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THE ROLE OF NATURAL AND SYNTHETIC CHELATING AGENTS IN THE ALIMENTARY ABSORPTION OF IRON AND OTHER METALS IN MAN

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SUMMARY

Taken on a world basis, the number of people affected by iron deficiency anaemia is second only to those affected by protein deficiency. Intensive work over many years has failed to answer satisfactorily some fundamental questions concerning the mechanism of gastrointestinal iron absorption on the homeostatic control of the extent of iron absorption. In particular, it is not understood why iron is absorbed predominantly in the duodenum, the most alkaline part of the intestinal tract, for at high pH values iron salts precipitate as extremely insoluble hydroxides which readily form inert high molecular weight aggregates.

Many plants growing in alkaline soil become iron deficient and develop iron chlorosis. Spectacular success in controlling this disease has been achieved recently by the use of synthetic chelates of iron which, unlike organic iron salts, are stable and soluble even under alkaline conditions. The first part of this investigation, therefore, was designed to establish whether synthetic chelating agents can affect iron absorption in man. Studies were carried out on healthy human volunteers. Radioactive iron-59 and a whole body counter were used to determine loss of ingested radioactivity after 14 days. It was found that both the synthetic chelating agents ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) could profoundly depress iron absorption in man. The effect of varying the molar ratios of these chelating agents to iron was studied. It was found that when administered in a 1:1 ratio neither agent had any effect, whereas the absorption depressing effect was marked at ratios of 10:1 and 50:1.

Similar studies were carried out using the keto hexose fructose which was found to have no effect in 1:1 or 10:1 ratios, but which enhanced iron absorption when given in a 50:1 molar ratio with respect to iron. No effect on iron absorption was found in parallel studies with glucose which, unlike fructose, does not chelate iron strongly. In vitro studies were undertaken to explain the differing chelating ability of these two sugars. It was shown that the iron chelating ability of some sugars is related to the presence in their molecules of a dihydroxy acetone structure. This is a property shared by fructose and all other keto sugars but lacking in aldo sugars and disaccharides. On the basis of these experiments the iron chelating ability of the sugars tagatose and sorbose was predicted and subsequently demonstrated. The differing iron chelating ability of these groups of substances was proposed as a fundamental difference in the chemical properties of keto and aldo sugars.

These findings led to the question "Do chelating agents normally affect iron absorption in man?" The next phase of the investigation, therefore, was a study of the extent to which man encounters chelating agents in his food, in the intermediate or end products of food digestion, or in drugs. The possibility had to be considered that endogenous chelating agents could be produced. Before undertaking this work it was necessary to develop new methods and adapt existing ones because of the complex nature of the biological samples involved. Laboratory methods for the detection and characterisation of chelating agents include ~~ir~~on-exchange and thin layer chromatography, visible and ultraviolet spectrophotometry and a simple and sensitive radioiron solubility test.

Studies of food were concentrated on the carbohydrate amylopectin. This important component of the human diet was shown to react with saliva to form within 1 to 2 minutes a product which is a powerful iron chelator, yet further salivary hydrolysis caused rapid disappearance of iron chelating ability. This led to the proposal of the hypothesis: ptyalin acts in the intestinal transport and absorption of metals, not in the initiation of carbohydrate digestion.

Allopurinol and salicylic acid were the two drugs studied for their effects on iron absorption, Allopurinol, a xanthine-oxidase inhibitor was found to have no effect on iron absorption under the conditions of the experiments. It was concluded that mucosal xanthine-oxidase is not an important controller of the iron valency state. Salicylic acid, a powerful iron chelator, was found to have no effect on the absorption of ferrous or ferric iron. In vitro studies showed gastric juice bound iron even more strongly than salicylic acid, thus explaining the ineffectiveness of this agent. Therefore, studies of endogenous chelating agents were concentrated on human gastric juice.

Resolution of gastric juice by molecular sieving on Sephadex columns and by polyacrylamide disc gel electrophoresis demonstrated the presence of a high molecular weight iron-binding protein.

At this point in the investigation it had become clear that iron can be absorbed readily by the duodenal mucosa because it is in true solution as a chelate either of food or food breakdown substances, or linked to an endogenous chelating agent. In this soluble form iron would be free to engage in exchange and transport reactions. The question to be answered then became: "Why is not an excessive amount of iron absorbed?"

An indication that the role of the gastric juice iron-binding protein is to inhibit iron absorption has been provided by the observation that this substance is greatly reduced or

absent in subjects with idiopathic haemochromatosis.

It is concluded that the chelating agents present in food and formed as intermediate products of food digestion are an important part of the physiological mechanism which permits the absorption of iron from the alkaline environment of the duodenum. The endogenous chelating agents present in such body fluids as gastric juice may well serve as part of the control mechanism limiting iron absorption by operating as a negative feedback mechanism.

DECLARATION

I declare that this thesis is of my own composition and that it 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 by another person, except when due reference is made to such material in the text.

December, 1966.

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CHAPTER I

INTRODUCTION

Understanding of the normal mechanism of gastrointestinal absorption of a number of essential trace metals is poor. Such metals include iron, cobalt, manganese, molybdenum, copper and zinc. In nature, in both plants and animals, a number of overload and deficiency conditions of these metals is recognised. These abnormal states have implications in health and disease for humans and in agriculture. For example, iron deficiency occurs both in human beings and in plants. In the case of humans, iron deficiency ranks second only to protein deficiency as a nutritional world health problem (Coons, 1964; Witts, 1966), and has been the object of intensive study by the World Health Organization (1959).

Iron deficiency anaemia is a disease which is not confined to the poorer countries where nutritional deficiencies are often aggravated by the chronic blood loss resulting from hookworm infestation. In highly developed western countries caloric requirements for both men and women decrease as increasing use is made of machines and this often results in an insufficient dietary intake leading to iron deficiency anaemia (Hallberg, Högdahl, Nilsson and Rybo, 1966).

Iron overload states are associated with diseases such as haemochromatosis, haemosiderosis, Bantu siderosis and Kaschin-Beck disease (Beutler, Fairbanks and Fahey, 1963). An important cause of accidental death in young children is poisoning from ingestion of attractively coloured sugar coated ferrous sulphate tablets.

The prevalence and seriousness of these diseases underlines the need for a greater understanding of the mechanism of gastrointestinal iron absorption in man, and in particular, of the control mechanism which regulates the extent of absorption. The need for a similar understanding of the absorption mechanism of other metals mentioned above is related not only to the diseases associated with their deficiency or excess but also to the existence of increasing quantities of their radioactive isotopes. This poses a potential hazard to populations exposed to these isotopes as nuclear reactors become more widespread and radioisotopes are more extensively used in research and industry.

Iron is a ubiquitous element, the fourth most abundant in the earth's crust, comprising 4.7 per cent of the lithosphere. It is the most abundant element if the total substance of the planet is considered. Iron is an essential nutrient not only for animals but for plants and microorganisms as well. A problem of great economic importance in horticulture is the inability of many plants growing on alkaline soils to obtain sufficient iron for the normal production of chlorophyll. The resultant disease of iron chlorosis in mild form greatly impairs the growth and fruit bearing capacity of the plants and if unchecked often kills the plant. It is rare for there to be an absolute shortage of iron in any soil type and iron chlorosis is therefore caused by an unavailability of iron, usually because of alkaline conditions in the soil (Wallace, 1962, 1966). At high pH all inorganic iron salts form the extremely insoluble ferrous or ferric hydroxides. These hydroxides readily undergo partial hydrolysis leading to "olation" in which the metal ions are joined by bridging -OH groups. In this way high molecular weight aggregates are built up and form polymer precipitates which become increasingly chemically inert as, with ageing, they form iron oxides which are not available to the living organism (Perrin, 1964).

A spectacular advance in the treatment of iron deficiency diseases in plants was pioneered by Stewart and Leonard and co-workers in 1952. Their work was concerned largely with the citrus crops in Florida and is discussed by Chaberek and Martell (1959). Control of iron chlorosis was achieved through application to the soil of synthetic chelates of iron such as ethylenediaminetetraacetic acid (EDTA), hydroxyethylethylenediaminetriacetic acid (HEDTA), and diethylenetriaminepentaacetic acid (DTPA). These compounds react with iron salts to form metal chelate compounds which remain soluble even at alkaline pH. When applied to the soil they are taken up by the plant roots and translocated to the leaves intact. In the leaf these metal chelate compounds are subject to photodecomposition resulting in the liberation of iron where it is needed to play its role in chlorophyll biosynthesis (Wallace, 1962, 1966).

Advances in the control of trace metal deficiency conditions in plants have been brought about in two ways by the use of synthetic chelate complexes. First of all, the synthetic metal chelate compounds of a variety of essential trace metals are being used experimentally and commercially for the successful control of metal deficiencies in plants. More important, however, has been the growing awareness and understanding of biological chelation phenomena which have come about through the use of these substances as experimental tools. Ultimately, one would hope to see the widespread agricultural use of these relatively expensive substances decrease as increasing understanding pointed the way to agricultural practices which made use of the natural biological chelates formed by soil microorganisms and plants themselves when grown correctly.

Surprisingly, although synthetic chelating agents have been used extensively in horticultural research and practice since 1952, comparatively little use has been made of these substances in medicine. Most of the work that has been done has been concerned with the parenteral administration of chelating agents such as British Anti-Lewisite (BAL: 2,3-dimercaptopropanol), Penicillamine (β,β-dimethylcysteine), and recently Desferrioxamine for the mobilisation and excretion of unwanted metals encountered in metal overload conditions such as haemochromatosis, Wilson's disease (a clinical condition characterised by copper overload), or poisoning by heavy metals or radioactive fission products. Little information is available on the effect of orally administered chelating agents on the gastrointestinal absorption of metals in man, and indeed, the few reports in the literature are in conflict. Some investigators have reported that EDTA will impair iron absorption (Brise and Hallberg, 1962) while others have proposed the use of iron-EDTA as a haematinic for the promotion of iron absorption (Lapinleimu and Wegelius, 1959; Hodgkinson, 1961; Herridge, 1958; Will and Vilter, 1954). Bothwell and Finch (1962, p. 96) say in this regard "Iron bound to synthetic chelates such as versenate is well absorbed by plants and a unicellular organism has been described that secretes into the surrounding medium a substance that facilitates iron absorption. It is, therefore, tempting to propose that a mechanism might exist in man by which some chelating substance is secreted into the lumen and binds the iron prior to its absorption. At present there is no experimental evidence to support or exclude such a concept."

A further observation which prompted the study reported in this thesis was the recent discovery by Saltman and his co-workers that the naturally occurring hexose sugar fructose is capable of forming a highly stable chelate complex with iron (Charley, Sarkar, Stitt and Saltman, 1963) and can facilitate the movement of iron across biological membranes (Stitt, Charley, Butt and Saltman, 1962). Because of their importance as foodstuffs the effect of fructose and other sugars on iron absorption in man is of obvious importance.

Because of the high incidence of diseases of iron metabolism and the basic questions concerning the mechanism of its absorption which remain unanswered, it was decided to concentrate the study on the metal iron. The developing objects of the investigation reported in this thesis were first of all to answer a number of specific questions:

i. Do orally administered synthetic chelating agents affect the gastrointestinal absorption of iron in man?

ii. How are the conflicting literature reports to be explained?

iii. Does fructose enhance iron absorption in man?

iv. Does glucose enhance iron absorption in man?

v. Why should fructose, but not glucose, strongly chelate iron?

vi. In practice, does man encounter metal chelating agents in his diet, in drugs, or does he manufacture and secrete them into the gut lumen?

It was hoped that answers to these questions might shed further light on the mechanism of iron absorption and on the physiological control mechanism regulating the extent of iron absorption.

CHAPTER II

LITERATURE REVIEW

1. Gastrointestinal Iron Absorption

Reviews of the voluminous literature on iron absorption have recently been published by Josephs (1958), Callender (1959), Beutler (1961), Bothwell and Finch (1962), Beutler, Fairbanks and Fahey (1963), Coons (1964) and Callender (1964). It is proposed here merely to draw attention to certain salient points concerning iron absorption which have had relevance to the present investigation.

In the healthy human body a series of physiological mechanisms operates to maintain homeostasis. Where metals such as sodium, potassium, calcium or magnesium are concerned any unwanted excess which may occur within the body is removed by excretion, usually via the kidneys or via the intestine in the faeces. In 1937 McCance and Widdowson made the classic observation that no mechanism existed for the excretion of an unwanted excess of iron from the body. They postulated that the amount of iron in the body is regulated by controlled absorption, and pointed out that parenteral injection of iron is not followed by increased urinary or faecal excretion of the metal. Because of this peculiar behaviour of iron much research work in the last 30 years has been concentrated on the role of mucosal absorption in regulating the body's iron content.

Published studies of iron absorption fall into three categories according to whether the sources used were simple ferrous or ferric compounds, iron incorporated into foodstuffs, or haemoglobin iron. It appears that there are important differences in the mode of absorption of these different forms of iron. In any event, it is to be noted that the normal dietary intake of iron is about 15 mg. per day, that is, about 5 mg. per meal. Much of the earlier experimental work was carried out with iron doses of 100 to 800 mg. of iron which lie well outside the normal dietary range, and results of such work must be applied with great caution to the normal physiological situation.

Furthermore much of the experimental work on iron absorption, including some experiments reported in this thesis, have been carried out using laboratory animals and not on man. Often these experiments have been performed on everted or isolated loops of intestine from which the natural excretions have been washed out. It cannot be too strongly emphasised that it might not be appropriate to extrapolate the results of such experiments to the human situation. The danger is especially real with experiments using iron as its chemical and therefore metabolic behaviour depends largely on its valency state and type of chemical combination. These in turn are governed by the luminal environment with respect to pH, redox potential, other chemical species present, residence time of intestinal contents in different regions of the gut and quantity of bacterial flora. There are great species variations in these factors.

