were placed in the tubes for counting in their solid state.

Preparation of Wheat Homogenate

One gram of the dried and threshed wheat was weighed, and then transferred to an iron-free 50 ml polythene test tube. To this, 4 to 5 ml of deionised water was added and the resultant mixture was homogenised with the Ultra-Turrax homogeniser for 30 seconds. The homogeniser was washed twice with deionised water, which was added to the initial homogenate. The final volume was made up to 10 ml with deionised water.

Physical Characterisation of Iron in Wheat

Physical characterisation of the iron in wheat was performed using the methods of centrifugation, dialysis and sephadex gel filtration.

Centrifugation

Homogenates of wheat were centrifuged at 10,000 g for 1 hour. Following centrifugation the supernatant was decanted into a 15 ml graduated glass tube and the volume adjusted to 10 ml with deionised water. The iron content was then determined directly by atomic absorption spectrophotometry.

The precipitate was wet ashed using the concentrated nitric, sulphuric and perchloric acid mixture as described in chapter III. The volume of the digest solution was adjusted to 25 ml and the iron concentration determined by atomic absorption spectrophotometry.

The soluble and precipitated iron was estimated in raw, cooked, acid-peptic digested, acid digested and cooked and acid-peptic digested wheat homogenate. All estimations were performed in triplicate.

Dialysis

Aliquots of wheat homogenate were dialysed against both deionised water and 0.16 M hydrochloric acid. The method was identical to that described in chapter IV. Ten ml of

Fe^{59} labelled wheat homogenate was placed within the cellulose dialysis tubing (Visking). The initial radioactivity was determined by duplicate counting in the large volume counter (Nuclear Data A.E.I.) described in chapter III. Dialysis against 300 ml of deionised water and an identical volume of 0.16 M hydrochloric acid was allowed to proceed for 24 hours at 4°C. After dialysis the homogenate was recounted in duplicate, and the dialysable iron was calculated by the difference between the initial activity and that remaining in the dialysis bag after 24 hours. Dialysable iron in raw, cooked, enzymatically and acid digested wheat homogenate was estimated. All experiments were performed in triplicate.

Sephadex Gel Filtration

Radioactive wheat homogenate in the raw, cooked and enzymatically digested states was centrifuged at 10,000 g for 1 hour. The supernatant was decanted and 4 ml aliquots were applied on sephadex gel columns. Fractionation of the wheat iron by sephadex gel filtration was possible only with those samples which had sufficient levels of iron in the supernatant to enable accurate detection. Thus even though fractionation of the raw and cooked wheat homogenate was attempted the results were not meaningful, since the further dilution of the small amount of soluble iron present in these preparations produced by the filtration method precluded

accurate detection of iron within the eluted fractions.

The method of sephadex gel filtration employed was the same as that described in chapter IV. Sephadex G200 (Pharmacia) was packed in a type K25/45 column and eluted by upward flow. The eluting buffer was 0.9% NaCl-0.01 M tris pH 8. The effluent from the column was collected as 5 ml fractions in an automatic fraction collector.

The fractions eluted from the sephadex G200 columns were monitored for radioactive iron, protein, and carbohydrate in a manner identical to that previously described in chapter IV.

Cooking and Digestive Procedures

Cooking. The Fe^{59} labelled wheat homogenate was heated at 100°C for 10 minutes with continuous stirring.

Acid-peptic digestion. Ten ml of the wheat homogenate was incubated at 37°C for 2 hours at pH 1.6 with pepsin. The procedure was performed in an identical manner to that described in chapter III, the only difference being the quantity of pepsin used. 12 to 20% of the wheat grain is made up of protein (Aykroyd & Doughty, 1970): for this study study it was assumed that wheat contained 20 per cent protein. Therefore the quantity of pepsin used was 1% w/w of the calculated protein in 1 g of wheat.

Acid digestion. The same procedure as described in chapter IV was employed, the pH of the wheat homogenate being adjusted to pH 1.6 with hydrochloric acid and the resultant solution incubated at 37°C for 2 hours.

Absorption of Wheat Iron

The rat was used as the experimental animal for the absorption studies. The animals used, and the care of the animals was identical to that described in chapter V. Furthermore, the method of administration of the test substance was identical to that described in chapter V, in that the rats were anaesthetised lightly with ether and then intubated with a 14 gauge polythene tube. The test substance was administered into the stomach through the polythene tube.

Test Substances

The test substance administered to all experimental animals contained 10 μg Fe, and the volume of the test dose in all cases was 1.5 ml; this was always followed by 0.25 ml of deionised water. The total amount of iron given was half that used in the absorption experiments described in chapter V, this reduction in the iron dose being necessary due to the low iron content of the wheat. The low levels of iron precluded the preparation of a wheat homogenate containing

20 μg Fe per 1.5 ml of such a consistency that it could be administered through the polythene tube. However the reduction of the test dose of iron and thereby the reduction of the wheat administered enabled the test substances to be given in the manner previously described.

(1) Raw wheat. 4.6 g of Fe^{59} labelled wheat was homogenised for 30 seconds in 10 ml of deionised water. The final volume of the homogenate was made up to 18 ml. The iron concentration of the final homogenate was 10 μg Fe per 1.5 ml.

(2) Cooked wheat. 6.9 g of radioactive wheat was homogenised for 30 seconds. The volume of the homogenate was made up to 27 ml with deionised water. The resultant mixture was heated at 100°C for 10 minutes with continuous stirring. The volume was then readjusted to 27 ml with deionised water so that the iron concentration was 10 μg per 1.5 ml of the homogenate.

(3) Wheat, acid-peptic digested. 4.6 g of Fe^{59} labelled wheat was homogenised, and the volume made up to 15 ml. This was then acid-peptic digested for 2 hours at 37°C as described earlier in this chapter. The enzymatic reaction was stopped by elevating the temperature of the digest mixture to 100°C for 60 seconds. The final volume of the solution

was adjusted to 18 ml. The iron concentration of the final solution was 10 μg per 1.5 ml.

Wheat, cooked and acid-peptic digested. Radioactive wheat homogenate was prepared as described earlier. The homogenate was then cooked, and acid-peptic digested for 2 hours. The iron concentration of the final wheat preparation was 10 μg Fe per 1.5 ml.

RESULTS

Total Iron Content

The iron content of the wheat ranged from 24.6 μg Fe per g of wheat to 28.0 μg Fe per g of wheat. The mean of the five determinations was 26.6 ± 1.5 μg Fe (mean ± 1 S.D.).

Incorporation of Radioactive Iron into Wheat

Under the conditions employed five per cent of the radioactivity added to the media was incorporated into the wheat. Sixty-seven per cent of the total activity taken up by the wheat was within the actual grain, and thirty-three per cent was in the husks.

Physical Characterisation of Iron in Wheat

Centrifugation. Virtually all of the iron in the wheat homogenate was present in the precipitate after centrifugation at 10,000 g for 1 hour. The soluble wheat iron, or the iron

in the supernatant, comprised only 6 per cent of the total wheat iron (table 19), However a marked increase in the soluble iron was demonstrated following enzymatic and acid digestion, and cooking in conjunction with enzymatic digestion (table 19). Thus after 2 hours of peptic hydrolysis of the wheat homogenate the soluble iron was increased to almost half of the total wheat iron. Acid digestion increased the soluble iron to approximately 40 per cent of the total wheat iron. Cooking in conjunction with peptic hydrolysis increased the level of soluble iron to approximately one-third of the total iron. Cooking alone had no effect on the soluble iron content of the wheat homogenate (table 19

Dialysis

Dialysis of raw wheat against deionised water resulted in a loss of 1 per cent of the radioactive iron (table 20). The dialysable fraction increased to 12.1 per cent when dialysed against 0.16 M hydrochloric acid. After cooking alone, 0.3 per cent of the activity was lost when the wheat homogenate was dialysed against deionised water, and this rose to 4 per cent when dialysed against dilute hydrochloric acid.

Acid digestion, acid peptic digestion and cooking in conjunction with the latter produced an approximately two-

TABLE 19. Centrifugation of wheat homogenate at 10,000 g for 1 hour

Sample	Fe in supernatant *expressed as % of total Fe	Fe in precipitate *expressed as % of total Fe
Wheat, raw	6	94
Wheat, cooked	nil	100
Wheat, acid-peptic digested for 2 hours	45	55
Wheat, acid digested for 2 hours	39	61
Wheat, cooked and acid-peptic digested for 2 hours	23	77

*Mean values obtained after three determinations in each group

TABLE 20. Dialysis of wheat homogenate against H_2O and 0.16 M HCl

Sample	*Dialysable wheat Fe, as % of total Fe	
	Dialysed against H_2O	Dialysed against HCl
Wheat, raw	1	12.1
Wheat, cooked	0.3	4
Wheat, peptic hydrolysed for 2 hours	4	6.2
Wheat, acid digested for 2 hours	3	5
Wheat, cooked and peptic hydrolysed for 2 hours	2	5

*Mean values obtained after three determinations in each group

fold increase in the dialysable fraction when dialysed against deionised water. However, when dialysed against dilute hydrochloric acid a reduction in the dialysable iron component was demonstrated when compared with raw wheat (table 20).

Sephadex Gel Filtration

The iron in the supernatant of the peptic hydrolysed wheat homogenate separated into one fraction which lay in the low molecular weight region, and the peak of radioactivity was associated with a small protein peak (fig. 19). A similar elution pattern was produced following fractionation of acid digested wheat homogenate, with the exception that a small intermediate molecular weight radioactive protein peak was also demonstrated (fig. 20). Cooking in conjunction with acid-peptic digestion of the wheat homogenate, resulted in a different elution pattern (fig. 21). Two radioactive protein fractions emerged, one being in the high molecular weight region and the other in the low molecular weight area.

Absorption of Wheat Iron

The absorption of wheat iron is detailed in table 21. The rats retained a mean of 19.9 per cent of the iron from uncooked wheat; 30.5 per cent from cooked wheat; 19.6 per

Fig. 19: Gel filtration of acid-peptic digested wheat by sephadex G200.