Iron is liberated from food during digestion in the stomach. Sanford (1960) presented results of a study of the effects of in vitro acid-peptic digestion on a number of cooked and uncooked food substances. The effects of saliva and substances present in gastric juice other than pepsin and hydrochloric acid were not considered in this work which was aimed at determining the amount of iron released from food under the experimental conditions used. Sanford's work shows clearly that acid-peptic digestion releases a large proportion of the iron from cooked foodstuffs but is relatively ineffective with raw foodstuffs.

Iron absorption by mucosal cells occurs predominantly in the duodenum (Gabrio and Salomon, 1950; Granick, 1946; Wheby and Crosby, 1963) which is the most alkaline part of the gastro-intestinal tract. Iron absorbed by mucosal cells is either retained in the body when it is passed on to transferrin, the iron binding protein of blood plasma, or it is lost to the body when the mucosal cell is exfoliated at the end of its life span (Crosby, 1963; Conrad and Crosby, 1963; Conrad, Weintraub and Crosby, 1964; Wheby and Crosby, 1963; Wheby, Jones and Crosby, 1964).

Ferrous iron is absorbed more readily than ferric iron in the rat (Groen, Van Den Brock and Veldman, 1947; Venkatachalam, Brading, George and Walsh, 1956). Brise and Hallberg (1962) investigated this phenomenon in man and found that with a dose of 5 mg. of iron there was no difference in the extent of absorption of ferrous and ferric iron. With increasing doses, progressively greater differences were noted (3, 4, 5 and 7 times as much ferrous as ferric iron were absorbed when doses of 30, 90, 150 and 210 mg. respectively were given).

With inorganic iron, the greater the dose administered, the more will be absorbed, although the percentage will drop (Smith and Pannacciulli, 1958).

When the absorption of inorganic and haemoglobin iron is compared in the same individuals it is found that haemoglobin iron is absorbed only about half as well (Callender, 1964). Haemoglobin iron is absorbed at a different rate from inorganic iron, the peak of absorption being considerably later than with inorganic iron (Turnbull, Cleton and Finch, 1962; Bannerman, 1965; Conrad, 1966). Substances which interfere with the absorption of inorganic iron do not appear to influence the absorption of haemoglobin iron, namely, phyates (Turnbull et alii, 1962), desferrioxamine (Hwang and Brown, 1965).

Iron in foodstuffs is, in general, not absorbed as well as inorganic iron even when the need for iron is great. The extent of absorption of food iron has been studied extensively by Moore and co-workers (Moore, 1960). Ascorbic acid has been shown to enhance the absorption of inorganic iron (Brise and Hallberg, 1962) and food iron (Chodos, Ross, Apt, Pollycove and Halkett, 1957; Moore and Dubach, 1951; Pirzio-Biroli et alii, 1958) but not haemoglobin iron.

In summary it may be said that regulation of the iron content of the body is effected not by excretion of excess but by restriction of absorption. There appears to be no physiological mechanism for ridding the body of unwanted excess iron.

The iron content of the body is 4 to 5 gm. Porphyrin compounds account for about 70 per cent (haemoglobin 65 per cent, myoglobin 3 to 5 per cent); storage iron 30 per cent (ferritin and haemosiderin); and the rest is found in enzymes (cytochromes, catalase, peroxidase, xanthine oxidase etc.) and in the serum bound to the transport protein transferrin (Bothwell and Finch, 1962).

In the healthy adult male the daily loss of iron - about 0.6 mg. - is through urine, sweat, exfoliated cells from the skin and gastrointestinal tract, and possibly bile. In the female, iron losses can be greatly increased through menstruation, pregnancy and lactation. Typical losses for a menstruating woman are about 1.2 mg. per day, although Price, Forsyth, Cohn and Cronkite (1964) have shown that menorrhagia can result in losses as great as 7 mg. per day. During pregnancy, iron losses increase to a maximum of about 2 mg. per day. Iron losses during lactation are related to the volume of milk and the duration of lactation. Human milk contains 0.5 mg. Fe per litre.

It should be emphasised that the outstanding source of iron loss is bleeding. The iron content of the blood is 500 mg. per litre and appreciable iron loss can occur because of chronic and unnoticed bleeding into the intestinal lumen.

Dietary iron. Tables of iron content of different foodstuffs are available from many sources. Such a one, after Bothwell and Finch (1962) is given in Table 1, and the figures for the iron content of carbohydrate materials are given in Table 2 (Diem, 1962). The value of such data is limited by the following considerations: the iron content of food materials varies greatly ~~greatly~~ according to the soil type and agricultural practice; the iron content of prepared food depends on the food processing, the parts discarded during

Table 1

Iron Content of Foodstuffs*

Foodstuff	Fe mg./100 gm.
Refined sugar	0
Milk	0.1
Fruit	0.4 to 0.8
Potatoes	0.7 to 1.0
Rice	0.9 to 1.4
Corn, maize	1.8 to 2.3
Wheat	1.0 to 4.0
Muscle meats	1.5 to 2.5
Leafy vegetables	1.1 to 2.9
Eggs	2.5
Legumes	4.0 to 8.0

*from Bothwell and Finch (1962)

Table 2

Iron Content of Carbohydrate Materials*

Foodstuff	Fe mg./100 gm.
Flour, corn, whole, unbolted	2.4
Flour, rice	0.9
Flour, rye, dark	4.8
Flour, wheat, whole	2.3
Flour, wheat, white, unenriched	0.7
Flour, soybean, full fat	12.1
Flour, soybean, medium fat	13.0
Macaroni, unenriched, dry	1.5
Noodles, unenriched, dry	2.1
Oatflakes	5.2
Cornflakes	1.0

*from Diem (Ed.) Documenta Geigy-Scientific Tables (1962) preparation, the type of cooking and the chemical composition of the cooking utensils used; the availability of iron appears to differ from one foodstuff to another (Moore, 1960).

In concluding this brief discussion of the literature of iron absorption it is worth noting the remarks of Bothwell and Finch (1962, p.104) concerning the areas of ignorance in this field: "Although the studies that have been done on the absorption of food iron provide a foundation for understanding some of the quantitative aspects of iron balance, present information is still fragmentary, and virtually nothing is known of the absorption of iron from the great staple foodstuffs of the world such as rice and wheat."

2. The Chemistry of Iron

Most of the essential trace metals including iron, manganese, cobalt, copper and molybdenum are transition elements and have many chemical properties in common. Some particular aspects of transition element and iron chemistry have been singled out for discussion below, either because they form the basis of experimental methods used throughout this study, or because they are of help in relating molecular structure to biological function.

The transition elements are defined as those elements in which the two outermost shells of orbital electrons are incomplete. More precisely, it can be said that they are the elements the atoms or ions of which contain partially filled d orbitals and which are characterised by the participation of d orbitals in chemical bond formation (Larsen, 1965).

The transition metals are denser and have higher melting points than the nontransitional metals such as potassium or calcium. They show a wide range of valency states and thus are an important source of oxidising and reducing agents. Their salts are usually coloured, unlike those of the non-transitional metals and their compounds exhibit the property of paramagnetism - that is they are drawn into a strong magnetic field. These elements also exhibit the property of combining with a wide variety of organic ligands to form coordination compounds and metal chelate complexes. The transition metals and their compounds possess marked catalytic properties in both chemical and biological systems. All these properties are related to the unpaired electrons in the unfilled d shell.

Iron has two common valency states, Fe^{++}-ferrous and Fe^{+++}-ferric. The ferric state is the more stable in solution. In aqueous solution metallic ions do not exist as such but are hydrated. The number of water molecules bound by the metal is the coordination number, which for both Fe^{++} and Fe^{+++} is six.

$$Fe\ Cl_3 + 6H_2O \rightarrow [Fe^{111}(H_2O)_6]^{+++}Cl^-{}_3$$

$$Fe\ Cl_2 + 6H_2O \rightarrow [Fe^{11}(H_2O)_6]^{++}Cl^-{}_2$$

These hydrated cations are acids because they can react with water to produce protons:

$$[Fe^{111}(H_2O)_6]^{+++} + H_2O \rightleftharpoons [Fe^{111}(OH)(H_2O)_5]^{++} + H_3O^+$$

As the pH of a ferric solution increases, the insoluble hydroxide is formed. The solubility product of ferric hydroxide is such that when the pH of a 0.001 M. solution of a ferric salt is raised above 2.4 precipitation occurs. Hydroxyl groups are able to act as bridges in the formation of polymeric species; this is the olation reaction. Such polynuclear complexes are formed relatively slowly but when formed are often extremely inert chemically.

The commonly encountered compounds of iron that have the lowest solubilities are the hydroxide and sulphide. These chemical properties of iron are summarised in Table 3.

Radioactive iron. The properties of the radioactive isotopes of iron are summarised in Table 4. In all the work reported in this thesis the radioactive isotope Fe^{59} has been used and has been detected and counted either by its β radiations

Table 3

Properties of Iron

Symbol: Fe   Atomic Number: 26   Atomic Weight: 55.847(1)

Natural abundance of elements in earth's crust:(2)

Element	% Abundance in lithosphere
O	49.4
Si	25.75
Al	7.51
Fe	4.7

Iron content of human body = 4 gm.

Iron concentration in human body = 4 gm./70 kg. = 0.006%.

Standard potential values of iron (2)

Reaction	E° (v)
$Fe^{2+} + 2e^- \rightleftharpoons Fe$	- 0.440
$Fe^{3+} + e^- \rightleftharpoons Fe^{2+}$	0.771
$Fe(OH)_3 + e^- \rightleftharpoons Fe(OH)_2 + OH^-$	- 0.56
$Fe(OH)_2 + 2e^- \rightleftharpoons Fe + 2\ OH^-$	- 0.877

Compounds of low solubility

Compound	Solubility product(2)
Ferrous sulphide	$3.8.10^{-20}$
Ferrous hydroxide	$1.64.10^{-14}$
Ferric hydroxide	$1.1.10^{-36}$

(1) Based on Carbon-12.

(2) Kolthoff and Elving (1962).

Table 4

Isotopes of Iron

Naturally occurring stable isotopes

Fe isotope	% Abundance
54	5.82
56	91.66
57	2.19
58	0.33

Radioactive isotopes of iron

Fe isotope	Half life	Mode of Decay, Energy (Mev), % of Disintegrations
52	8.3 hr.	β^+ 0.80(2.63)[40%], K capture [60%]; γ 0.17 [100%
53	8.9 min.	β^+ ~2.8,7.4,1.6; γ 0.38
55	2.7 yr.	K capture 0.0059 No γ
59	45 d.	β^- 0.46,0.27,1.56 γ 1.10,1.29,0.19
60	~10^5 yr.	β^- 0.13(0.3) γ 0.027(1.33,1.17,0.059)
61	6.0 min.	β^- 2.8; γ 0.29

( ) = Radiations from short lived daughter.

[%] = % of disintegrations.

in a gas flow proportional counter or by its γ photons using a sodium iodide crystal scintillation counter.

Coordination chemistry of iron. Much of the biological importance of iron is related to its ability to form coordination complexes. Present day knowledge of the structure and nature of these complexes has been gained over the last 75 years from the work of many chemists. Outstanding among these have been Werner, Sidgwick, Pauling and Orgel. Monographs which give good accounts of recent developments have been written by Pauling (1960) and Orgel (1960). Once again, a few topics will be discussed here because of their importance; no attempt will be made to give a comprehensive account.

Iron has a coordination number of six and the valence bonds are directed to the six corners of a regular octahedron - this feature serves to orient the molecules that interact while bound to iron and thus influences the stereochemistry of such reactions. A typical coordination compound of iron is potassium hexacyanoferrate^{III} $K_3Fe(CN)_6$. In this compound the trivalent iron atom is bound by coordinate covalent links to the six $(CN)^-$ groups. As a result, the iron atom is incorporated into an anion $[Fe(CN)_6]^{---}$. This is an important consequence of complex formation and, according to the nature of the ligands concerned, so iron may be present in solution as a cation, an anion or as a molecule with no charge.

The atomic number of iron is 26 and the configuration of its orbital electrons is $1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^6\ 3d^6\ 4s^2$. This electronic arrangement for the iron atom and those for ferrous and ferric ions are set out in Table 5. Such an electronic configuration of a partially filled d orbital coupled with the small ionic radii of Fe^{++} and Fe^{+++} (Table 5) makes for ready formation of stable complexes. The electrons in the 3d shell are in five orbitals, each of which is capable of accommodating two electrons of opposite spin, so that a total of ten electrons would be found in a completed 3d shell. In iron the 3d shell is incomplete, having only five electrons in Fe^{+++} and six electrons in Fe^{++}. The five orbitals in which these electrons are placed differ in their directional properties in such a way that only two orbitals point in the direction of the corners of the coordination octahedron. The electrons in these two orbitals will point in the direction of the ligands and will repel the electrons on the ligands. The electrons in the remaining three orbitals will have zero electron density in the direction of the ligands and there will be consequently relatively little repulsion between these electrons and the ligands.

Of the five orbitals available to the iron 3d electrons, two of them will therefore have a higher energy than the other three.

Table 5 - Electronic Configuration

	1s	2s	2p			3s	3p			3d					4s	No. of unpaired electrons
Fe	2	2	2	2	2	2	2	2	2	2	1	1	1	1	2	4
Fe^{++}	2	2	2	2	2	2	2	2	2	2	1	1	1	1	0	4
Fe^{+++}	2	2	2	2	2	2	2	2	2	1	1	1	1	1	0	5

Radii (1)

Atomic Covalent Radius Fe	1.16 Å
Ionic Radius Fe^{++}	0.83 Å
Ionic Radius Fe^{+++}	0.67 Å

(1) Heslop and Robinson, p 463.

Electrons will consequently tend to fill the three lower energy orbitals before any of them go into the two higher energy orbitals.