The sample was 4 ml of supernatant obtained by centrifugation of acid-peptic digested wheat homogenate for 1 hour at 10,000 g.

The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all measured values have been included.

▽------▽ radioactivity

●———● absorbance 280 mμ

FIGURE 19

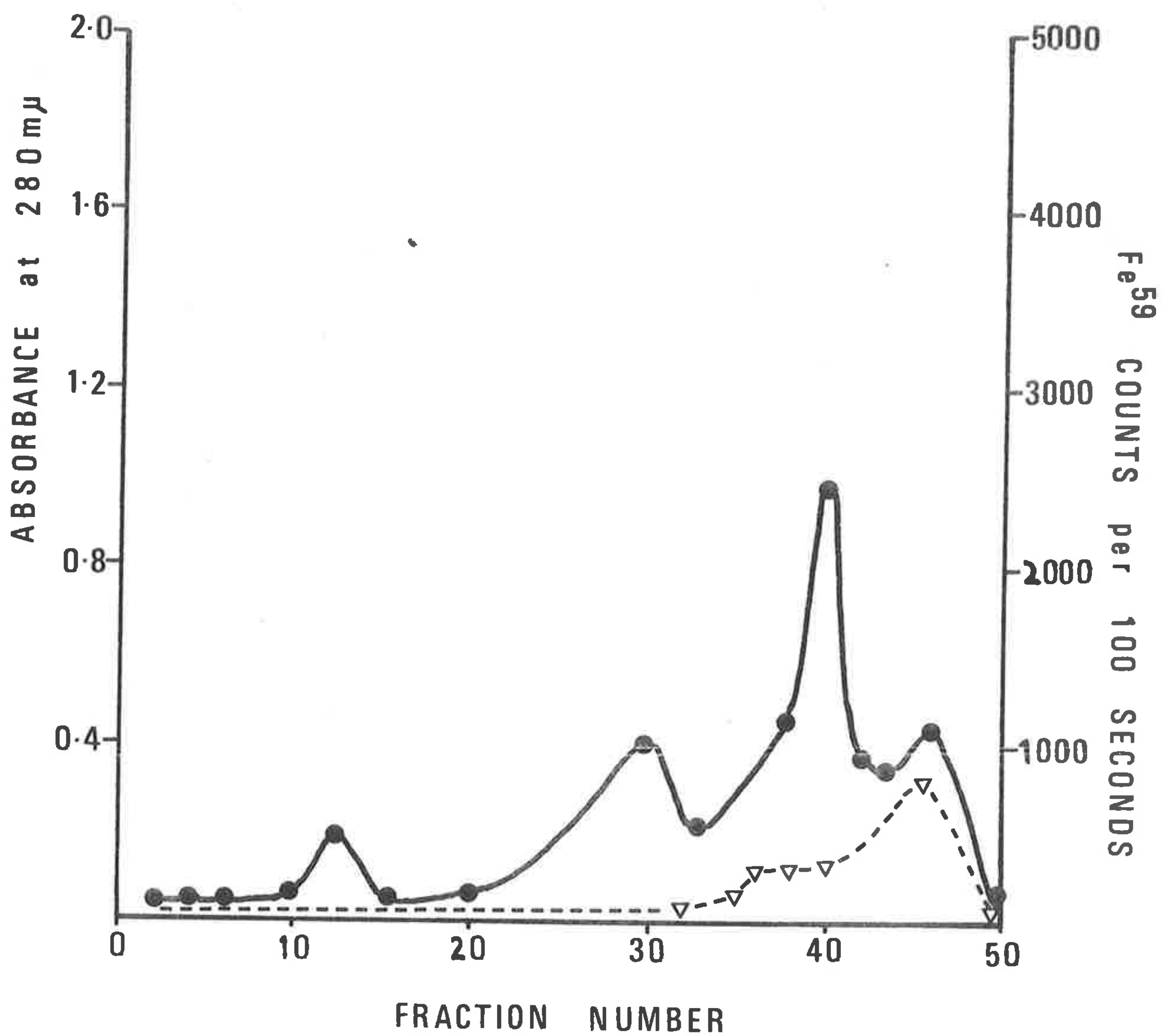


Fig. 20: Gel filtration of acid digested wheat by sephadex G200.

The sample was 4 ml of supernatant obtained by centrifugation of acid digested wheat homogenate for 1 hour at 10,000 g.

The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all values measured have been included.

∇------∇ radioactivity

●———● absorbance 280 mμ

FIGURE 20

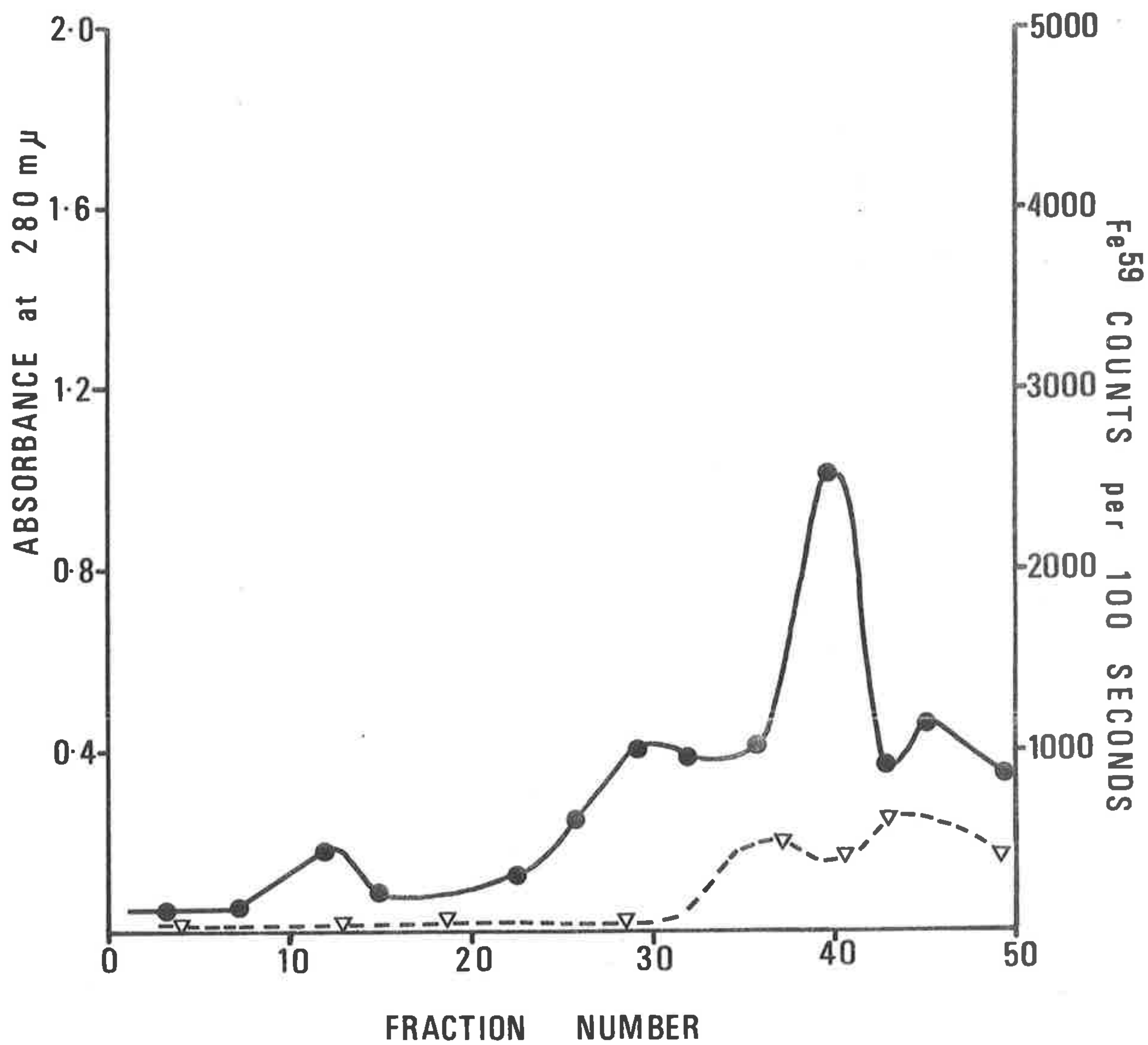


Fig. 21: Gel filtration of cooked and acid-peptic digested wheat by sephadex G200.

The sample was 4 ml of supernatant obtained by centrifugation of cooked and acid-peptic digested wheat homogenate for 1 hour at 10,000 g.

The eluant was 0.9% NaCl-0.01 M tris pH 8.

For simplicity not all values measured have been included.