Another effect, which is counter to the ligand repulsion phenomenon described above, must be considered. According to Hund's rule, two electrons in the same orbital repel each other so that all orbitals tend to be singly occupied before any of them are filled by two electrons. The resolution of these effects is explained by the Ligand Field Theory (Orgel, 1960). According to the ligand field theory ligands can be arranged in increasing order of ligand field splitting energy. Such an arrangement of ligands is found to accord closely to the spectrochemical series: I^-, Br^-, Ce^-, OH^-, F^-, H_2O, NH_3, CN^-. If the ligands are held strongly enough, the repulsion between the ligand electrons and the higher energy orbitals is sufficient to overcome the Hund repulsion between electrons in the same orbital and a strong field or spin-paired complex is formed. If, however, the ligand repulsion is weaker than the Hund effect, a weak field or spin-unpaired complex is produced instead. The electronic structures of the strong field and weak field complexes of ferrous and ferric iron are shown in Table 6 which is taken from Eichorn (1964).

The practical importance of this approach is that the

Table 6

Electronic Configuration of 3d Orbital in Weak and Strong Field Iron Complexes(1)

	Fe^{++}		Fe^{+++}	
	Weak	Strong	Weak	Strong
High energy orbitals	(X	-	X	-
	(			
	(X	-	X	-
Low energy orbitals	(X	X X	X	X
	(			
	(X	X X	X	X X
	(			
	(X X	X X	X	X X

(1) Eichorn (1964).

weak and strong field complexes of ferrous and ferric iron all have different numbers of unpaired 3d electrons. By empirical determination of the magnetic moment of any iron compound it is possible to determine its electronic configuration and thus correlate molecular structure with biological function.

3. Iron Chelation Chemistry

When in a coordination compound the ligand is attached to the metal through two or more atoms, so that the metal forms part of a heterocyclic ring structure, the compound is called a metal chelate complex. This graphic term was coined by Morgan and Drew in 1920 who derived it from the Greek χηλαι - the great claw of the lobster and other crustaceans. A chelating agent, therefore, is a molecule that is capable of seizing and holding a metal ion in a clawlike grip, the clutching structure forming a ring in which the ion is held by a pair of pincers. The pincers consist of ligand atoms (usually oxygen, nitrogen or sulphur), each of which donates an electron pair to form a coordinate bond with the metal ion. A chelate may be bi-, tri-, quadri-, quinqui- or multidentate according to the number of donor atoms complexing the metal ion. Bidentate chelates with 5- or 6-atom member rings are the most stable, fewer atoms in a ring imposing strain because of distortion of bond angles.

Metal chelate compounds differ profoundly in their properties from simple salts of metals and even from metal coordination complexes (that is, complexes with unidentate ligands). The most outstanding property of the metal chelates is their great stability compared with that of other compounds of the metals. This increase in stability derives from the "chelate effect", explanations of which are given by Chaberek and Martell (1959) and Mellor (1964).

Specific effects of chelation such as alteration of absorption spectrum, effects on solubility, redox potential and charge on the metal will be considered later when the design of particular experiments and the development of experimental methods is discussed.

Early studies of chelation phenomena were concerned for the most part with the simple compounds that could be synthesized by the chemist. Monographs which give good accounts of various areas of non-biological chelation chemistry include those of Martell and Calvin (1962), Welcher (1961), Dwyer and Mellor (1964), and Perrin (1964). More recently it has been realised that metal chelate bonding by small and macromolecules occurs widely in nature; examples include chlorophyll, haemoglobin, cyanocobalamine, transferrin, caeruloplasmin and many enzymes. Biologists have increasingly been using synthetic chelating agents for a variety of purposes in living systems. These biological aspects of chelation phenomena are reviewed by Chaberek and Martell (1959), Seven (1960), Johnson and Seven (1961), Gross (1964) and Schubert (1966).

The stability of a metal chelate complex is expressed quantitatively by a stability or formation constant. In the simplest case of a reaction between a metal ion M and a ligand L: $M + L \rightleftharpoons ML$

the stability constant K_{ML} is given by:

$$K_{ML} = \frac{[ML]}{[M][L]}$$

where the bracketed symbols represent the activities of the product and the reactants at equilibrium. Under physiological conditions the extent to which a given metal chelate complex is actually formed depends not only on the concentrations of the metal ion [M], and the ligand [L] and the stability oonstant K_{ML} but is affected by competition for the ligand by other metals (predominantly calcium) and, possibly, by competition for the metal M by other ligands. Frequently other reactions have to be considered, such as insoluble metal hydroxide formation. In this complex situation use is made of an effective formation constant which is calculated by the use of alpha coefficients or side reaction coefficients which afford a measure of the competing side reactions. An excellent treatment of the use of stability constants and effective formation constants is given by Schubert (1964). A more formal account of the subject is given by Martell and Calvin (1962).

Another characteristic of chelation reactions is the rate of formation which is dependent on the lability or inertness of the complex and is independant of the thermodynamic stability or instability of the complex. For example, it is an observed fact that most chromium complexes are very inert in contrast to most ferric complexes which are labile. The significance of this in the practical situation is that whereas a given metal may rapidly exchange from one chelating agent to another for which it has a greater affinity, another metal may fail to exchange in the time available for the reaction.

The complexities introduced by competing side reactions and varying reaction rates are compounded when the situation in the gut lumen is being studied for here the pH is continually varying, the nature and concentration of the reactants alters as digestion proceeds and the time available for reaction is related to intestinal motility. For these reasons it has been found necessary to resort to more empirical methods in the present investigation and it has rarely been possible to predict the outcome from a consideration of relative stability constants.

CHAPTER III

THE EFFECT OF SYNTHETIC CHELATING AGENTS ON IRON ABSORPTION

1. Purpose and Plan of the Investigation

Variable results have been reported of the effect of orally administered chelating agents on the gastrointestinal absorption of iron in man. Lapinleimu and Wegelius (1959), Hodgkinson (1961), Herridge (1958) and Will and Vilter (1954) studied the effect of EDTA on the absorption of iron in man and concluded that the iron-EDTA complex was absorbed as well as ferrous sulphate or ferrous gluconate. Indeed, proprietary preparations of this compound are sometimes used in the treatment of iron deficiency anaemia. Brise and Hallberg (1962) using their elegant double isotope technique also studied this problem and found that iron absorption was depressed by chelation with EDTA.

Animal experiments have done little to resolve the confusion. Larsen et alii in two studies (1960a, 1960b) found that iron absorption was depressed when EDTA was fed to rats; however, Seeberg, Hidalgo and Wilken (1954) found that anaemia was corrected in rats equally well whether Fe-EDTA or $FeSO_4$ was used.

Another chelating agent, desferrioxamine, has been used to prevent or diminish the absorption of iron in accidental poisoning by iron compounds (Henderson, Vietti and Brown, 1963). Although desferrioxamine has been shown greatly to reduce the absorption of ferrous sulphate (Bannerman and Malpas, 1965), it is ineffective in blocking the absorption of radioiron incorporated in haem (Hwang and Brown, 1965).

An understanding of the chemical behaviour of chelate compounds in biological systems might provide an explanation for these variable results. For example, many iron chelates will dissociate at the low pH encountered in the stomach (Welcher, 1961), and can only reform when the luminal pH rises on passing into the duodenum. If the liberated iron were free to reform in the duodenum only a stoichiometric ratio of iron to chelating agent is needed. However, if other ligands are present in gastric juice or other intestinal secretions these could compete with the original chelating agent for iron. In this competitive situation it is to be expected than an excess of chelating agent over iron would be needed for any effect of the chelating agent on absorption to be produced.

The present study was designed to determine the effect on absorption of increasing amounts of chelate in relation to iron. The synthetic chelates EDTA and DTPA were used.

2. Material and Methods

Forty-four radioiron absorption tests were carried out on 11 haematologically normal adult volunteers, each of whom served as his own control. Blood was taken from each subject for a complete blood picture, serum iron and latent iron binding capacity.

A dose of 2 μCi of Fe^{59} with 5 mg. of iron as ferrous sulphate, 50 mg. of ascorbic acid, the test substance when used, and 200 ml. of water were given orally after an over-night fast. Each person had four studies over a period of 10 weeks in which a 0:1, 1:1, 10:1, and 50:1 molar ratio of chelate to iron was administered. The chelating agents used were the disodium dihydrogen salt of EDTA and the calcium trisodium salt of DTPA. The four tests were carried out on each subject in a random sequence.

The total amount of radioactive iron administered to each subject was 8 μCi. If all four doses were completely absorbed the total irradiation of the marrow would be 0.35 rads (Bothwell and Finch, 1962).

The absorption of Fe^{59} was measured in the whole body counter of the Anti-Cancer Foundation of the University of Adelaide (Figures 1 and 2). The technique of measuring iron absorption by whole body counting offers great advantages of

Figure 1: Whole Body Counter

View shows steel room with door open and at right multichannel analyser and electronic equipment racks.

Figure 2: Whole Body Counter

View of interior showing scintillation detectors mounted on circular carriage and sliding bed for the subject.

simplicity and accuracy over the earlier chemical iron balance methods, the method of determining the appearance of absorbed radioactivity in circulating red cells, or the measurement of the faecal excretion of unabsorbed radioisotope. The whole-body counting technique measures directly the body's retention of orally administered Fe^{59}. The technique has been described by Price, Cohn, Wasserman, Reizenstein and Cronkite (1962) and in a number of papers presented at a symposium on whole body counting held in Vienna in 1962 (International Atomic Energy Agency, 1962). A description of the Adelaide whole body counter has been given by Deller (1965) and by Deller, Worthley and Martin (1965).

Absorption of Fe^{59} was measured in the whole-body counter after 14 days had been allowed for the excretion of unabsorbed radioactivity. The subject being "counted" was placed on a couch surrounded by four sodiumiodide (thallium activated) scintillation crystals of diameter 4.5 inches and length 2 inches, each viewed by a 5 inch photomultiplier tube. The four scintillation detectors were mounted on a carriage which was motor driven to scan the full length of the subject with pauses at the head and feet. The crystal carriage movement was controlled by a timer so that after a

1.6 minute pause at the head the detectors scanned the length of the subject's body in the next 12 minutes and the count was completed by a 1.6 minute pause at the feet. The end-pause scan method produces a quasi-4π detector system which makes the counts per scan independent of the distribution of radioactive material within the body. As a result the "100% value" can be determined immediately after ingestion of the radioactive test substance (Deller, 1965).

Background radioactivity was minimised by housing the whole assembly in a 6 inch thick steel castle of internal dimensions 8 feet by 5 feet 5 inches.

Pulses from the four detectors were passed to an adding circuit, linear amplifier, single channel analyser and scaler/timer. The setting used for the single channel analyser was such as to admit pulses of emergy 0.8 - 1.7 MeV.

The assumption made in multiple tests of absorption using radioiron and a whole body counter is that Fe^{59} absorbed from a previous test is not excreted from the body during subsequent tests. Because of the relative novelty of the method used in this investigation of measuring absorption on multiple occasions on the same subject, precise details of the technique adopted are given here (Davis and Deller, 1966a).

Calculation of iron absorption in multiple tests. On the first day (Day 0) the subject was counted as described above prior to the administration of the labelled iron. This first counting result (DO.A) was taken as a measure of natural body radioactivity and instrumental background and was assumed not to alter throughout the period of the experiment. After the oral administration of the isotope, the whole body activity was again measured (DO.B) to establish the 100% value. After 14 days, when the unabsorbed radioactivity had been excreted, the subject returned and was again counted (D14.A).

In order to correct for day to day variations in overall counting efficiency, a Co^{60} standard source was counted before and after counting the subject at each visit. The mean Co^{60} standard count (Co^{60}.DO) was used to give a measure of the machine counting efficiency at each visit, and counts obtained at subsequent visits were corrected to conform with the counts obtained at the first visit.

$$D14.A = \underline{D14.A} \times \frac{Co^{60}.0}{Co^{60}.14}$$

or in general $$Dn.A = \underline{Dn.A} \times \frac{Co^{60}.0}{Co^{60}.n}$$

The absorption of radioiron, and therefore total iron, administered on Day 0 (%R) is obtained from the relationship:

$$\%R = \frac{(D14.A - DO.A)}{(DO.B - DO.A)} \times M^{0/14} \times 100\%$$

where $M^{A/B}$ is the decay correction for Fe^{59} ($t_{\frac{1}{2}} = 45.1$ days) for the elapsed period A to B (days).

After the measurement D14.A is carried out, the subject is immediately given the second test dose of radioiron and is again counted (D14.B). He returns on Day 28 and is again counted (D28.A). The absorption of the second dose of radioiron, administered on Day 14, is given by the relationship:

$$\%R = \frac{D28.A - \dfrac{DO.A + D.14A - DO.A}{M^{14/28}}}{(D14.B - D14.A)} \times M^{14/28} \times 100\%$$

In similar fashion the absorption of the third dose, administered on Day 28, is given by:

$$\%R = \frac{D42.A - DO.A + \dfrac{D28.A - DO.A}{M^{28/42}}}{(D28.B - D28.A)} \times M^{28/42} \times 100\%$$

The absorption of the fourth dose, administered on Day 42, is given by:

$$\%R = \frac{D56.A - DO.A + \dfrac{D42.A - DO.A}{M^{42/56}}}{(D42.B - D42.A)} \times M^{42/56} \times 100\%$$

<u>Urinary excretion of absorbed radioiron.</u> Urinary excretion of radioiron was measured in four volunteers as the fraction of the test dose excreted in each of the two 24 hour periods following the oral ingestion of 5 mg. of iron labelled with 7 µCi of Fe^{59} and present as the appropriate chelate. In each case the iron was accompanied by a fifty-fold molar excess of the chelating agent used and 50 mg. of ascorbic acid. The total volume of the test dose was 200 ml.