▽------▽ radioactivity

o———o absorbance 280 mμ

FIGURE 21

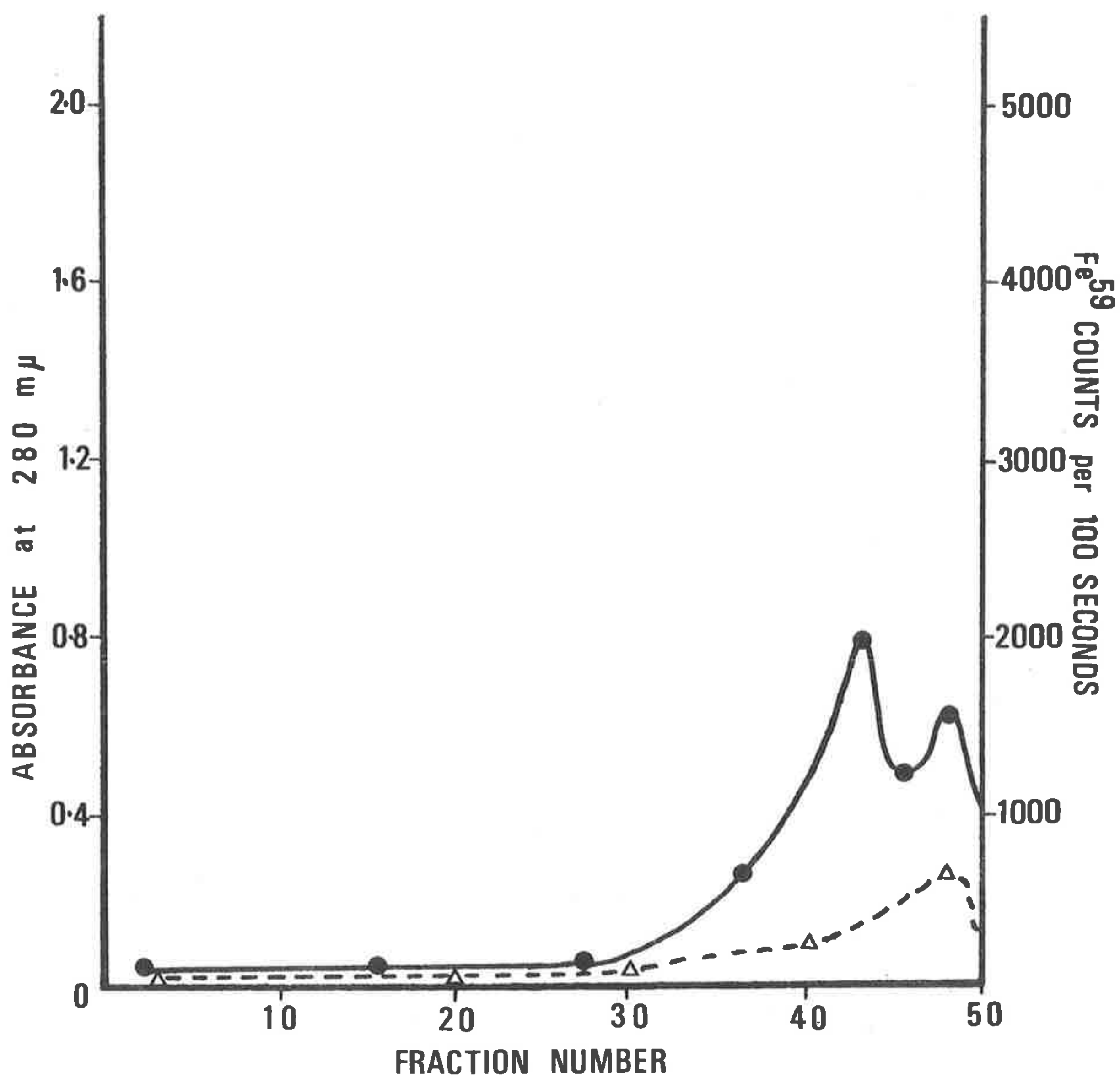


TABLE 21. Abosrption of wheat iron by rats

Test dose		% retention of Fe^{59} Mean ± S.E.
Whole wheat	*(6)	19.9 ± 1.7
Wheat, cooked	(6)	30.5 ± 3.7
Wheat, acid-peptic digested for 2 hours	(6)	19.4 ± 2.7
Wheat, cooked and acid-peptic digested for 2 hours	(8)	14.2 ± 2.5

*Number of experimental animals

per cent from peptic hydrolysed wheat and 14.2 per cent from cooked and peptic hydrolysed wheat. Although cooking produced some increase in wheat iron absorption when compared with raw wheat, this difference was probably not significant ($p < 0.05 > 0.02$). Cooking plus peptic hydrolysis, by contrast, appeared to reduce iron retention when compared to raw wheat. This difference also was of doubtful significance.

COMMENT

The method devised for the isotopic labelling of wheat iron proved to be simple and effective in that sufficient radioactivity was incorporated into the growing wheat to allow fractionation and characterisation of the wheat iron complexes. However only about 5 per cent of the iron added to the incubation system was incorporated into the grain due to the wastage associated with the regular changing of the sucrose medium. For this reason it is only feasible where there is easy access to radioactive isotopes of iron.

Another shortcoming of this method of labelling the wheat iron stores is that the maturation of the grain in the laboratory environment probably does not proceed to the same end-point as in the fields. The starch formation has been shown to continue unimpeded (Jenner, 1970) under these conditions. However, sunlight is essential for the final

stages of maturation which thus do not occur in the present system. During this final stage of ripening phosphorus is incorporated into the phytate compounds (Jenner, 1970). As phytates may remarkably impede iron absorption (Kuhn et al. 1968) this is a very important step in the ripening of the grain. Direct correlations between the behaviour of wheat produced in the medium described with that of wheat ripened in the field are only possible if phytate contents are similar. This information is not yet available.

The value obtained for the total iron in wheat was in agreement with the figures published by McCance & Widdowson (1960). It thus appears unlikely that the small quantity of high specific activity ferric citrate added to the sucrose medium appreciably altered the total iron content of the grain.

Most of the iron in raw wheat was present in an insoluble form. Furthermore the dialysis experiments showed that the preponderance of iron in raw wheat occurred as a large molecular weight fraction. The very low content of low molecular weight iron complexes in raw wheat prohibited further fractionation of these iron components by sephadex gel filtration due to inability to detect the low levels accurately.

A change in the nature of wheat iron was demonstrated following cooking and digestion. Both acid digestion alone, and peptic hydrolysis increased the soluble and dialysable iron, the latter procedure proving more effective than the former, indicating that both acid and pepsin play a role in altering wheat iron. By contrast, acid seemed to be the most important factor in modifying liver iron. Cooking alone decreased the soluble and dialysable iron components when compared with raw wheat. Cooking plus enzymatic digestion decreased the soluble and dialysable wheat iron fractions when compared with digested wheat.

Sephadex gel filtration of peptic hydrolysed wheat confirmed that the iron containing fraction was present only as low molecular weight complexes; whereas acid digested wheat contained an intermediate molecular weight component as well, and cooked, digested wheat contained high and low molecular weight iron fractions.

The way in which cooking interferes with the release of small molecular weight iron complexes is not yet determined. However it is possible that heating of the wheat homogenate for 10 minutes may result in increased iron-phosphorus complex formation, with the result that the iron exists in a very stable and insoluble form. Secondly, the cooking may alter the protein in such a manner that the iron is enveloped

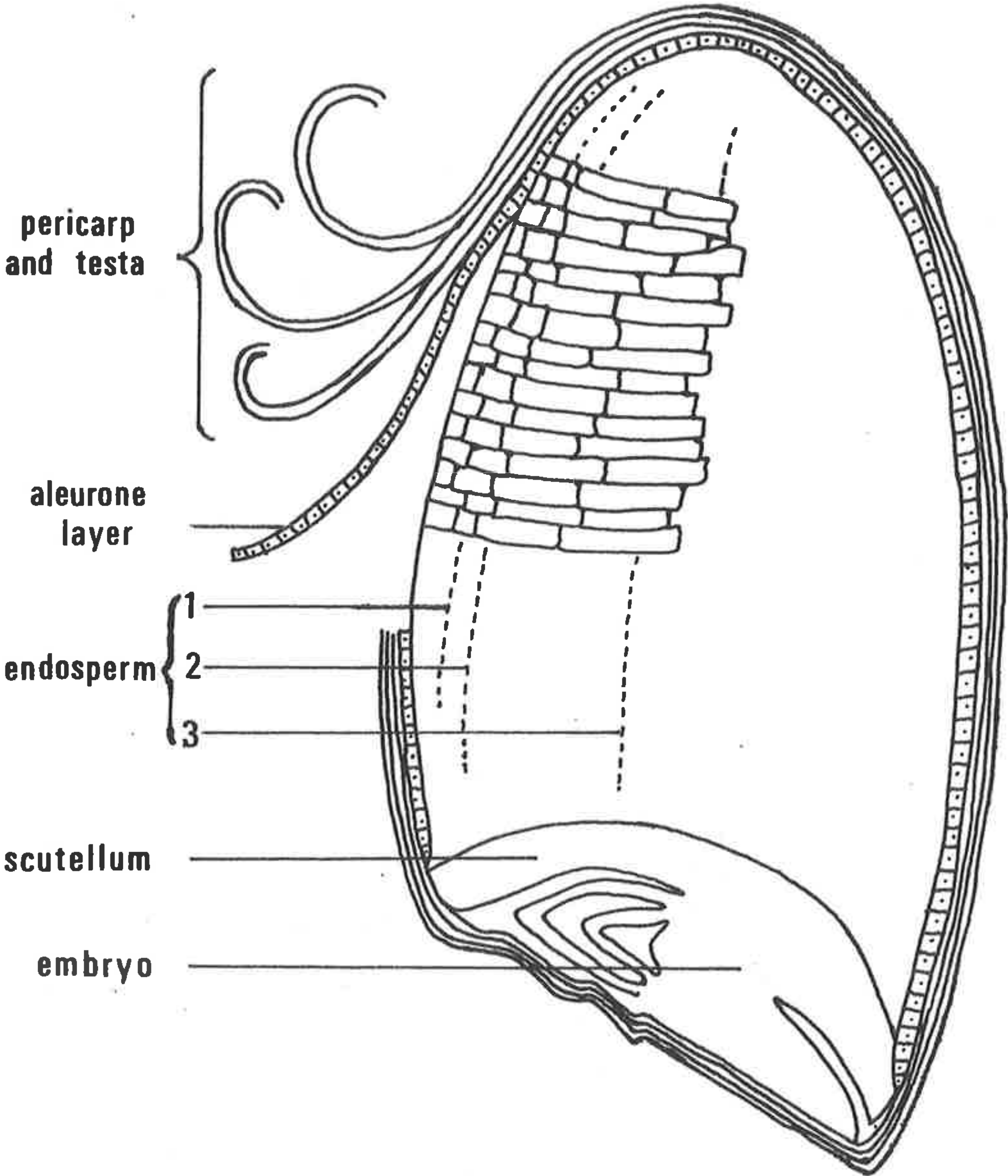
by the protein coagulum and is not therefore readily released by enzymatic digestion.

The sephadex filtration studies also showed that the wheat iron complexes were associated with protein fractions. This finding is in agreement with the known facts on wheat iron. It is accepted that the iron in wheat occurs largely in the aleurone layer of the grain (diagram I) which is also rich in protein, phytate and other minerals (Aykroyd & Doughty, 1970; Jenner, 1970).

This nutritious part of the grain is lost almost completely in the milling of fine white flour; however, most is retained in the flour used for baking brown bread. The importance of the aleurone layer as a source of dietary iron is doubtful as it is also a rich source of phytate. Certainly, it has been shown that the iron content of brown bread is twice that of white bread (Widdowson & McCance, 1942). However these same authors demonstrated that the absorption of iron from white bread was almost ten-fold greater than from brown bread. They attributed this marked difference in the levels of absorption to the higher phytate content of the brown bread.

DIAGRAM 1

DIAGRAMMATIC LONGITUDINAL SECTION OF WHEAT



The absorption of wheat iron by the rat was of the same order as for liver iron. These studies tend to indicate that there may be a phytate deficiency in the wheat, thus enabling more of the iron to be absorbed. Certainly the absorption of wheat iron by man has been reported to be 1.4 to 5 per cent (Elwood, Newton, Eakins & Brown, 1968; Hussain et al. 1965), a figure which is much lower than that obtained for animal iron absorption by man (Layrisse et al. 1968). However it is difficult to compare the results of the earlier workers (Elwood et al. 1968; Hussain et al. 1965) with those in the present study, since several variables are present.

The iron content of wheat used by Hussain and his co-workers (1965) for absorption studies in man was almost four-fold higher than the wheat used in the present study, and also than the values published by McCance & Widdowson (1960). Furthermore the method used for the isotopic labelling of the wheat iron differs from that described by Hussain and his associates (1965). These workers grew the wheat from seed in a hydroponic medium; this technique is both very difficult and time consuming. The labelling process is, however, more efficient when the wheat is grown from seed since an uptake of 25 per cent of the radioactivity added to the medium was reported, whereas in this study a 5 per cent uptake was achieved.

Also their absorption studies were done in man, whereas in this study, due to difficulties in obtaining sufficient quantities of radioactive wheat, absorption studies were limited to pilot studies carried out in rats.

The high level of wheat iron absorption by the rat could be attributed to the ability of this animal to utilize wheat iron more efficiently than man, or to differences in the levels of the phytate content of wheat labelled by different methods. Laboratory matured wheat has in all probability a lower phytate content than field wheat since sunlight is necessary for the final stages of maturation. The phytate complexes are formed in these final stages. In addition, it has long been recognised that phytates reduce iron absorption in man (McCance & Widdowson, 1935; Kuhn et al. 1968) and so the high levels of absorption most probably reflect the low levels of phytate in the wheat used in this study.

There was no correlation between increase in the soluble iron fraction or the low molecular weight dialysable material and the subsequent retention of the iron by the rat. Peptic hydrolysis resulted in the highest levels of dialysable and soluble iron complexes, but the absorption of wheat iron following peptic hydrolysis did not alter significantly when

compared to absorption from raw wheat. Furthermore, cooking reduced the soluble and dialysable iron fractions in wheat, and yet absorption was probably significantly higher than the other wheat preparations tested. Jacobs et al. (1969) likewise found that acid-peptic digestion increased both soluble iron and ionisable iron in wheat in the form of white and brown bread. They did not, however, assess the effect of cooking on wheat iron distribution nor test the the absorption of each specific component.

CHAPTER VIII

GENERAL DISCUSSION

In this thesis the experimental findings and their interpretation have been discussed in detail at the end of each section. It is therefore not intended that the present chapter should contain further consideration of all results, but rather review the main features of the study with relation to the current knowledge of iron absorption.

The total iron content and distribution of the iron in guinea pig liver between haem and non-haem compartments was first assessed. It was found that the total iron content and the type of iron present was very similar to that found in other species (McCance & Widdowson, 1960; Jacobs et al. 1969; Van Wyck et al. 1971). As the iron content of guinea pig liver was so closely similar to that of larger animals, it seemed that guinea pigs provided an acceptable working model to study the handling of dietary liver iron.

A number of parameters were used to monitor the effects of cooking and enzymatic digestion on the dietary iron complexes. These included physical sizing, documentation of changes in the chemical nature of the iron, and a comparison of the degree of absorption of the iron fractions in animals and man. These studies have expanded considerably the understanding of the importance of preparative and digestive processes on dietary iron absorption. They have also

complemented the studies of earlier workers (Kaldor, 1957; Sandford, 1960; Jacobs et al. 1969) who largely measured the amount of ionic iron in various foodstuffs and the increase in this fraction produced by cooking and enzymatic digestion.

The processes of cooking and enzymatic digestion profoundly altered the dietary iron complexes. Thus cooking and digestion modified the complex size as assessed both by dialysis and sephadex gel fractionation. However, there was no change in the haem,non-haem iron partition during these degradation procedures indicating that ionic iron had not been released from the haem fraction. This conflicted with previous reports (Kaldor, 1957; Sandford, 1960) which claimed that levels of up to 12 per cent of haemoglobin iron were released in the ionic state by acid-peptic digestion both alone and in conjunction with cooking.

The in vitro studies on liver iron suggested strongly that gastric acid played the more important role in reducing the size of the iron complexes since dialysis and sephadex gel filtration of acid, and acid-peptic digested liver homogenate produced similar results. Furthermore the effect of acidic or acid-peptic digestion in reducing the complex size was far greater than prior tryptic digestion. These in vitro studies gave some support to the postulate (Jacobs, Rhodes & Eakins, 1967; Jacobs & Owen, 1969) that gastric acid is the important luminal

factor in iron absorption.

It was anticipated that a progressive increase in absorption would result as the iron compounds were reduced in size by the cooking and digestive processes. However the correlation was not absolute,and the absorption of iron from raw and enzymatically digested liver was the same, in spite of the presence of a lower molecular weight iron fraction in the latter preparation. More precise experiments did, however, show that absorption was broadly related to the size of the iron complexes. Thus cooking in conjunction with acid-peptic digestion produced the greatest increase in dialysable liver iron, and the absorption from this preparation was the highest of all the liver fractions, and comparable to that of the inorganic ferric chloride. These findings suggested that the presence of a large proportion of the test dose of iron as low molecular weight complexes enhanced absorption. The importance of the size of the iron complexes in relation to their absorbability was further demonstrated by the increased absorption of the dialysable liver iron fraction when compared with absorption from the non-dialysable fraction and raw liver homogenate.

The absorption of iron from the main liver iron components haem and ferritin was found to be similar to that in other

studies. Chemically prepared haem was absorbed extremely poorly. This confirmed the findings of Conrad and his associates (1966a) who showed that the absorption of crystalline haem was very low; by contrast absorption of a mixture of haem and globin breakdown products was much more efficient. Hussain et al. (1965) investigated the absorption of iron from ferritin along with several other iron compounds and found that this was readily available to man. The values for ferritin absorption, presented in this thesis, confirm this finding.

The in vitro studies indicate that the degradation procedures largely affect the ferritin molecule. It is therefore likely that the increased absorption of treated liver is due to release of iron from the ferric hydroxide micelles of the iron-protein compound ferritin. This theory was further supported by the two-fold increase in ferritin iron absorption produced by cooking and acid-peptic digestion of the ferritin solution before administration. Furthermore Granick (1942, 1946) showed that temperatures in excess of 90°C denatured ferritin and that peptic hydrolysis of ferritin released iron from this molecule.

Although alteration in the size of iron complexes with the emergence of a greater proportion of low molecular weight

species certainly enhances iron absorption, it does not explain all the observed facts. This study has shown that iron from liver which has been cooked in conjunction with peptic hydrolysis is absorbed as well as iron from ferric chloride solution. More than 50 per cent of the iron in liver was dialysable following the combined degradation procedures, and the remainder was of such a size that it was retained by the cellulose dialysis tubing. This contrasts with ferric chloride where all the iron is of low molecular weight. It may be that enhancing factors are present to account for the high absorption levels of cooked digested liver which contained a mixed species of iron complexes. Several workers (Kroe et al. 1963; Klavins et al. 1962; Martinez-Torres & Layrisse (1970) have demonstrated that proteins, peptides and amino acids play an important role in enhancing iron absorption in man and animals. It may be that both the degradation of the liver proteins as well as the degradation of the iron complexes are responsible for the increased absorption.

In man it was possible to measure absorption only from cooked liver and from liver which was cooked and peptic hydrolysed. Although there was a marginal increase in the absorption of the in vitro digested liver the levels were not significantly different. It may be that the human process of digestion further modifies the cooked liver to release iron

complexes which are comparable to those produced by in vitro digestion. If so the effect differs from that in the rat where apparently no significant in vivo digestive changes occurred.

The observations on the physical and chemical changes induced in wheat iron during digestion differed from those of liver. Approximately 60 per cent of the liver iron was water soluble, and this fraction was not increased by cooking and enzymatic digestion. By contrast, virtually no soluble iron was detected in raw and cooked wheat homogenate, but soluble iron increased greatly following acid digestion, acid-peptic digestion and cooking in conjunction with acid-peptic digestion. The increase in the soluble wheat iron fraction after the degradation processes is in agreement with the results of Jacobs et al. (1969) who studied the changes in soluble iron in a number of common foodstuffs. The greatest increase in soluble iron resulted from acid-peptic digestion of the wheat, indicating acid-pepsin may affect the form of wheat to a greater degree than acid alone.

Furthermore minimal changes in the dialysable wheat iron fractions were detected following the digestive process when compared with raw wheat. These findings are also contrary to those obtained with liver iron complexes, in that a great

increase in the dialysable iron fraction was produced by both enzymatic digestion and cooking plus enzymatic digestion. However, in spite of the minimal changes demonstrated by dialysis, the sephadex gel fractionation studies (G200) clearly revealed the presence of a low molecular weight iron fraction in digested wheat and in wheat both cooked and digested. Therefore it can be assumed that some reduction in the size of the complexes was produced by the cooking and digestive procedures, but the effect appears to be less marked than for liver.