3. Results

Effect of orally administered EDTA on iron absorption

Four tests of radioiron absorption were carried out on each of six subjects. In these tests the molar ratios of EDTA:iron used were 0:1 (control), 1:1, 10:1 and 50:1. The results are shown in Table 7. All subjects showed a significant reduction of iron absorption when the iron was accompanied by 50:1 EDTA and 10:1 EDTA ($p > 0.05$). 1:1 EDTA:iron did not significantly reduce iron absorption.

Effect of orally administered DTPA on iron absorption

Four tests of radioiron absorption were carried out on each of five subjects. In these tests the molar ratios of DTPA:iron used were 0:1 (control), 1:1, 10:1 and 50:1. The results are shown in Table 8. As was the case with EDTA, a 1:1 DTPA:iron preparation had no significant effect on iron absorption, whereas when the iron was accompanied by a 10:1 or 50:1 molar ratio of DTPA, iron absorption was significantly depressed ($p > 0.02$).

Urinary Excretion of Orally Administered Iron Chelates

In order to exclude the possibility that the iron absorption results given above were affected by a urinary excretion of chelated iron, an experiment was carried out to determine the extent of this excretion with each of the chelating agents used. The results listed in Table 9 show that in no case did the 48 hour urinary excretion of Fe^{59} exceed 1% of the dose administered.

4. Conclusion

The results presented here show quite clearly that in man the gastrointestinal absorption of inorganic iron can be profoundly depressed by the synthetic chelating agents EDTA and DTPA provided that a sufficient molar excess of chelating agent is present. The excess of chelating agent would seem to be essential if the iron-chelate is to be reformed after the iron leaves the acid milieu of the gastric lumen. This important requirement, which does not appear to have been recognised hitherto, could explain the conflicting results which have been published. The inability of workers such as Bannerman and Malpas (1965) and Hwang and Brown (1965) to demonstrate any blocking effect of desferrioxamine on the absorption of haemoglobin iron could well be related to the apparently low dosage of desferrioxamine. It would appear that in none of their experiments was a molar ratio of desferrioxamine to iron in excess of eight to one used.

Table 7

Effect of Orally Administered EDTA on Absorption of Iron

Subject No.	Sex	Molar Ratio EDTA:Iron	Absorption %			
			0:1	1:1	10:1	50:1
1	F		73	21	2	7
2	F		57	45	12	0
3	F		4	21	0	0
4	F		52	13	6	11
5	F		9	3	0	2
6	F		4	3	0	-*
	Mean absorption (%)		33	18	3	4

*Test not completed.

Table 8

Effect of Orally Administered DTPA on Absorption of Iron

Subject No.	Sex	Molar ratio DTPA:Iron	Absorption %			
			0:1	1:1	10:1	50:1
7	F		11	26	4	6
8	F		16	-*	8	0
9	F		20	9	3	3
10	F		8	33	0	3
11	F		27	7	23	2
Mean absorption (%)			16	19	8	3

*Test not completed.

Table 9

Urinary Excretion of Orally Administered Radioiron Chelates

Subject No.	Sex	Chelating agent	% of Oral Dose of Radioiron in urine	
			1st 24 hours	2nd 24 hours
12	M	EDTA	0.2	0.0
13	M	EDTA	0.8	0.2
14	M	DTPA	0.0	0.0
15	M	DTPA	0.4	0.1

CHAPTER IV

THE EFFECT OF FRUCTOSE AND GLUCOSE ON IRON ABSORPTION

1. Purpose and Plan of the Investigation

The keto hexose sugar fructose has recently been shown by Charley, Sarkar, Stitt and Saltman (1963a) to form a highly stable chelate complex with iron, and to increase iron absorption in experimental animals (Stitt, Charley, Butt and Saltman, 1962). No studies of the effect of fructose on iron absorption in man have been reported and because of the dietary importance of fructose as a constituent of honey, ripe fruits and sweet wines, it was decided to study the effect of increasing amounts of fructose on iron absorption in man.

Glucose does not form a stable chelate with iron but in many other ways is metabolically similar to fructose. In order to determine whether the effect of fructose was a general metabolic effect, perhaps through stimulation of an active transport mechanism, or was related to its chelating ability, a further experiment using glucose was carried out.

2. Material and Methods

Thirty-eight radioiron absorption tests were carried out on 13 haematologically normal volunteers. In the experiment with fructose a dose of 2 µCi of Fe^{59} with 5 mg. of iron as ferrous sulphate, 50 mg. of ascorbic acid, fructose where used and 200 ml. of water was given orally after an overnight fast. Each of the six volunteers had four studies over a period of 10 weeks in which a 0:1, 1:1, 10:1 and 50:1 molar ratio of fructose to iron was administered. The method of testing was randomised and each subject served as his own control. In the experiment with glucose each of the seven subjects studied had only two radioiron absorption tests: one, a control, in which a dose of 2 µCi of Fe^{59} with 5 mg. of ferrous sulphate and 50 mg. of ascorbic acid was given; the other, in which a fiftyfold molar excess of glucose to iron was added to a dose which was otherwise the same as the control dose.

Two radioiron urinary excretion tests were carried out on two volunteers who had been given 5 mg. of iron as ferrous sulphate labelled with 7 µCi of Fe^{59}, 50 mg. of ascorbic acid, and a fiftyfold molar excess of fructose in a total volume of 200 ml.

The method of carrying out the tests and calculating the results was identical to that used for the studies reported in Chapter III.

3. Results

Effect of orally administered fructose on iron absorption. In Table 10 are presented results of the four radioiron absorption tests carried out on each of six subjects. The molar ratios of fructose:iron used were 0:1. 1:1. 10:1 and 50:1 and the mean iron absorptions in these tests were 21, 21, 21 and 48% respectively. The increase in iron absorption with the 50:1 fructose:iron was highly significant ($p > 0.01$).

Urinary excretion of orally administered iron-fructose. In order to determine whether the stability and metabolic behaviour of the stable iron-fructose chelate was such that some urinary excretion of radioiron might take place, the extent of this excretion was measured in two subjects. In neither subject was any radioactivity above background detected in the urine collected in the first or second 24 hour period following administration of the radioiron-fructose chelate.

Effect of orally administered glucose on iron absorption. Two tests of radioiron absorption were carried out on each of seven subjects, a control test and a test with glucose in a 50:1 molar ratio with respect to iron. The results, which are presented in Table 11, fail to show that glucose has any statistically significant effect in enhancing iron absorption under the conditions used.

4. Conclusion

The results reported in this chapter lend further support to the thesis that gastrointestinal absorption of inorganic iron in man can be profoundly affected by chelation provided that a sufficient molar excess of chelating agent is present. Whereas EDTA and DTPA were shown to diminish the absorption of iron, the sugar fructose significantly enhanced iron absorption. These experiments show that the observations of Stitt et alii (1962) apply to humans when fructose is present in a 50:1 molar excess.

That the effect of fructose is connected with its ability to chelate iron is supported by the experiments which show that glucose failed to enhance iron absorption when given under the conditions which had been most effective with fructose.

The highly soluble iron fructose was not excreted in the urine to any detectable degree. This suggests that the iron-fructose chelate complex can be metabolised within the body.

Table 10

Effect of Orally Administered Fructose on Absorption of 5 mg. Iron

Subject No.	Sex	Molar ratio Fructose:Iron	Absorption %			
			0:1	1:1	10:1	50:1
16	F		19	26	23	60
17	F		33	25	30	44
18	F		0	12	13	31
19	F		33	33	26	62
20	F		19	19	5	56
21	F		21	13	26	35
	Mean Absorption (%)		21	21	21	48

Table 11

Effect of Orally Administered Glucose on Absorption of 5 mg. Iron

Subject No.	Sex	Molar ratio Glucose:Iron	Absorption % 0:1	Absorption % 50:1
24	M		3	4
25	M		4	9
26	M		11	13
27	M		20	17
28	M		17	19
29	M		29	33
30	M		11	23
	Mean absorption (%)		14	17

CHAPTER V

METHODS FOR DETECTING AND CHARACTERISING METAL CHELATING AGENTS

1. Purpose and Plan of the Investigation

The results of the investigations reported in the first part of this thesis showed that chelating agents could affect the extent of gastrointestinal iron absorption in man and, according to the nature of the chelating agent used, either enhance or depress absorption. This led to two questions: in fact, does man encounter chelating agents in food, drugs or in body secretions? and, secondly, why is fructose but not glucose able to form a stable chelate complex with iron? Before these questions could be answered suitable methods for detecting the presence of chelating agents in biological samples had to be established. Much of our knowledge of chelation chemistry comes from the study in vitro of the behaviour of the increasingly wide range of new chelate molecules synthesised by the organic chemist. However, most such studies have been carried out in systems of known composition, and containing accurately measured amounts of the substances under study. Consequently the methods usually employed have not lent themselves without modification to the study of biological samples which are usually of complex, and often of unknown composition.

Proof of chelate formation can only be obtained with certainty by the isolation and analysis of the pure compound according to the classical techniques of organic chemistry (Martell and Calvin, 1962). In biological samples such as saliva, gastric juice, bile or food undergoing digestion the problem of the identification and characterisation of chelates is considerably more complicated. In order to facilitate such studies the four methods reported here have been developed for in vitro investigations of those chelating agents, natural and synthetic, which may be encountered in biological samples.

Ferric iron has been used as the test metal in the studies described in this chapter not only because of the interest in iron metabolism but because of other properties of the metal: it has a great affinity for most chelating agents; it has an extremely insoluble hydroxide at the pH encountered in the duodenum; and because the radioactive isotope Fe^{59} has a convenient half life, is easy to count and is available in high specific activity.

2. Materials and Methods

Reagents. Ferric chloride stock solution (0.01 M.$FeCl_3$ in 0.02 M.HCl). 0.5585 gm. of rust free A.R. iron wire was dissolved in about 30 ml. of HCl (1:1). Complete oxidation was obtained by adding 2 ml. of concentrated HNO_3 and boiling. The excess of HNO_3 was removed by evaporating just to dryness under an infrared lamp. The product was transferred quantitatively to a one litre volumetric flask and made up to volume with 0.02 M.HCl. From this stock solution fresh working solutions were prepared as required by dilution with 0.01 M.HCl.

$Fe^{59}Cl_3$ stock solution. Radioactive iron solution was prepared from $Fe^{59}Cl_3$ so as to contain 0.01 µCi Fe^{59} per ml. and 10 µg Fe^{+++} per ml. in 0.01 M.HCl.

Counting apparatus. Solutions containing Fe^{59} were counted using a well-type sodium iodide crystal scintillation counter and single channel analyser (Philips type PW4003 automatic gamma counter). A 2 ml. sample in a 6 x 5/8" test tube was counted with an overall counting efficiency of 20% and a background counting rate of 2 c.p.s.

Spectrophotometer. All spectrophotometric examinations were made with a Beckman D.B. recording spectrophotometer fitted with a log/linear potentiometric recorder and scale expansion device. Unless otherwise stated measurements were made in 1 cm. silica cells and the narrow slit program was used. All scans were carried out at a speed of 40 mµ per minute.

Figure 2a: Automatic well-type γ scintillation counter (Phillips Type PW4003). Used for radioiron solubility tests.

pH measurements and titrations. All pH measurements and titrations to a given pH were carried out electrometrically with a Radiometer pH meter and automatic titration apparatus.

Thin layer chromatograms. Flexible thin layer chromatograms (Eastman chromagram sheet) were used throughout the study. The chromatograms were run in the Eastman chromagram developing apparatus.*

Detailed accounts of the methods used will be included in the following section.

3. Radioiron Solubility Test

Perhaps the most outstanding property which chelation frequently confers on a metal ion, is solubility in a situation where it would otherwise be quite insoluble. Ferric iron is extremely insoluble at high pH, the solubility product constant of ferric hydroxide at 18°C is 1.1×10^{-36} (Hodgman, 1963). These properties are made use of in a simple solubility test of chelating ability which has been developed in the course of this investigation. The test depends on the fact that most metal chelates dissociate at

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*Distillation Products Industries, Rochester, N.Y., U.S.A.

low pH, the metal being replaced by hydrogen ion, and that as the pH is raised the chelating agent reunites with available metals and will combine preferentially with the metal ion for which it has the greatest avidity.

The test is set up in 25 ml. beakers as shown in Table 12. The sample is mixed with carrier iron and radioactive iron and the pH is adjusted to below 2.0 with HCl. Blank and control samples (0 and 100% values) containing distilled water in place of sample are included. The contents of the sample and blank beakers are titrated to pH 8.0 with NH_4OH and the final volume is adjusted to 8.0 ml. (the pH in the control beaker is left at 2.0). A suitable aliquot from each beaker is then centrifuged at 2,000 r.c.f. for 30 minutes to deposit any ferric hydroxide which may have been formed. A 2 ml. aliquot of the supernatant is pipetted off for determination of Fe^{59} gamma activity in the scintillation counter. The results are expressed as:

$$\begin{array}{l}\text{Fe solubility}\\ \text{index of sample}\end{array} = \frac{\text{Sample Activity} - \text{Blank Activity}}{\text{Control Activity} - \text{Blank Activity}} \times 100\%$$

The sensitivity of the test is such that a 1% value indicates that 1 ml. of sample has complexed 0.01 µM. of Fe^{+++}. The sensitivity of the test may be increased if necessary by decreasing the concentration of carrier iron.

Table 12

Radioiron Solubility Test for Detection of Chelating Agents

Reagents	Samples	Control	Blank
1. 0.001 M.$FeCl_3$	2 ml.	2 ml.	2 ml.
2. 0.01 µCi Fe^{59}/ml.	2 ml.	2 ml.	2 ml.
3. Sample Solution	2 ml.	-	-
4. Distilled Water	-	2 ml.	2 ml.
5. pH adjustment (HCl, NH_4OH + H_2O to volume)	2 ml.	2 ml.	2 ml.