Absorption studies of wheat iron were performed only in rats. Retention of wheat iron was of the order of 20 per cent which was considerably in excess of levels reported in man (Hussain et al. 1965; Elwood et al. 1968). This difference may represent simply a species variation in handling of cereal iron, or more likely, a difference in the phytate content of the grain used in this study compared with that used by others. Certainly a low phytate content is compatible with increased levels of iron absorption (Sharpe et al. 1950; Apte & Venkatachalam, 1962). The phytate content of grain is sensitive to conditions under which the grain matures (Jenner, 1970). The wheat used in this study was ripened without direct sunlight, which would lower the phytate content.

There was no direct correlation between changes in size

of wheat iron complexes, the chemical form of wheat iron, and absorption of iron from the various preparations. In fact, manoeuvres such as enzymatic digestion, which in vitro were associated with most degradation of wheat iron complexes, failed to increase iron absorption when compared with a raw wheat control group, whereas cooking enhanced absorption two-fold despite a minimal effect on complex size. Thus maximal absorption of iron occurred from the preparation of wheat which by in vitro testing was thought to contain the least readily available iron complexes.

It is apparent that dietary iron absorption is a very complex process, and that the results from the study of a single food item cannot be extrapolated to the absorption of iron from other dietary sources. However, the present work has clearly shown that the cooking and digestive procedures to which foodstuffs are subjected before and after ingestion markedly alter the iron complexes. Unfortunately no simple well defined relationship between complex size and absorption has been demonstrated. In liver, iron absorption certainly relates to complex size, whereas with wheat no correlation has been found.

However, it has been amply demonstrated that whatever the physical and chemical changes involved, prior cooking and

enzymatic digestion enhances iron absorption from diets which are rich in animal proteins such as liver. Conrad and his co-workers (1966a, 1966b) had previously shown that digestion of haemoglobin to haem, and globin by intestinal secretions was necessary for efficient haem iron absorption. This study further indicates that ferritin is likewise degraded during digestion and that degradation of the ferritin molecule makes the iron more readily available for absorption.

The limited studies on wheat iron do not give any clear indications that enzymatic digestion alone or in conjunction with cooking are critical to wheat iron absorption; cooking alone may have such an effect. It is recognised (Patwardhan, 1962; W.H.O. Report, 1959, 1968) that iron deficiency is a major problem in countries with a high cereal dietary content. This may reflect partly absolute dietary lack of iron (Patwardhan, 1962) and partly unavailability of iron due to complexing with cereal phytate (Ramalingaswami & Patwardhan, 1949). A further factor which has been suggested by the present study is the relative resistance of cereal iron to digestive processes.

In summary, liver iron complexes have been identified and quantitated. The degradation of these iron complexes to smaller molecular size fractions by cooking and enzymatic

digestion has been demonstrated. In the test case of liver, it would appear that the degradation processes enhance absorption by reducing the size of the iron complexes and also by degradation of proteins. The combined effects of cooking and peptic hydrolysis were such that absorption of liver iron was comparable with inorganic iron. Similarly, wheat iron has been quantitated and partially physically characterised, and the changes in the nature of the complexes following the digestive procedures observed. No clearly defined effect on absorption of wheat iron could be determined, except that cooking alone enhanced absorption. The data were insufficient to postulate the manner in which cooking produced the increased wheat iron absorption.

This study has further highlighted the complexity of dietary iron handling. It is apparent that detailed studies on the nature and availability of iron in other foodstuffs are essential for a clearer understanding of dietary iron absorption. Furthermore, these investigations should be performed on mixtures of foodstuffs as well as on single items, so that the effects of interaction of foodstuffs on the nature and absorption of dietary iron can be more fully assessed.

APPENDIX 1

STATISTICAL METHODS

The statistical methods employed for the analysis of results have been outlined by Bailey (1959).

STATISTICAL FORMULAE

A. Calculation of mean, standard deviation and standard error

$$\text{mean } (x) = \frac{1}{n\Sigma x}$$

$$\text{Standard deviation } (S) = \sqrt{\Sigma \frac{(\bar{x}-x)^2}{n-1}}$$

$$\text{Standard error of mean} \quad \bar{x} \pm \frac{S}{\sqrt{n}}$$

B. Comparison of mean of two samples

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{\beta_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

List of abbreviations used in statistical formulae:

n = number of observations in sample

x = observed measurements in a sample

$\bar{x}$ = mean of measurements x

Σ = summation symbol

S = estimated standard deviation

t = t test

p = significance level actually achieved by data

The significance levels were obtained from published statistical tables (Bailey, 1959).

The following notations have been utilized in this thesis.

$p > 0.05$ not significant

$p < 0.01$ significant

$p < 0.001$ highly significant

BIBLIOGRAPHY

ALLAN, J.E. (1959) Determination of iron and manganese by atomic absorption. Spectrochimica Acta 15: 800-806.

ANDREWS, P. (1965) The gel filtration behaviour of proteins related to their molecular weights over a wide range. J. Biochem. 96: 595-606.

APTE, S.V. & VENKATACHALAM, P.S. (1962) Iron absorption in human volunteers using high phytate cereal diet. Indian J. Med. Res. 50: 516-520.

APTE, S.V. & VENKATACHALAM, P.S. (1964) The influence of dietary calcium on absorption of iron. Indian J. Med. Res. 52: 213-218.

AYKROYD, W.R. & DOUGHTY, J. (1970) Wheat in human nutrition. F.A.O. Nutritional Studies, 23: 1-163.

BAIRD, I.M. & WILSON, G.M. (1959) The pathogenesis of anaemia after partial gastrectomy. 2. Iron absorption after partial gastrectomy. Quart. J. Med. 28: 35-41.

BALCERZAK, S.P., PETERNEL, W.W. & HEINLE, E.W. (1967) Iron absorption in chronic pancreatitis. Gastroenterology, 53: 257-264.

BANNERMAN, R.M. (1965) Quantitative aspects of haemoglobin iron absorption. J. Lab. Clin. Med. 65: 944-950.

BENJAMIN, B.I., CORTELL, S. & CONRAD, M.E. (1967) Bicarbonate-induced iron complexes and iron absorption: one effect of pancreatic secretions. Gastroenterology, 53: 389-396.

BERGEIM, O. & KIRCH, E.R. (1948) Reduction of iron in the human stomach. J. Biol. Chem. 177: 591-596.

BERLIN, N.I., HYDE, G.M., PARSONS, R.J. & LAWRENCE, J.H. (1949) The blood volume of the adult rat, as determined by Fe^{59} and P^{32} labelled red cells. Proc. Soc. Exp. Biol. (N.Y.) 71: 176-178.

BEUTLER, E., FAIRBANKS, V.F. & FAHEY, J.L. (1963) Clinical disorders of iron metabolism. Grune & Stratton Inc. New York, p. 37.

BIGGS, J.C., BANNERMAN, R.M. & CALLENDER, S.T. (1961) Iron absorption in achlorhydria. Proc. 8th Congress of European Society of Medicine, Vienna, 1: 236.

BIGGS, J.C. & DAVIS, A.E. (1963) Relationship of diminished pancreatic secretions to haemochromatosis. Lancet, 2. 814.

BOCKUS, H.L. (1965) In 'Gastroenterology', vol. 3: 576-577. W.B. Saunders Co. Philadelphia.

BOTHWELL, T.H., PIRZIO-BIROLI, G. & FINCH, C.A. (1958) Iron absorption I: Factors affecting absorption. J. Lab. Clin. Med. 51: 24-36.

BRADFIELD, R.B., JENSEN, M.V., GONZALES, L. & GARRAYAR, C. (1968) Effect of low-level iron and vitamin supplementation on a tropical anaemia. Amer. J. Clin. Nutr. 21: 57-67.

BRANSBY, E.R., DAUBNEY, C.G. & KING, J. (1948) Comparison of nutrient values of individual diets found by calculation from food tables and chemical analysis. Brit. J. Nutr. 2: 232-236.

BRISE, H. (1962a) Influence of meals on iron absorption in oral iron therapy. Acta Med. Scand. 171 (supp. 376): 39-45.

BRISE, H. (1962b) Effect of surface-active agents on iron absorption. Acta Med. Scand. 171 (supp. 376): 47-50.

BROWN, E.B. (1963) The absorption of iron. Amer. J. Clin. Nutr. 12: 205-213.

BROWN, E.B., HWANG, Y.F. & NICOL, S. (1966) Absorption of haemoglobin iron. Clin. Res. 14: 312 (abstract).

BROWN, E.B. & JUSTUS, B.W. (1958) In vitro absorption of radioiron by everted pouches of rat intestine. Amer. J. Physiol. 194: 319-326.

BRUCKMANN, G. & ZONDEK, S.G. (1940) An improved method for the determination of non-haemin iron. J. Biol. Chem. 135: 23-30.

CALLENDER, S.T., MALLET, B.J. & SMITH, M.D. (1957) Absorption of haemoglobin iron. Brit. J. Haemat. 3: 186-192.

CALLENDER, S.T. & WARNER, G.T. (1968) Iron absorption from bread. Amer. J. Clin. Nutr. 21: 1170-1174.

CHARLEY, P.J., SARKAR, B., STITT, C.F. & SALTMAN, P. (1963) Chelation of iron by sugars. Biochim. Biophys. Acta 69: 313-321.

CHARLEY, P.J., STITT, C., SHORE, E. & SALTMAN, P. (1963) Studies in the regulation of intestinal iron absorption. J. Lab. Clin. Med. 61: 397-410.

CHODOS, R.B., ROSS, J.F., APT, L., POLLYCOVE, M. & HALKETT, J.A.E. (1957) The absorption of radioiron labelled foods and iron salts in normal and iron-deficient subjects and in idiopathic haemochromatosis. J. Clin. Invest. 36: 314-326.

CHOUDHURY, M.R. & WILLIAMS, J. (1959) Iron absorption and gastric operations. Clin. Sci. 18: 527-532.