The test has been found useful because of its sensitivity and the simplicity with which large numbers of samples can be screened. A possible cause of a false positive result would be the increase of iron solubility at pH 8.0 by a sample which did not chelate but which reduced ferric to ferrous iron. However, glucose, a substance with marked reducing power but no chelating ability, does not give a positive result with this technique.

4. Ion-exchange Resin Test

Most chelating agents form negatively charged or neutral complexes with iron. When a small amount of $Fe^{59}Cl_3$ is allowed to react with the unknown sample under suitable conditions, the Fe^{+++} because of its great affinity for chelating agents compared with other ions usually encountered in biological samples is converted to an anionic or neutral complex. If the solution is passed through a cation exchange resin column of sufficient capacity to retain the added Fe^{+++}, the appearance of radioactivity in the effluent can be used as an index of the chelating ability of the sample. It further indicates that the iron complex which has been formed is neutral or negatively charged (Samuelson, 1953).

In the ion exchange test described below the chelation step is carried out in the resin bed itself, the carrier and radioactive iron having been absorbed on the resin column before the sample being tested was run through.

Method. The strongly acid cation exchange resin Dowex AG-50W-X8 Analytical Grade Resin* was used. This resin contains 8% divinyl benzene crosslinking and the beads are in the 40-80 mesh range. The bed volume was 5 ml. of resin in the hydrogen form and the maximum flow rate was 1 ml. per minute except for water washes, where a flow rate of about 5 ml. per minute was found to be adequate. The columns were prepared for use by conversion to the sodium form (50 ml. of 3 M. NaCl followed by a 50 ml. water wash) and then loaded with iron. 1 ml. of a solution of 0.1 μCi Fe^{59} and 0.01 mg. carrier Fe^{+++} in 0.01 M. HCl was applied to the top of the column which was then rinsed through with 50 ml. of water. No radioactivity was found in the eluate from this rinse, indicating that all the iron had been adsorbed on the resin column. (At this point the loaded column may be conditioned with buffers or given any other treatment as required in a particular experiment.)

The results of an experiment using as test substance the chelating agent EDTA are shown in Figure 3 where the percentage

*Bio-Rad Laboratories, Richmond, California, U.S.A.

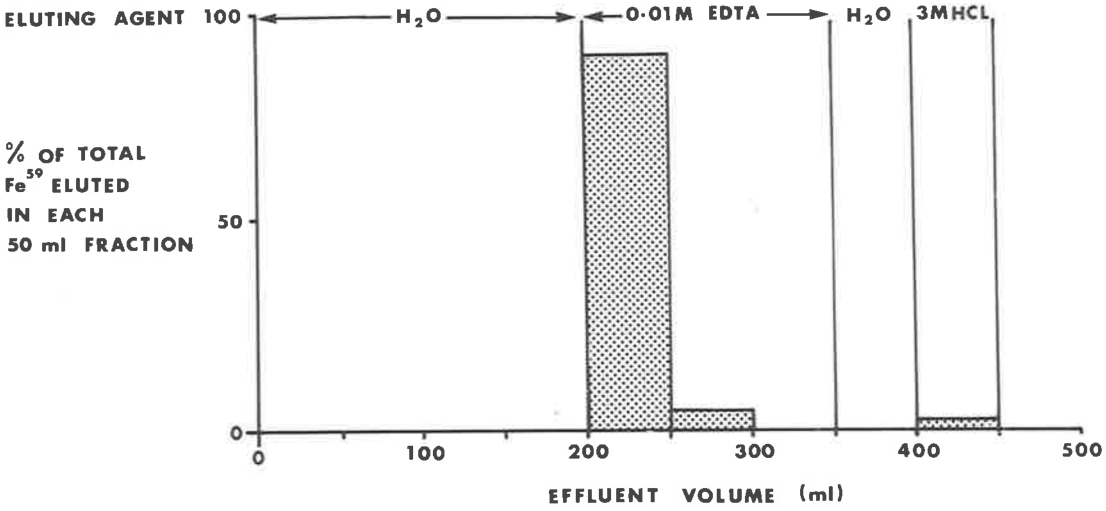


Figure 3: Effect of 0.01 M. EDTA on the ion-exchange behaviour of $Fe^{59}Cl_3$ on the cation exchange resin Dowex AG-50W-X8.

of the total radioiron activity which is removed at each stage is plotted as a function of volume of eluant. It can be seen from these results that 0.01 M. EDTA effectively elutes iron from the column, and the eluted iron can be detected and estimated by scintillation counting of the tracer Fe^{59}. At the end of the experiment when all the sample has run through the column, the resin bed is washed with 50 ml. of water to flush out the last of the sample and the column is then eluted with 50 ml. 3 M. HCl. This acid wash has been found to remove quantitatively any radioactive iron remaining on the column and converts the column back to hydrogen form ready for a new cycle of operations with the next sample.

5. Thin Layer Chromatography

The use of thin layer chromatography (TLC) as a tool in the investigation of chelating ability of biological samples is based on the fact that the chromatographic mobility of metal chelate complexes is different from that of the free metal ion in most solvent/adsorbent systems.

TLC on iron-free silica gel using radioactive Fe^{59} as a test metal possesses the following advantages:

1. It is well suited to an experiment which requires serial sampling, such as following the variation of chelating ability of a sample during the course of an enzymic digestion.
2. It is a quick and simple method requiring very little sample (1-5 µl.).
3. Qualitative information can be obtained by designing a system in which chelating agents of known Rf values are included on the plate.

An example of the use of TLC for the resolution of a mixture of chelating agents is given in the following experiment.

Materials: Eastman Chromagram Sheet (Type K301R) 20 x 20 cm. Eastman Chromagram developing apparatus.

Reagents: Migrating solvent n-butanol: 95% ethanol: acetic acid: H_2O = 40 : 25: 25: 35.
Solvent pH 2.35.
Visualisation reagent 0.5% w/v 8-hydroxyquinoline in 60% v/v aqueous ethanol.
Chromatograms sprayed, dried and examined under ultraviolet light.

Samples: 2 µl. of 1% w/v solutions of iron chelates of desferrioxamine (DFO), EDTA, DTPA and ethylenediamine di (0-hydroxyphenyl) acetic acid (EDDHA). The samples were spotted 2 cm. from the lower edge of the chromatogram sheet and 2 cm. apart.

Result: Complete resolution of the mixed sample was obtained. Rf values of the iron chelates found by this system were DFO - 0.42, EDTA - 0.49, DTPA - 0.56 and EDDHA - 0.89.

In the experiment described above the 20 µg. of iron chelate used could be located directly by examination of the plate under a short wavelength ultraviolet lamp. Greater sensitivity and quantitation of results can be obtained by the use of a thin layer radiochromatogram scanner which locates the position and determines the amount of radioactive iron which has been chelated and moved to a different position on the plate from ionic iron.

6. Visible and Ultraviolet Spectrophotometry

It has long been recognised that a characteristic colour is one of the most important distinguishing features of the metal chelate compounds (Chaberek and Martell, 1959). When a metal is chelated its colour is both intensified and the wavelength of maximum absorption is shifted (Martell and Calvin, 1962; Hill-Cottingham, 1957). This property of the metal chelate compounds has been used in a number of ways in this study to give information about their formation and properties.

(a) Chelate detection by the effect on the absorption spectrum of Fe +++

In Figure 4 (Curve II) is shown the visible absorption spectrum of 0.0005 M. $FeCl_3$, sodium salicylate having no absorbance in the visible region. When iron is chelated by salicylic acid and the absorption spectrum of a 0.0005 M. solution of iron salicylate is run (Curve I) the overall increase in absorbance and the altered spectral curve can be seen. Qualitative changes in spectral characteristics can often be simply revealed by running the difference spectrum between the ionic and chelated forms of the metal. In Curve III a peak at 360 mμ is clearly shown as well as the major peak at 520 mμ.

The effect on the visible absorption spectrum of iron is further illustrated in Figures 5 and 6 where the effects of chelation by EDDHA and by fructose are shown.

The detection of chelation formation by the change of

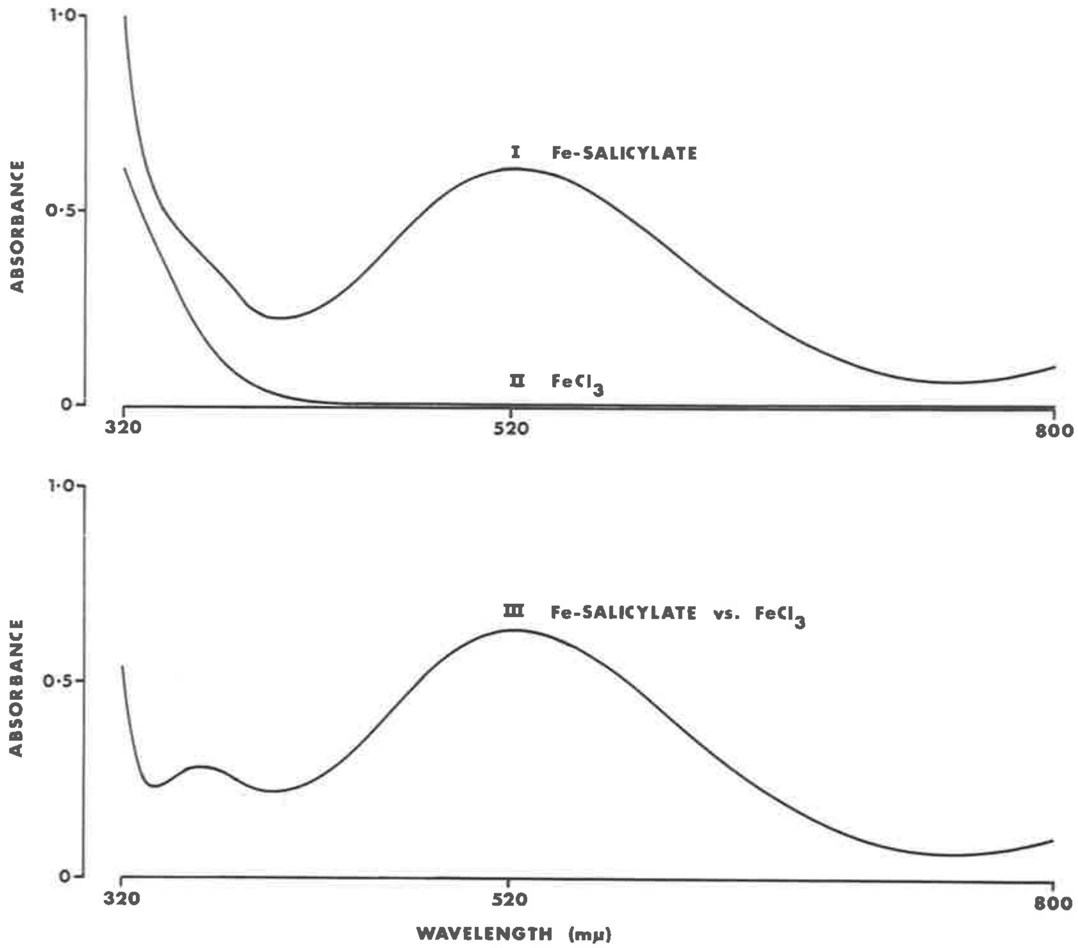


Figure 4: Spectral transmittance curves of iron salicylate chelate.

I. 0.0005 M. Fe-salicylate at pH3.

II. 0.0005 M. $FeCl_3$ at pH 3.

III. Difference spectrum between I and II.

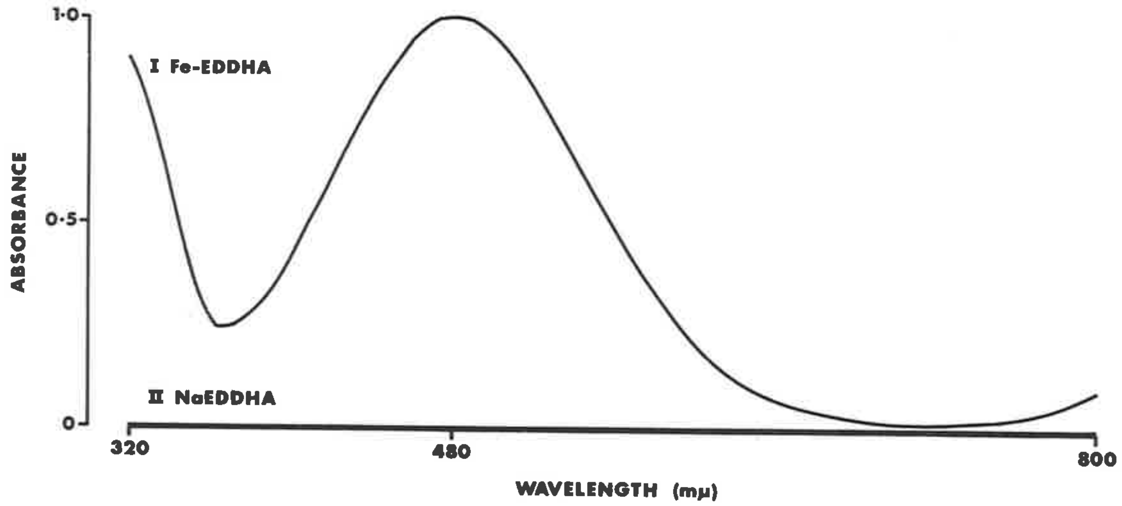


Figure 5: Spectral transmittance curve of EDDHA.
I. 0.00025 M. Fe-EDDHA at pH 5.
II. 0.00025 M. Na-EDDHA at pH 5.

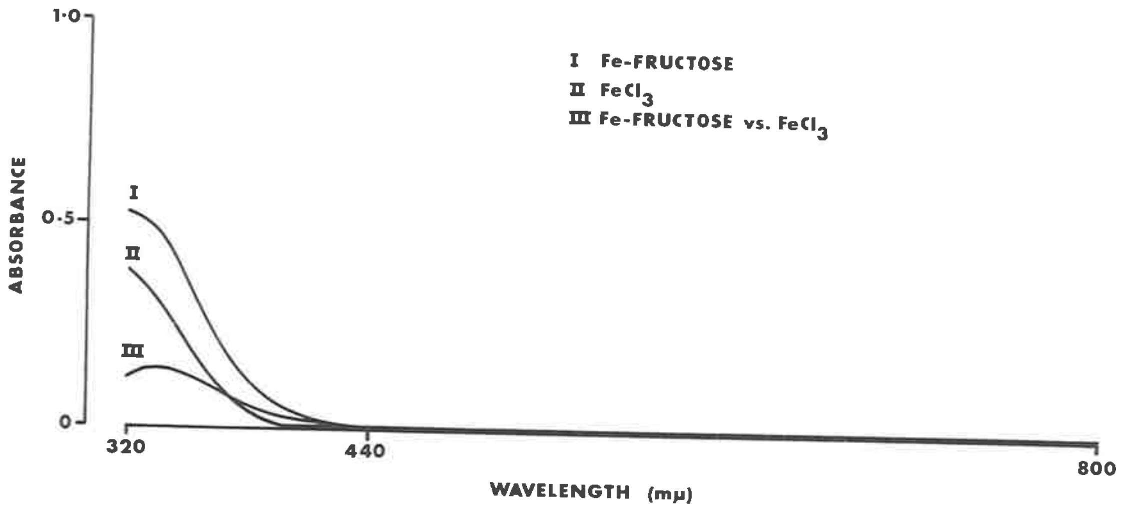


Figure 6: Spectral transmittance curves of iron-fructose chelate.

I. Iron-fructose, 0.0005 M. $FeCl_3$ in 0.1 M. fructose at pH 2.5.

II. 0.0005 M. $FeCl_3$ at pH 2.5.

III. Difference spectrum between I and II.

the ultraviolet absorption spectrum is illustrated in Figure 7 where the effect of iron on the absorption spectrum of EDTA is shown.

(b) Ligand:metal ratio.

A simple spectrophotometric method for the determination of the composition of a metal chelate compound in solution is Job's method of continuous variation (Chaberek and Martell, 1959). This depends on the fact that for a constant total concentration of metal ion and chelating agent, the concentration of metal chelate will be greatest when the metal and chelating are brought together in the same ratios in which they exist in the compound. The use of this method is illustrated in Figure 8 where the results of an experiment with Fe^{+++} and DTPA show that ferric iron forms a 1:1 chelate with DTPA under the conditions used.

(c) Estimation of relative binding strengths of chelating agents for a given metal

It is often desirable to know which of two ligands has the greater affinity for a given metal in a situation where no quantitative data are available for a prediction to be made. Such a case occurs when we are considering the variety of ligands to which dietary iron is exposed in its passage through the gut. An empirical test which may be helpful in

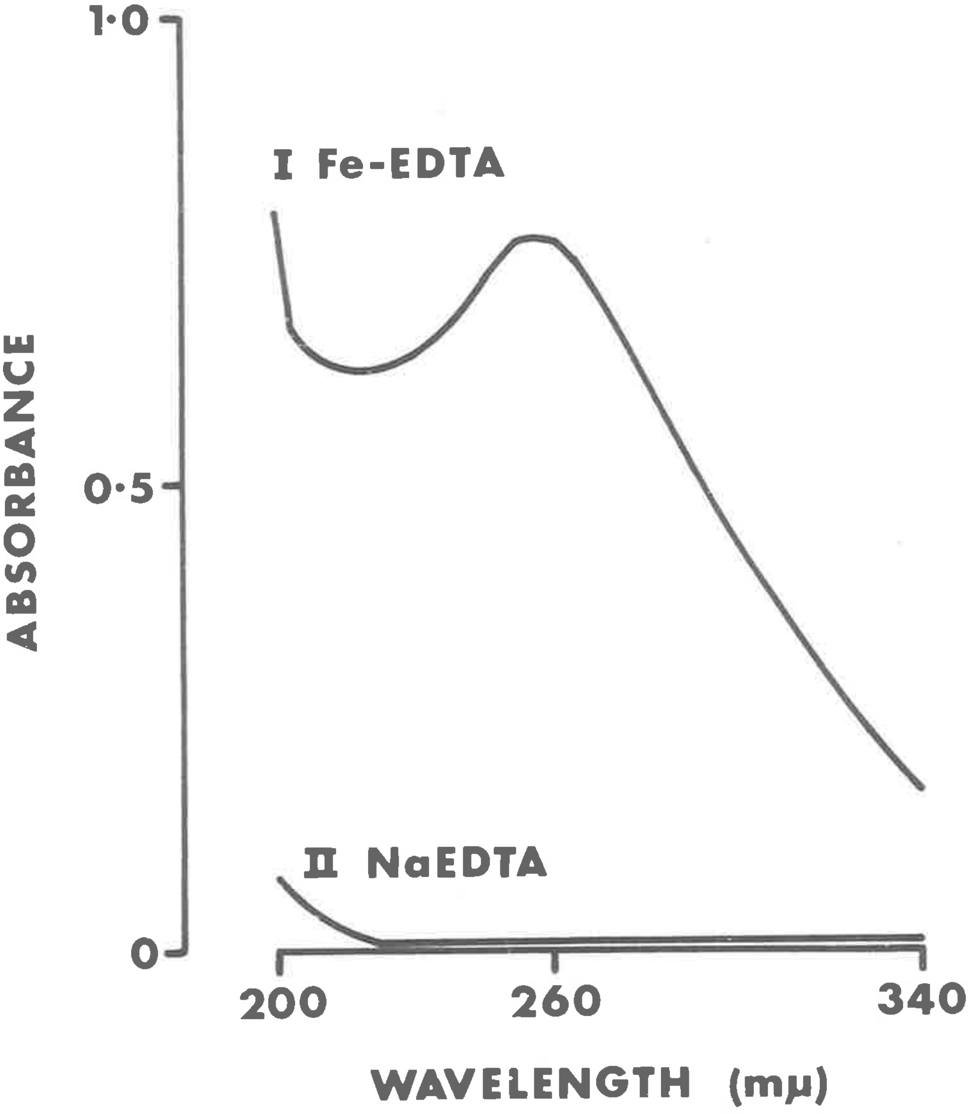


Figure 7: Spectral transmittance curves of EDTA.
I. 0.0001 M. Fe-EDTA at pH 5.
II. 0.0001 M. Na-EDTA at pH 5.

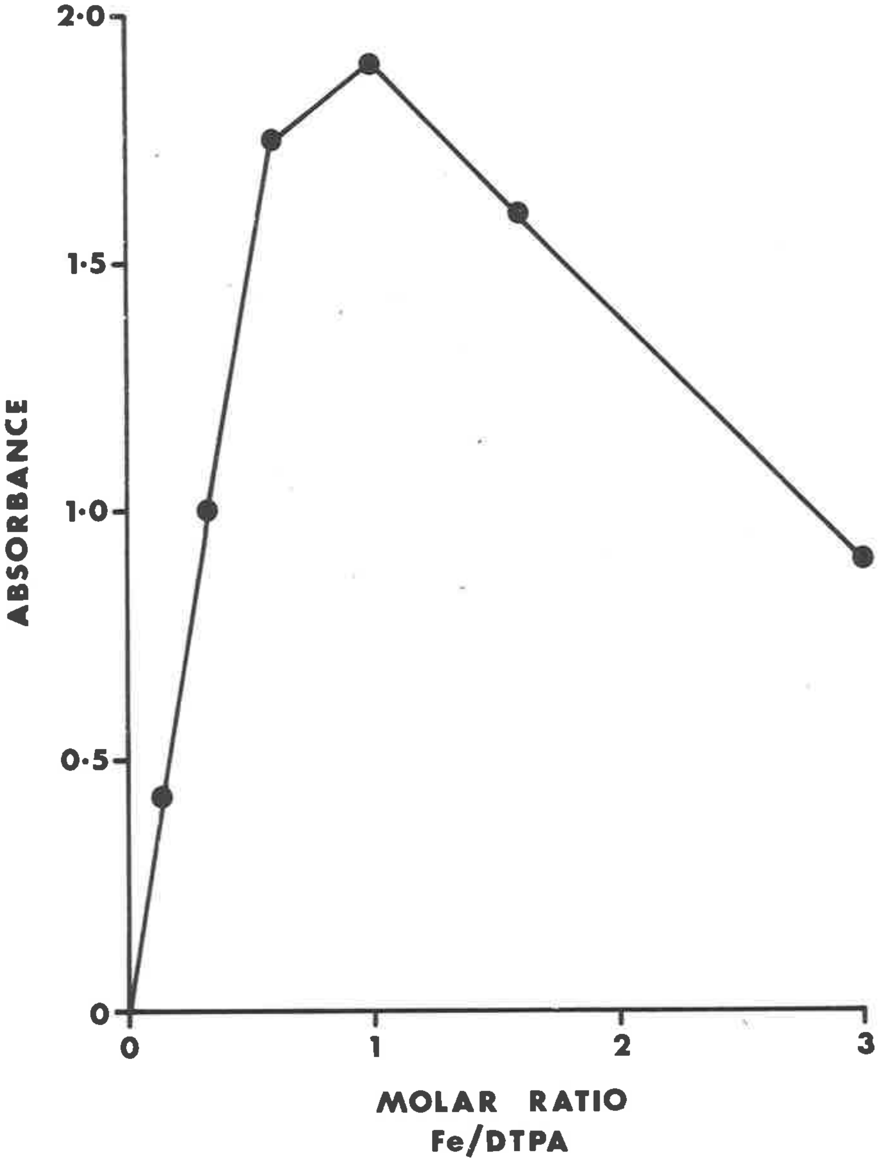


Figure 8: Absorbance readings at 340 mμ of mixtures of $FeCl_3$ and DTPA. Total molar concentration of reacting species = 0.0005. pH = 5.

these cases, particularly as it can be carried out under any desired conditions of concentration, pH, reaction time and temperature, is illustrated in Figure 9. For the purposes of this experiment the two synthetic compounds EDTA and salicylic acid were used, although the method is of general applicability whenever there is a difference in the absorption spectrum of the two metal chelates being studied.

The absorption spectrum of iron salicylate has a maximum at 520 mμ, at which wavelength Fe-EDTA has minimal absorbance, and all readings were carried out at this wavelength. A series of 0.001 M. iron salicylate solutions was prepared having EDTA concentrations increasing in steps from zero to 0.001 M. The absorbance of these solutions was measured and the result plotted (Curve I, Figure 10). A series of 0.002 M. Fe-EDTA solutions was prepared having sodium salicylate concentrations increasing in steps from zero to 0.02 M. The result of absorbance measurements on these solutions is shown in Curve II, Figure 10. From these results it is concluded that at pH3, EDTA binds ferric iron much more strongly than does salicylic acid. Even a tenfold molar excess of salicylic acid over EDTA is insufficient to cause the formation of any appreciable amount of iron salicylate. The results also show that the exchange of iron between salicylic acid

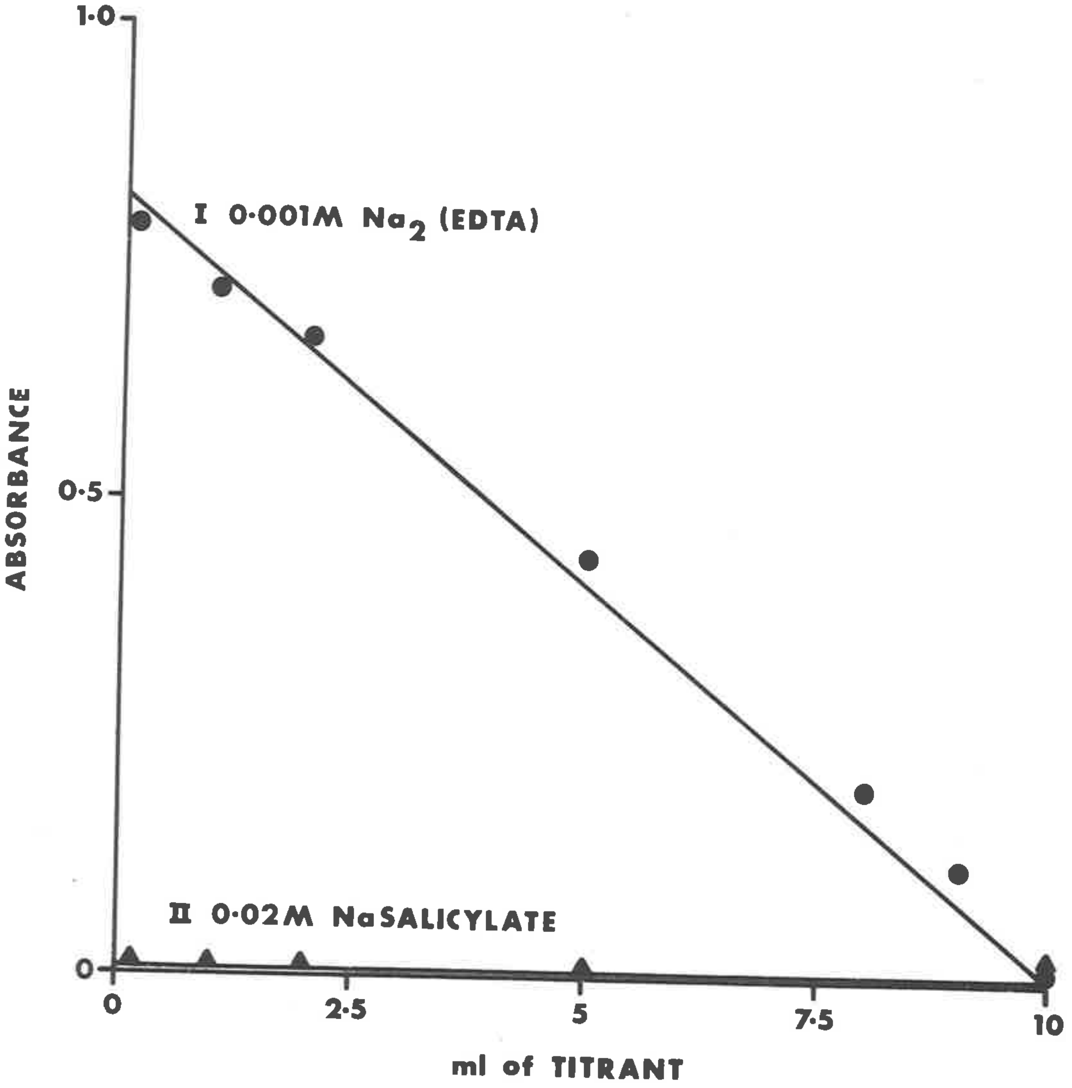


Figure 10: Relative stabilities of salicylate and EDTA complexes of Fe^{+++}. Absorbance measurements at 520 mμ, pH = 3 throughout experiment.