CONRAD, M.E., BENJAMIN, B.I., WILLIAMS, H.L. & FOY, A.L. (1967b) Human absorption of Hb-iron. Gastroenterology, 53: 5-10.

CONRAD, M.E., CORTELL, S., WILLIAMS, H.L. & FOY, A.L. (1966a) Polymerisation and intraluminal factors in the absorption of haemoglobin iron. J. Lab. Clin. Med. 68: 659-668.

CONRAD, M.E. & CROSBY, W.H. (1963) Intestinal mucosal mechanisms controlling iron absorption. Blood 22: 406-415.

CONRAD, M.E., FOY, A.L., WILLIAMS, H.L. & KNOSPE, W.L. (1967a) Effect of starvation and protein depletion on ferrokinetics and iron absorption. Amer. J. Physiol. 213: 557-565.

CONRAD, M.E. & SCHADE, S.G. (1968) Ascorbic acid chelates in iron absorption: a role for hydrochloric acid and bile. Gastroenterology 55: 35-45.

CONRAD, M.E., WEINTRAUB, L.R. & CROSBY, W.H. (1964) The role of the intestine in iron kinetics. J. Clin. Invest. 43: 963-974.

CONRAD, M.E., WEINTRAUB, L.R., SEARS, D.A. & CROSBY, W.H. (1966b) Absorption of Hb-iron. Amer. J. Physiol. 211: 1123-1130.

COOK, J.D., ALVARADO, J., GUTNISKY, A., JAMRA, M., LABARDINI, J., LAYRISSE, M., LINARES, J., LORIA, A., MASPES, V., RESTREPO, A., REYNAFARJE, C., SANCHEZ-MEDAL, L., VELEZ, H. & VITERI, F. (1971) Nutritional deficiency and anaemia in Latin America: a collaborative study. Blood 38: 591-603.

COOK, J.D., BROWN, G.M. & VALBERG, L.S. (1964) The effects of achylia gastrica on iron absorption. J. Clin. Invest. 43: 1185-1191.

COONS, C.M. (1964) Iron metabolism. Ann. Rev. Biochem. 33: 459-480.

COURTICE, F.C. (1943) The blood volume of normal animals. J. Physiol. 102: 290-305.

CROSBY, W.H. (1968) Control of iron absorption by intestinal luminal factors. Amer. J. Clin. Med. 21: 1189-1193.

CROSBY, W.H. & FURTH, F.W. (1956) A modification of the benzidine method for measurement of haemoglobin in plasma and urine. Blood 11: 380-383.

DAVIS, A.E. (1964) Iron absorption in haemochromatosis and cirrhosis of the liver. Aust. Ann. Med. 13: 201-203.

DAVIS, A.E. and BADENOCH, J. (1962) Iron absorption in pancreatic disease. Lancet 2: 6-8.

DAVIS, A.E. & BIGGS, J.C. (1965) The pancreas and iron absorption. Gut 6: 140-142.

DAVIS, P.S., LUKE, C.G. & DELLER, D.J. (1966) Reduction of gastric iron-binding protein in haemochromatosis. Lancet, 2: 1431-1433.

DICKMAN, S.R. & CLOUTIER, A.A. (1951) Factors affecting the activity of aconitase. J. Biol. Chem. 188: 379-388.

DIXON, M. & WEBB, E.C. (1966) Enzyme techniques. In 'Enzymes' 2nd edition, p. 8-10.

DUBACH, R., CALLENDER, S.T. & MOORE, C.V. (1948) Studies in iron transportation and metabolism VI. Absorption of radioactive iron in patients with fever and with anaemias of varied aetiology. Blood 3: 526-540.

DUBACH, R., MOORE, C.V. & CALLENDER, S.T. (1955) Studies in iron transportation and metabolism IX. The secretion of iron as measured by the isotope technique. J. Lab. Clin. Med. 45: 599-615.

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A. & SMITH, F. (1956) Phenol-sulphuric acid method of carbohydrate estimation. Anal. Chem. 28: 350.

EICHRAN, G.L. (1964) In 'Iron Metabolism'. Ciba Symposium, p. 9-21. Springer-Verlag, Berlin.

ELWOOD, P.C. (1970) Some epidemiological aspects of iron deficiency relevant to its evaluation. Proc. Roy. Soc. Med. 63: 1230-1232.

ELWOOD, P.C., NEWTON, D., EAKINS, J.D. & BROWN, D.A. (1968) Absorption of iron from bread. Amer. J. Clin. Nutr. 21: 1162-1169.

ELWOOD, P.C., WATERS, W.E. & GREEN, W.J.W. (1970) Evaluation of iron supplements in prevention of iron deficiency anaemia. Lancet 2: 175-177.

ENDICOTT, K.M., GILLMAN, T., BRECKER, G., NESS, A.T., CLARKE F.A. & ADAMIK, E.R. (1949) Study of histochemical iron using tracer methods. J. Lab. Clin. Med. 34: 414-421.

FINCH, C.A. (1965) Iron balance in man. Nutr. Rev. 23: 129-131.

FISCHER, D.S. & PRICE, D.C. (1964) A simple serum iron method using the new sensitive chromagen tripyridyl-s-triazine. Clin. Chem. 10: 21-31.

FOOD AND NUTRITION BOARD. National Academy of Sciences, National Research Council (1970). Recommendation for increased iron levels in the American diet. Nutr. Rev. 28: 108-109.

FORRESTER, R.H., CONRAD, M.E. & CROSBY, W.H. (1962) Measurement of total body Fe in animals using whole body liquid scintillation detectors. Proc. Soc. Exp. Biol. 111: 115-119.

FOY, H. & KONDI, A. (1956) Nutritional and intestinal factors and iron losses in the genesis of tropical anaemias; preliminary communication. Lancet 1: 423-424.

GABRIO, B.W., SHODEN, A. & FINCH, C.A. (1953) A quantitative fractionation of tissue ferritin and haemosiderin. J. Biol. Chem. 204: 815-821.

GOLDBERG, A., LOCHHEAD, A.C. & DAGG, J.H. (1964) Histamine-fast achlorhydria and iron absorption. Lancet, 1:848-850.

GRACE, W.J., DOIG, R.K. & WOLFF, H.G. (1954) Absorption of iron from the gastrointestinal tract. Amer. J. Clin. Nutr. 2: 162-167.

GRANICK, S. (1942) Ferritin I. Physical and chemical properties of horse spleen ferritin. J. Biol. Chem. 146: 451-461.

GRANICK, S. (1943) Ferritin IV. Occurrence and immunological properties of ferritin. J. Biol. Chem. 149: 157-167.

GRANICK, S. (1946) Ferritin: its properties and significance for iron metabolism. Chem. Rev. 38: 379-403.

GRANICK, S. (1949) Iron metabolism and haemochromatosis. Bull. N.Y. Acad. Med. 25: 403-428.

GRANICK, S. (1951) Structure and physiological functions of ferritin. Physiol. Rev. 31: 489-511.

GRANICK, S. (1954) Iron Metabolism. Bull. N.Y. Acad. Med. 30: 81.

GRANICK, S. & MICHAELIS, L. (1943) Ferritin II. Apoferritin of horse spleen. J. Biol. Chem. 147: 91-97.

GROEN, J. VAN DEN BROEK, W.A. & VELDMAN, H. (1947) Absorption of iron compounds from the small intestine of the rat. Biochim. Biophys. Acta 1: 315-326.

GUBLER, C.J., LAHEY, M.E., ASHENBRUCKER, H., CARTWRIGHT, G.E. & WINTROBE, M.M. (1952) Studies on copper metabolism. A method for the determination of copper in whole blood, red blood cells and plasma. J. Biol. Chem. 196: 209-220.

HAHN, P.F., BALE, W.G., LAURENCE, E.O. & WHIPPLE, G.H. (1939) Radioactive iron and its metabolism in anaemia; its absorption, transportation and utilisation. J. Exp. Med. 69: 739-753.

HAHN, P.F., BALE, W.G., ROSS, J.F., BALFOUR, W.M. & WHIPPLE, G.M. (1943) Radioactive iron absorption by the gastro-intestinal tract: influence of anaemia, anoxia and ante-cedent feeding; distribution in growing dogs. J. Exp. Med. 78: 169-188.

HALLBERG, L. & SÖLVELL, L. (1967) Absorption of haemoglobin iron in man. Acta med. Scand. 181: 335-354.

HALLBERG, L., SÖLVELL, L. & ZETTERFELDT, B. (1966) Iron absorption after partial gastrectomy. Acta med. Scand. (Suppl. 445): 269-275.

HARRISON, P.M. (1964) In 'Iron Metabolism'. Ciba Symposium: 40-59. Springer-Verlag, Berlin.

HEMMATI, A. (1968) Determining the site of absorption of iron in the intestinal tract by means of a remote controlled intestinal capsule. Dtsch. Med. Wschr. 93: 1468-1471.

HÖGLUND S. & REIZENSTEIN, P. (1969) Studies in iron absorption V. Effect of gastrointestinal factors on iron absorption. Blood 34: 486-504.

HUSSAIN, R., WALKER, R.B., LAYRISSE, M., CLARK, P. & FINCH, C.A. (1965) Nutritive value of food iron. Amer. J. Clin. Nutr. 16: 464-471.

HWANG, Y. & BROWN, E. (1963) Studies of the effect of desferri-oxamine on human iron absorption and excretion. J. Lab. Clin. Med. 62: 885 (abstract).

HWANG, Y. & BROWN, E. (1965) Effect of desferrioxamine on iron absorption. Lancet 1: 135-137.

JACOBS, A. (1968) Gastric factor in iron absorption. Lancet 1: 1313-1314.

JACOBS, A. (1969) Availability and absorption of dietary iron. Proc. Roy. Soc. Med. 63: 1215-1216.

JACOBS, A., BOTHWELL, T. & CHARLTON, R.W. (1964) Role of hydrochloric acid in iron absorption. J. App. Physiol. 19: 187-188.

JACOBS, A. & MILES, P.M. (1968) Role of gastric secretion in iron absorption. Gut, 10: 226-229.