I. Effect on absorbance of solutions 0.001 M. with respect to Fe salicylate when the concentration of EDTA is increased in steps from 0 to 0.001 M.

II. Effect on absorbance of solutions 0.002 with respect to Fe-EDTA when the concentration is increased in steps from 0 to 0.02 M.

and EDTA under the conditions of the experiment is instantaneous.

This method is useful in obtaining comparative information about the ligands present in samples of unknown composition.

7. Comment

Four methods have been described in this chapter for the detection and partial characterisation of metal chelating agents. The first, the radioiron solubility test, was developed by the author for use in this investigation; the others are standard techniques which have been applied to the further studies to be described below.

The use of these methods gives powerful indirect evidence of the presence of chelating agents in biological samples. Although the only unequivocal method of proving the existence of a chelating molecule is the isolation and analysis of the pure compound, this is usually not practicable or even desirable in the clinical situation because of the effort required and because a given ligand may serve only to pass on a metal ion to another ligand which binds it more strongly. The combination of methods is proposed because the complex samples being studied may well contain interfering agents which could lead to a false conclusion if only one method were used. For example, a powerful reducing substance in a sample could give a falsely high result in the radioiron solubility test by reducing ferric to ferrous iron. However, this situation would not impair the results obtained with the other three tests. Similarly, intensely coloured substances present in the sample might interfere with the spectrophotometric tests for chelate formation, yet such substances should not interfere with the other tests.

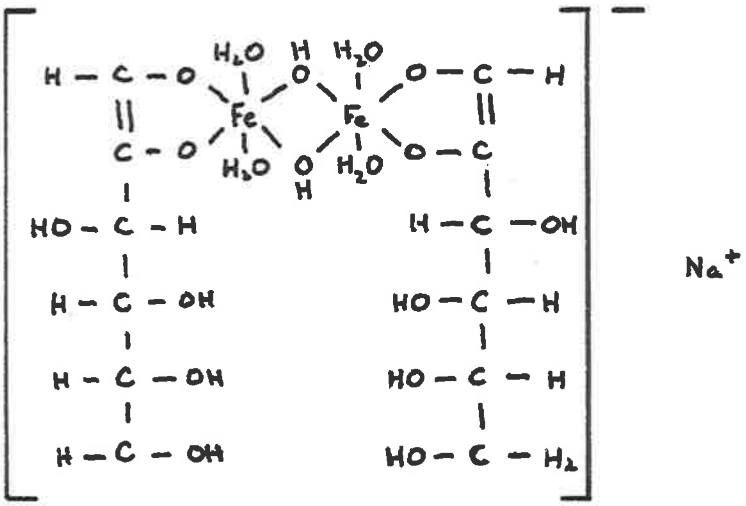
The methods described in this chapter are well suited to screening large numbers of samples and are sufficiently sensitive for many biological investigations. They may usually be applied directly to complex samples without any prior separation or fractionation. They give quantitative information permitting comparisons to be made between subjects and the range of normal values and their variation in diseased states to be determined. Finally, they enable some characterisation of the chelate compounds to be made.

CHAPTER VI

THE NATURE OF THE IRON-FRUCTOSE CHELATE COMPLEX

1. Purpose and Plan of the Investigation

Fructose is capable of forming a highly stable chelate complex with iron, in contrast to some other hexose sugars and disaccharides which have little or no such ability (Charley, Sarkar, Stitt and Saltman, 1963). Recently, attention has been focused on the iron-fructose complex because of its ability to move across biological membranes (Stitt, Charley, Butt and Saltman, 1962) and to enhance gastrointestinal iron absorption in man (Davis and Deller, 1966a) as has been shown in Chapter IV above. The structure which has been proposed for this compound is one in which iron, incorporated into a five-membered ring, is bound through the hydroxyl and carbonyl oxygen atoms to carbon atoms one and two of the fructose molecule (Charley et alii, 1963; Aasa, Malmström, Saltman and Vänngård, 1964).



In an endeavour to explain why this iron-complexing ability is not shared by all sugars, an attempt was made to determine the exact molecular configuration in fructose responsible for this ability and to formulate a general rule which would allow prediction of the iron chelating potential of sugars and polyols.

2. Methods and Results

Simple sugars and polyols possess between them only three functional groups - hydroxyl, aldehyde and ketone. A consideration of the saturated compounds of one, two or three carbon atoms containing only these functional groups shows that 24 such compounds can theoretically exist. As many of these 24 compounds as could be obtained were examined and their ability to form a chelate complex with ferric iron was studied.

Chelating ability was assessed by the radioiron solubility test described in Chapter V. In each case 2 ml. of a 0.01 M. solution of the substance under test was used. When positive results indicative of chelation were obtained they were always confirmed independently by other techniques such as alteration of ultraviolet absorption spectrum. Results are given in Table 13 and it can be seen that of the compounds tested only three show any significant chelating ability as measured by this technique.

The two compounds 1-3,dihydroxypropane and glycerol which show moderate chelating ability, and dihydroxy acetone which shows strong chelating ability all share the common structural feature of hydroxyl groups on carbon atoms one and three, and are the only compounds among the twenty-four possibilities which do so. The dihydroxy acetone structure is found in the open chain fructose molecule, but is not present in any aldohexoses or disaccharides. On the basis of this evidence it seemed reasonable to propose that the ability of fructose to chelate iron is related to the dihydroxy acetone structure found in the open chain fructose molecule. The superiority of dihydroxy acetone over 1-3, dihydroxy propane and glycerol is presumably caused by the electron withdrawing effect of the carbonyl group which enables the hydroxyl groups to dissociate revealing oxygen atoms which can chelate iron and form a stable six-membered ring structure.

If the above hypothesis were correct, other sugars possessing the dihydroxy acetone structure, and only those, would chelate iron as powerfully as fructose does. On this basis it was predicted that the other keto hexose sugars sorbose and tagatose would demonstrate iron chelating ability and that no aldohexoses or disaccharides would chelate iron to the same extent. In Table 14 results are presented of tests carried out on the keto hexoses fructose, sorbose, tagatose and on a number of aldohexoses and disaccharides. Of all the sugars tested only sorbose and tagatose chelate iron as powerfully as does fructose.

3. Conclusion

As a result of the work discussed in this chapter it is concluded that the interesting biological phenomenon of iron chelation by sugars and polyols is exhibited most strongly by those compounds which contain the dihydroxy acetone structure in their molecule; that this property is one which distinguishes keto from aldo sugars; and that the structure of the iron-fructose chelate proposed by Saltman's group. depicted on page 54 should be modified to one in which the iron atom is incorporated into a six-membered ring, bound through the hydroxyl carbon atoms to carbon atoms one and three of the fructose molecule, as follows:

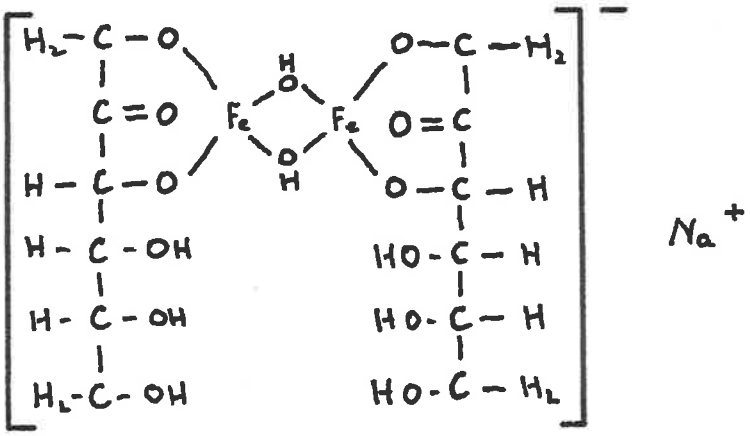


Table 13. Iron Chelating Ability of 1, 2 and 3 Carbon Atom Compounds with Hydroxyl, Aldehyde and Ketone Functional Groups

Compound	Iron chelating ability*	Compound	Iron chelating ability
Methanol	< 1	Propane dialdehyde	†
Ethanol	< 1	Acetone	< 1
Ethylene glycol	< 1	Glycollic aldehyde	< 1
n-Propanol	< 1	3-Hydroxy propanol	†
iso-Propanol	< 1	Lactaldehyde	†
Propylene glycol	< 1	Glyceric aldehyde	†
Propane-1,3-diol	17.5	2-Hydroxy propan-1,3-dial	†
Glycerol	12	1-Hydroxy acetone	< 1
Formaldehyde	< 1	Dihydroxy acetone	100
Acetaldehyde	< 1	Methyl glyoxal	< 1
Glyoxal	†	2-Keto propan-1,3-dial	†
Propionaldehyde	< 1	3-Hydroxy-2 keto propanal	†

*Iron chelating ability = % of Fe^{3+} held in solution at pH 8.0 when equal volumes of 0.1 M. solution of the substance under test and 10^{-2} M. $FeCl_3$ are mixed and the pH raised to 8.0.

†Compound not available or too unstable for testing by this procedure.

Table 14

Iron Chelating Ability of Some Keto Hexoses, Aldo Hexoses and Disaccharides

Type	Compound	Iron chelating ability*
Keto hexose	Fructose	100
	Sorbose	100
	Tagatose	100
Aldo hexose	Glucose	< 1
	Galactose	< 1
	Mannose	< 1
Disaccharide	Sucrose	< 1
	Lactose	< 1
	Maltose	< 1

CHAPTER VII

CHELATING AGENTS IN FOOD AND FOOD DIGESTION PRODUCTS

1. Purpose and Plan of the Investigation

After it became evident that under certain conditions chelating agents could markedly affect the extent of gastrointestinal iron absorption, it was decided to investigate whether food or food digestion products contained substances which could chelate dietary metals. Any such substances, in order to exert maximal effect, would have to be present in the foodstuff itself or else be produced as a result of salivary or gastric digestion so as to be able to react with the free metal ion as it was released from its food conjugate by the acid/peptic digestion in the stomach (Sanford, 1960). Exchange from one chelating agent to another will occur even in alkaline conditions, where labile complexes are involved, but chelation of an insoluble metal compound in alkaline conditions is usually very slow. For these reasons it was decided to examine some typical basic foodstuffs and look for chelating ability in the substances themselves and in their digestion products.

Fats were not examined in detail as the monocarboxylic fatty acids do not possess the ligand groups for chelation, and as has been shown above, glycerol has only moderate iron chelating ability in 0.1 M. concentration. Egg albumin was taken as an example of a typical dietary protein and it was examined together with the products of its tryptic and peptic hydrolysis. The most important carbohydrate constituents of man's diet are the two polysaccharides amylose and amylopectin. Both of these were studied together with the intermediate and end-products of their salivary digestion.

2. Materials and Methods

Reagents:

Albumin, Egg (Ovalbumin) Crude Powder, Grade II, Sigma.

Trypsin, Laboratory Reagent, B.D.H.

Pepsin, 1:10,000, Sigma.

Amylose, from Corn, Grade II, Sigma.

Amylopectin, from Corn, Grade II, Sigma.

Saliva: after rinsing the mouth the saliva sample was collected in a beaker and used immediately.

Apparatus: Enzymic digestions were carried out in a thermostatically controlled water bath at either 25 or $38^{\circ} \pm 0.2^{\circ}$C. The digestion mixtures were agitated by means of a mechanical flask shaker.

Molecular Sieving: Hydrolysate mixtures were resolved by downward passage through a 90 x 1.5 cm. column of Sephadex G-50 fine (exclusion limit 10,000 molecular weight).

Effluent was collected in 40 x 3.75 ml. fractions. Solvent used was physiological saline.

Protein estimations: Protein concentration was estimated by the standard biuret reaction. The absorbance of the product was measured spectrophotometrically at 320 mμ.

Carbohydrate Estimation: The orcinol reaction as described by Kabat and Mayer (1961) was used. This is a general reaction for the estimation of carbohydrates which is suitable for use even in the presence of protein. The coloured product is measured spectrophotometrically at 540 mμ.

Starch-iodine reaction: One drop of hydrolysate was mixed on a white spotting tile with a drop of 0.1 M. iodine solution (Merck-Titrisol).

3. Results

A. Protein. Preliminary radioiron solubility tests were carried out on albumin solutions of various strengths. These tests showed that albumin solutions of strength 0.01% or less had negligible chelating ability. Albumin solutions of strength greater than 0.01% gave positive results.

The effect of peptic and tryptic hydrolysis of albumin was then studied. A mixture of 20 ml. of 1% albumin and 20 ml. of 0.02% pepsin in 0.1 M. HCl was agitated in the water bath at 25°C. Samples were removed at one minute intervals for the first ten minutes and then at five minute intervals for the next hour. The experiment was repeated using 20 ml. of 1% albumin and 20 ml. of 0.02% trypsin in saline and agitation was carried out at 40°C. pH throughout the experiment remained between 6.8 and 6.9.