JACOBS, A. & OWEN, G.M. (1969) Effect of gastric juice on iron absorption in patients with gastric atrophy. Gut, 10: 488-490.

JACOBS, A., PATH, M.C. & GREENMAN, D.A. (1969) Availability of food iron. Brit. Med. J. 1: 673-676.

JACOBS, A., RHODES, J. & EAKINS, J.D. (1967) Gastric factors influencing iron absorption in anaemic patients. Scand. J. Haemat. 4: 105-110.

JACOBS, A., RHODES, J., PETERS, D.K., CAMPBELL, H. & EAKINS, J.D. (1966) Gastric acidity and iron absorption. Brit. J. Haemat. 12: 728-736.

JENNER, R.B. (1970) Wheat and composition. Personal communication.

JOHNSTON, F.A., FRENCHMAN, R. & BURROUGHS, E.D. (1948) The absorption of iron from beef by women. J. Nutr. 35: 453-465.

KALDOR, I. (1957) Haemoglobin as a source of iron in nutrition; some in vitro experiments. Aust.Ann.Med. 6: 244-246.

KAVIN, H., CHARLTON, R.W., JACOBS, P., GREEN, P., TORRANCE, J.D. & BOTHWELL, T.H. (1967) Effect of the pancreatic exocrine secretions. Gut, 8: 556-564.

KEILIN, J. (1960) Nature of haem linking groups in native and denatured haemoglobin and myoglobin. Nature, 187: 365-371.

KIMBER, C., PATTERSON, J.F. & WEINTRAUB, L.R. (1967) Pathogenesis of iron deficiency anaemia following partial gastrectomy. Study of iron balance. J. Amer. Med. Ass. 20: 935-938.

KIRCH, E.R., BERGEIM, O., KLEINBERG, J. & JAMES, S. (1947) Reduction of iron by foods in gastric artificial digestion. J. Biol. Chem. 171: 687-694.

KLAVINS, J.V., KINNEY, T.D. & KAUFMAN, N. (1962) The influence of dietary protein on iron absorption. Brit. J. Exp. Path. 43: 172-180.

KOEPKE, J.A. & STEWART, W.B. (1964) Role of gastric secretion in iron absorption. Proc. Soc. Exp. Biol. 115: 927-929.

KROE, D.J., KAUFMAN, N., KLAVINS, J.V. & KINNEY, T.D. (1966) Interrelation of amino-acids and pH on intestinal iron absorption. Amer. J. Physiol. 211: 414-418.

KROE, D.J., KINNEY, T.D., KAUFMAN, N. & KLAVINS, J.V. (1963) The influence of amino-acids on iron absorption. Blood, 21: 546-552.

KUHN, I.N., LAYRISSE, M., ROCHE, M., MARTINEZ-TORRES, C. & WALKER, R.B. (1968) Observations on the mechanisms of iron absorption. Amer. J. Clin. Nutr. 21: 1184-1188.

LABBE, R.F. & NISHIDA, G. (1957) A new method of haem isolation. Biochim. Biophys. Acta 26: 437.

LAUFBERGER, V. (1937) Sur le cristallisation de la ferritine. Bull. Soc. Chim. Biol. 19: 1575-1582.

LAYNE, E. (1957) Calculations in protein estimations. In 'Methods in Enzymology' 3: 452. Eds. Kolowick S.P. and Kaplan, N.O. Academic Press, N.Y.

LAYRISSE, M., MARTINEZ-TORRES, C. & ROCHE, M. (1968) Effect of interaction of various foods on iron absorption. Amer. J. Clin. Nutr. 21: 1175-1183.

LEMBERG, R. & LEGGE, J.W. (1949) Hematin and bile pigments. New York, Interscience Pub. Inc.

LEVERTON, R.M. & ROBERTS, L.J. (1937) A comparison of values obtained by calculation and by analysis for iron content of 85 mixed diets. J. Amer. Diet. Ass. 13: 139-143.

LINDER-HOROWITZ, M., RUETTINGER, R.T. & MUNRO, H.N. (1970) Iron induction of electrophoretically different ferritins in rat liver, heart and kidney. Biochim. Biophys. Acta 200: 442-448.

LOTTRUP, M.C. (1934) Treatment of anaemia in children with ferric and ferrous compounds, reduced iron and cupric sulphate. Amer. J. Dis. Child. 47: 1-8.

LUKE, C.G., DAVIS, P.S. & DELLER, D.J. (1967) Change in gastric iron-binding protein (gastroferrin) during iron-deficiency anaemia. Lancet 1: 926-927.

McCANCE, R.A. & WIDDOWSON, E.M. (1935) Phytin in human nutrition. Biochem. J. 29: 2694-2699.

McCANCE, R.A. & WIDDOWSON, E.M. (1937) Absorption and excretion of iron. Lancet 2: 680-684.

McCANCE, R.A. & WIDDOWSON, E.M. (1938) The absorption and excretion of iron following oral and intravenous administration. J. Physiol. 94: 148-154.

McCANCE, R.A. & WIDDOWSON, E.M. (1960) Copy of tables giving composition of most foods. H.M. Stationery Office.

MacDONALD, R.A. (1963) Idiopathic haemochromatosis. Genetic or acquired? Arch. Intern. Med. 112: 184-190.

McMILLAN, T. & JOHNSTON, F.A. (1951) The absorption of iron from spinach by six young women, and the effect of beef upon absorption. J. Nutr. 44: 383-398.

MARTINEZ-TORRES, C. & LAYRISSE, M. (1970) Effect of amino-acids on iron absorption from a staple food. Blood, 35: 669-682.

MAZUR, A. & SHORR, E. (1950) A quantitative immunochemical study of ferritin and its relation to the hepatic vaso-depressor material. J. Biol. Chem. 182: 607-627.

MEHLER, A.H. (1962) In 'Oxygenases'. Ed. Mayaishi, O. Academic Press, N.Y. p. 87.

MIGNON, M., RUSSELL, M.C., SEMB, L.S., MORGAN, E.H., FINCH, C.A. & NYHUS, L.M. (1965) Effect of gastric juice on the absorption of iron. Surg. Forum 16: 319-321.

MINISTRY OF HEALTH (1968) Iron in flour. Reports on public health and medical subjects. No. 117. H.M.S.O., London.

MINOT, G.R. & HEATH, C.V. (1932) The response of the reticulocytes to iron. Amer. J. Med. Sci. 183: 110-121.

MOESCHLIN, S., SCHMID, J.R. and SCHNIDER, T. (1965) Increased absorption of radioiron in gastrectomised patients by the addition of hydrochloric acid. Acta Haemat. 33: 200-209.

MONSEN, E.R., KUHN, I.N. & FINCH, C.A. (1967) Iron status of menstruating women. Amer. J. Clin. Nutr. 20: 842-849.

MOORE, C.V. (1955) The importance of nutritional factors in the pathogenesis of iron-deficiency anaemia. Amer. J. Clin. Nutr. 3: 3-10.

MOORE, C.V. & DUBACH, R. (1951) Observations on the absorption of iron from food tagged with radioactive iron. Trans. Ass. Amer. Physicians 64: 245-256.

MOORE, C.V. & DUBACH, R. (1956) Metabolism and requirements of iron in the human: report to the Council on Foods and Nutrition. Amer. Med. Ass. J. 162: 197-204.

MOORE, C.V., DUBACH, R., MINNICH, V. & ROBERTS, H.K. (1944) Absorption of ferrous and ferric radioactive iron by human subjects and by dogs. J. Clin. Invest. 23: 755-767.

MORGAN, E.H. & WALTERS, M.N.I. (1963) Fractionation of hepatic and splenic iron into ferritin and haemosiderin with histochemical techniques. J. Clin. Path. 16: 101-107.

MORTENSEN, L.E., VALENTINE, R.C. & CARNAHAN, J.E. (1962) An electron transfer factor from Clostridium pasteurianum. Biochem. Biophys. Rev. Comm. 7: 448-452.

MURRAY, M.J. & STEIN, N. (1966) Does the pancreas influence iron absorption? A critical review. Gastroenterology, 51: 694.

MURRAY, M.J. & STEIN, N. (1967) Effect of ligation of pancreatic duct on absorption of radioiron by rats. Gastroenterology, 53: 38-41.

MURRAY, M.J. & STEIN, N. (1968a) Effect of pancreatin on absorption of Fe^{59} in rats with fatty liver. Amer. J. Dig. Dis. 13: 527-529.

MURRAY, M.J. & STEIN, N. (1968b) The effects on iron absorption of gastrointestinal secretions from patients with iron deficiency anaemia. Brit. J. Haemat. 15: 87-91.

NATIONAL RESEARCH COUNCIL (1948) Recommended dietary allowances. Reprint and Circular Series, No. 129.

NATVIG, H., BJERKEDAL, T. & JONASSEN, O. (1963) Studies on haemoglobin values in Norway II. The effect of a supplementary intake of ascorbic acid and iron on the haemoglobin level of schoolchildren and men. Acta med. Scand. 174: 341-350.

O'HOGAN, J.E. (1960) The haem-globin linkage. Biochem. J. 74: 417-423.

OUCHTERLONY, Ö. (1949) Antigen-antibody reactions in gels and the practical applications of this phenomenon in the laboratory diagnosis of diphtheria. Med. Diss. (Stockholm).

OUCHTERLONY, Ö. (1958) Diffusion-in-gels methods for immunological analysis. Progr. Allergy 5: 1-78, (Karger, Basel/New York.)

PATWARDHAN, V.N. (1961) In 'Nutrition in India'. 2nd ed. Bombay, Indian Journal of Med. Sciences, p. 515.

PERUTZ, M.F. & MUIRHEAD, H. (1963) Structure of haemoglobin. Nature 199: 633-638.

PERUTZ, M.F., ROSSMAN, M.G., CULLIS, A.F., MUIRHEAD, H., WILL, G. & NORTH, A.C.T. (1960) Structure of haemoglobin. Nature, 185: 416-422.

PINKERTON, P.H., SPENCE, I., OGILVIE, J.C., RONALD, W.A., MARCHANT, P. & RAY, P.K. (1970) An assessment of the Coulter counter model S. J. Clin. Path. 23: 68-76.

PIRZIO-BIROLI, G., BOTHWELL, T.H. & FINCH, C.A. (1958) Iron absorption II. The absorption of radioiron administered with a standard meal in man. J. Lab. Clin. Med. 51: 37-48.

PIRZIO-BIROLI, G. & FINCH, C.A. (1960) Iron absorption III. The influence of iron stores on iron absorption in the normal subject. J. Lab. Clin. Med. 55: 216-220.

PORATH, J. (1955) Fractionation of polypeptides on dextrose gels. Clin. Chim. Acta 4: 776-778.

POWELL, L.W. & WILSON, E. (1970) In vivo intestinal mucosal uptake of iron, body iron absorption and gastric juice iron-binding in idiopathic haemochromatosis. Aust. Ann. Med. 19: 226-231.

PRICE, D.C., COHN, S.H., WASSERMAN, L.R., REIZENSTEIN, P.G. & CRONKITE, E.P. (1962) The determination of iron absorption and loss by whole body counting. Blood 20: 517-531.

PRICE, D.C., REIZENSTEIN, P., COHN, S.H., CRONKITE, E.P. & WASSERMAN, L.R. (1961) A method for studying iron absorption and loss by whole body counting. Clin. Res. 9: 165. (Abstract).

RAMALINGASWAMI, V. & PATWARDHAN, V.N. (1949) Diet and health of South Indian plantation labour. Indian J. Med. Res. 37: 51-60.

RAMSAY, W.N.M. (1957) The determination of iron in blood plasma or serum. Clin. Chim. Acta 2: 214-220.

RUDZKI, Z. (1970) Nature of gastric iron-binding glycoprotein. Ph.D. Thesis, University of Adelaide.

SALTMAN, P. (1965) The role of chelation in iron metabolism. J. Chem. Educ. 42: 682-687.

SALTMAN, P., FISKIN, R.D. & BELLINGER, S.B. (1956) The metabolism of iron by rat liver slices. The effect of physical environment and iron concentration. J. Biol. Chem. 220: 741-750.

SALTMAN, P., FRISH, H., FISKIN, R.D. & ALEX, T. (1956) The kinetics of iron metabolism in rat liver slices. J. Biol. Chem. 221: 777-780.

SANDFORD, R. (1960) Release of iron from conjugates in food Nature, 185: 533-534.

SARGENT, T. (1962) Metabolic studies with Fe^{59}, Ca^{47} and C^{11} in various diseases. Proc. I.A.E.A. Symposium on Whole Body Counting, Vienna, p. 447.

SAYLOR, L. & FINCH, C.A. (1953) Determination of iron absorption using two isotopes of iron. Amer. J. Physiol. 172: 372-376.

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

SCHADE, S.G., COHEN, R.J. & CONRAD, M.E. (1968) Effect of hydrochloric acid on iron absorption. New Engl. J. Med. 279: 741-750.

SCHLAPHOFF, D. & JOHNSTON, F.A. (1949) The iron requirement of six adolescent girls. J. Nutr. 39: 67-82.

SCHULTZ, J. & SMITH, N.J. (1958) Absorption of food iron - a quantitative study in infants and children. A.M.A. J. Dis. Child. 95: 109-119.

SCOTT, E.M. & HELLER, C.A. (1964) Iron deficiency in Alaskan Eskimos. Amer. J. Clin. Nutr. 15: 282-286.

SHACKLETON, L. & McCANCE, R.A. (1936) Ionisable iron in foods. Biochem. J. 30: 580-585.

SHARPE, L.M., PEACOCK, W.C., COOKE, R. & HARRIS, R.S. (1950) The effect of phytate and other food factors on iron absorption. J. Nutr. 41: 433-446.

SHERMAN, W.C., ELVEHJEN, C.A. & HART, E.B. (1934) Further studies on the availability of iron in biological materials. J. Biol. Chem. 107: 383-394.

SMITH, M.D. & MALLETT, B. (1957) Iron absorption before and after partial gastrectomy. Clin. Sci. 16: 23-34.

SMITH, P.M. (1968) Gastric iron binding in haemochromatosis. Lancet, 2: 1143.

SMITH, P.M., STUDLEY, F. & WILLIAMS, R. (1969) Postulated gastric factor enhancing iron absorption in haemochromatosis. Brit. J. Haemat. 16: 443-450.

SMITH, P. & WILLIAMS, R. (1968) Gastric factor in iron absorption. Lancet 1: 824.

SORENSON, E.W. (1967) Studies on iron absorption. The effect of bile and pancreatin on the absorption of iron. Acta Med. Scand. 181: 707-716.

STEINKAMP, R., DUBACH, R. & MOORE, C.V. (1955) Studies in iron transportation and metabolism VIII. Absorption of radioiron from iron enriched bread. Arch. Intern. Med. 95: 181-193.

TURNBERG, L.A. (1968) Gastric factor in iron absorption. Lancet, 1, 921.

TURNBULL, A., CLETON, F. & FINCH, C.A. (1962) Iron absorption IV. The absorption of haemoglobin iron. J. Clin. Invest. 41: 1897-1907.

U.S. COUNCIL ON FOODS AND NUTRITION (1968) J. Amer. Med. Ass. pp 109 & 203.

U.S. DEPARTMENT OF AGRICULTURE, AGRICULTURAL RESEARCH SERVICE, CONSUMER & FOOD ECONOMICS RESEARCH DIVISION (1969) Food intake and nutritive value of diets of men, women and children in the U.S., Spring 1965: A preliminary report. Washington D.C. (A.R.S. 62:18).

van HOEK, R. & CONRAD, M.E. (1961) Iron absorption. Measurement of ingested iron^{59} by a whole-body liquid scintillation counter. J. Clin. Med. 40: 1153-1159.

van WYCK, C.P., LINDER-HOROWITZ, M. & MUNRO, H.N. (1971) Effect of iron loading on non-heme iron compounds in different liver cell populations. J. Biol. Chem. 246: 1025-1031.

VAUGHAN, J.M. (1932) Anaemia following gastric operations. Lancet, 2: 1264.

WACK, J.P. & WYATT, J.P. (1959) Studies of ferrodynamics I. Gastrointestinal absorption of Fe^{59} under differing dietary states. Arch. Path. 67: 237-242.

WALKER, A.R.P. & ARVIDSSON, U.B. (1953) Iron 'overload' in the South African Bantu. Trans. Roy. Soc. Trop. Med. Hyg. 47: 536-548.

WALLENSTEN, S. (1954) Results of the surgical treatment of peptic ulcer by partial gastrectomy according to Billroth I and II methods. Acta Chir. Scand. Supp. 191.

WALSH, R.J., KALDOR, I., BRADING, I. & GEORGE, E.P. (1955) The availability of iron in meat: some experiments with radioactive iron. Aust. Ann. Med. 4: 272-276.

WATT, B.K. & MERRILL, A.L. (1963) 'Composition of Foods'. Agricultural Handbook No. 8. U.S. Dept. of Agriculture, Washington.

WAXMAN, S., PRATT, P. & HERBERT, V. (1968) Malabsorption of haemoglobin iron inpernicious anaemia; correction with intrinsic factor containing substances. J. Clin. Invest. 47: 1819-1825.

WEINTRAUB, L.R., CONRAD, M.E. & CROSBY, W.H. (1964) The significance of iron turnover in the control of iron absorption. Blood 24: 19-24.

WEINTRAUB, L.R., WEINSTEIN, M.B., HUSER, H. & RAFAL, S. (1968) Absorption of haemoglobin iron: the role of a heme-splitting substance in the intestinal mucosa. J. Clin. Invest. 47: 531-539.

WHEBY, M.S., SUTTLE, G.E. & FORD, K.T. (1970) Intestinal absorption of Hb iron. Gastroenterology, 58: 647-654.

WHITE, H.S. & WHITE, P.L. (1968) Food and Nutrition News, 39: No. 7, p. 1.

WIDDOWSON, E.M. & McCANCE, R.A. (1942) Iron exchanges of adults on white and brown bread diets. Lancet 1, 588-590.

WINTROBE, M.M. (1968a) The erythrocyte response to increased demands. In 'Clinical Haematology' 6th ed. 95-96. Lea & Febiger.

WINTROBE, M.M. (1968b) Storage iron. In 'Clinical Haematology' 6th ed. 136. Lea & Febiger.

WINTROBE, M.M. (1968c) Myoglobin. In 'Clinical Haematology' 6th ed. 138-141. Lea & Febiger.

WITTS, L.J. (1930) Simple achlorhydric anaemia. Guy's Hosp. Rep. 80: 253.

WORLD HEALTH ORGANIZATION (1970). Technical Report Series 452.

WYNTER, C.V.A. & WILLIAMS, R. (1968) Iron-binding properties of gastric juice in idiopathic haemochromatosis. Lancet, 2: 534-537.

ZETTNER, A., SYLVIA, L.C. & CAPACHO-DELGADO, L. (1966) The determination of serum iron, and iron binding capacity, by atomic absorption spectroscopy. Amer. J. Clin. Path. 45: 533-540.

ADDITIONAL REFERENCES

BAILEY, N.T.J. (1964) In 'Statistical Methods in Biology'. English Universities Press.

BRISE, H. & HALLBERG, L. (1962a) Absorbability of different iron compounds. Acta Med. Scand. 171, (suppl. 376), 23-37.

BRISE, H. & HALLBERG, L. (1962b) Effect of ascorbic acid on ferrous iron absorption. Acta Med. Scand. 171, (suppl. 376), 51-58.

FINCH, C.A. (1959) Body iron exchange in man. J. Clin. Invest. 38: 392-396.

WORLD HEALTH ORGANIZATION (1959) Iron deficiency anaemia. Technical Report Series 182.

WORLD HEALTH ORGANIZATION (1968) Nutritional anaemias. Technical report series 405.