The one ml. samples removed during these experiments were diluted so as to bring the concentration of original protein substrate to 0.01%. Radioiron solubility tests were carried out on all these samples, and protein concentration was measured.

No increase in chelating ability occurred during the course of peptic or tryptic hydrolysis of egg albumin as measured by the radioiron solubility test.

B. Carbohydrate. As determined by the radioiron solubility test the iron chelating abilities of amylose and amylopectin are small. Solutions of amylose of strength 0.02, 0.03, 0.04, 0.05, 0.1, and 0.5% w/v were tested by the radioiron solubility test and found to have no iron chelating ability. When amylopectin solutions of the same strengths were tested it was found that 0.5% and 0.1% strengths were able to chelate iron, but with further dilution chelating ability fell off and below a concentration of 0.03% w/v chelating ability could not be detected. These results are summarised in Table 15.

When these two carbohydrates were hydrolysed by the salivary amylase ptyalin and the end-products of the hydrolysis examined it was found that they had no chelating ability in the concentrations used. This result was to be expected from previous work (Chapter VI, Table 13) which showed the inability of 0.1 M. glucose and maltose to chelate iron.

It was then decided to measure the chelating ability of the hydrolysis mixture throughout the course of salivary digestion. Accordingly, the mixture was sampled at one minute intervals for the next 20 minutes.

Amylose. Hydrolysis mixture: 200 ml.of 0.03% amylose in 10% w/v physiological saline, pH 6.8-6.9 (checked at beginning and end of experiment), plus 0.05 ml. saliva. Hydrolysis conditions: gentle agitation in a water bath at 25°C.

Result: No iron chelating ability was exhibited by the hydrolysis mixture at any point throughout the reaction.

Amylopectin. Hydrolysis mixture: 200 ml. of 0.03% amylopectin in 10% w/v physiological saline, pH 6.8 - 6.9 (checked at beginning and end of experiment), plus 0.05 ml.

Table 15

Iron Chelating Ability of Amylose and Amylopectin Solutions of Various Concentrations

Concentration % w/v	Amylopectin	Amylose
	Iron Solubility Index	
0.5	100%	< 1%
0.1	100%	< 1%
0.05	85%	< 1%
0.04	49%	< 1%
0.03	8%	< 1%
0.025	< 1%	< 1%
0.02	< 1%	< 1%

saliva. Hydrolysis conditions: gentle agitation in a water bath at 25°C.

Result: A striking increase in iron chelating ability was observed within one minute of adding the saliva, the values rose to a maximum within two to four minutes of adding the saliva and then sharply declined to the baseline value within another 6 to 8 minutes. Results of a typical experiment are given in Figure 11.

A freshly prepared amylopectin solution had been used in the experiments just described and the possibility had to be considered that the increase in chelating ability was caused by a slow reaction of water with the amylopectin macromolecule. Further experiments were carried out on amylopectin solutions which had been prepared 24 hours earlier. Results of a typical experiment are shown in Figure 12. It can be seen that the 24 hour contact with water is not sufficient to increase the chelating ability of amylopectin, but it shortens the time required for saliva to increase chelating ability and also shortens the time for the induced chelating ability to return to the baseline level.

That this phenomenon was caused by enzymic digestion was suggested by experiments with heat denatured saliva (boiling water bath 5 minutes) which failed to increase chelating

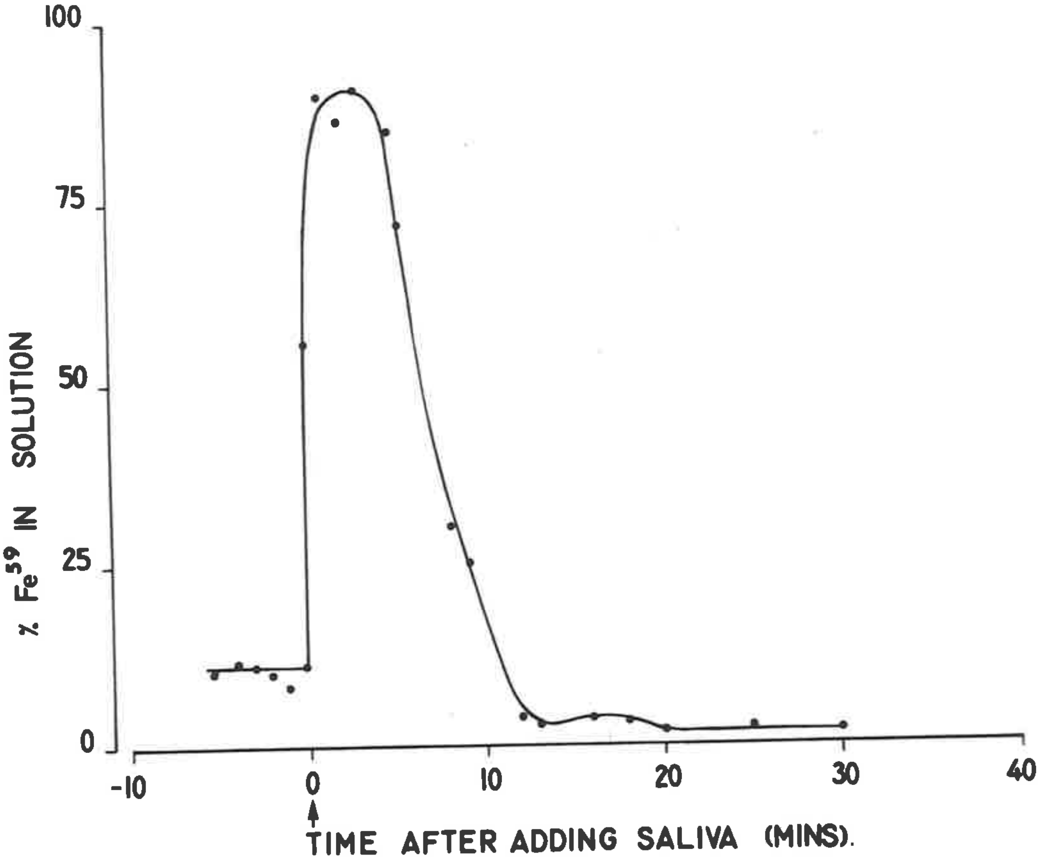


Figure 11: Chelating ability (measured by radioiron solubility test) of a freshly prepared 0.03% amylopectin solution throughout the course of salivary hydrolysis.

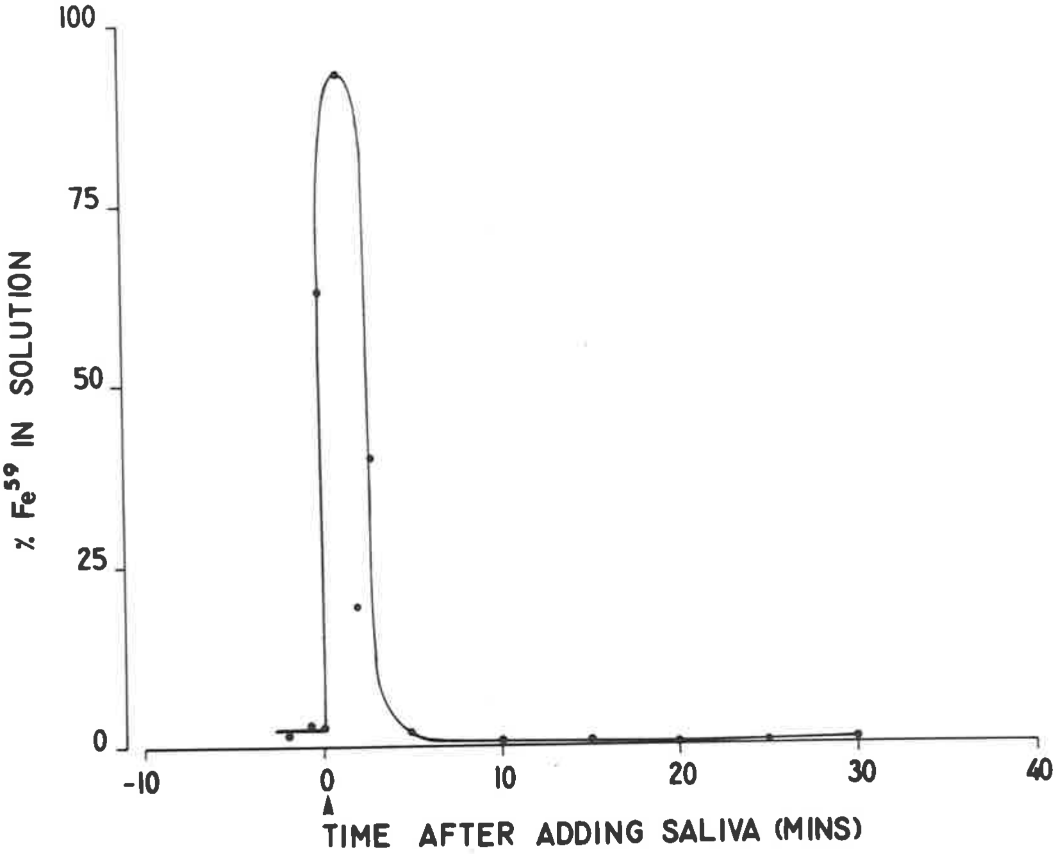


Figure 12: Chelating ability (measured by radioiron solubility test) of 0.03% amylopectin solution prepared 24 hours previously throughout the course of salivary hydrolysis.

ability.

In a further experiment the hydrolysis was stopped after one minute by HCl inactivation of ptyalin and radio-iron was added. 200 ml. of 3% w/v amylopectin solution in 10% w/v saline at pH 6.8 was added to 5 ml. of saliva and the mixture agitated at 25°C. for one minute. A 1 ml. aliquot of the hydrolysate was then removed and mixed with 0.15 µCi $Fe^{59}Cl_3$ and the pH lowered to below 2.0 by the addition of HCl. An electrometric titration to pH 8.0 was then carried out with NH_4OH and the solution centrifuged (2000 r.c.f. for 30 min.) to deposit any $Fe(OH)_3$ formed. 0.5 ml. of the supernatant was resolved according to molecular size by downward passage through a Sephadex column and the eluant collected in 40 x 3.75 ml. fractions in an automatic fraction collector. The fractions were analysed for radio-iron and carbohydrate content. The results of this experiment showed that the iron chelating fraction was totally excluded by the Sephadex and was therefore of a molecular weight in excess of 10,000. The results of this experiment are shown graphically in Figure 13 A and B.

4. Comment

The role of the human salivary glands in digestion appears somewhat anomalous. No protease or lipase of any consequence

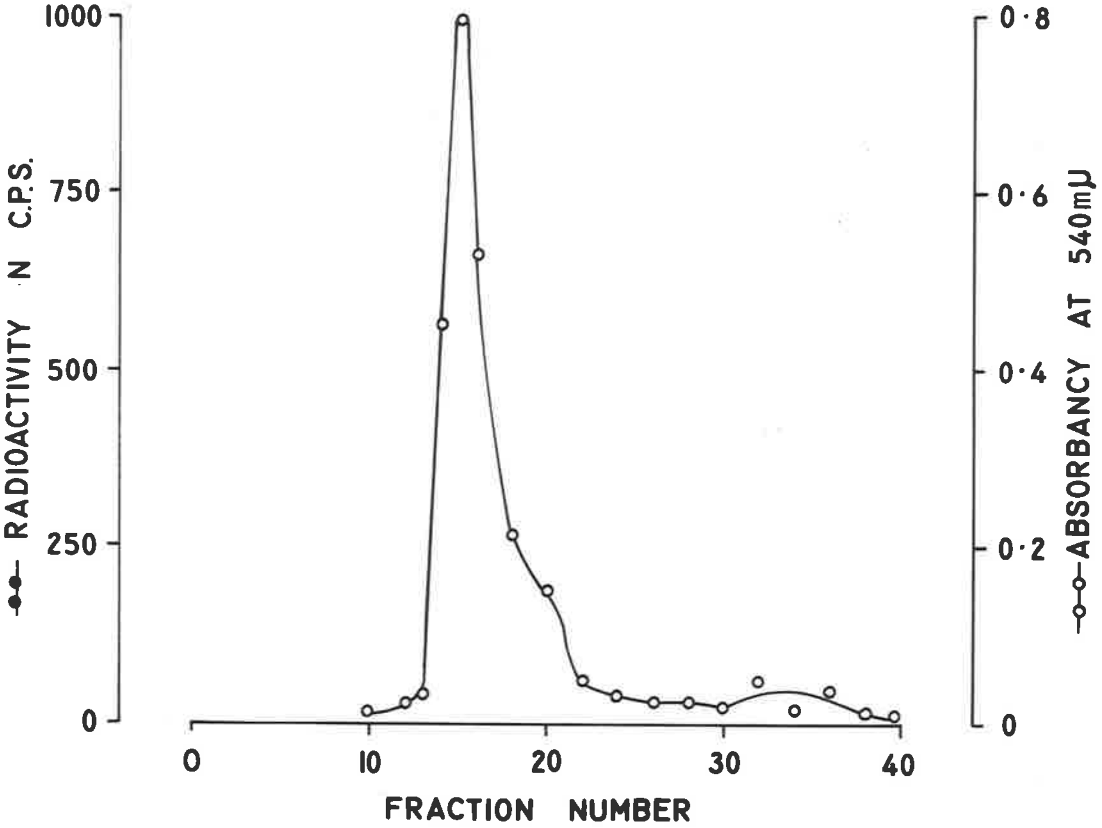


Figure 13A: Molecular sieving elution pattern of amylopectin.
Sample = 0.5 ml. of 3% amylopectin.
Column = 90 x 1.5 cm. Sephadex G-50 fine.
Fraction vol. 3.75 ml. Carbohydrate content (orcinol reaction) is proportional to absorbance at 540 mμ.

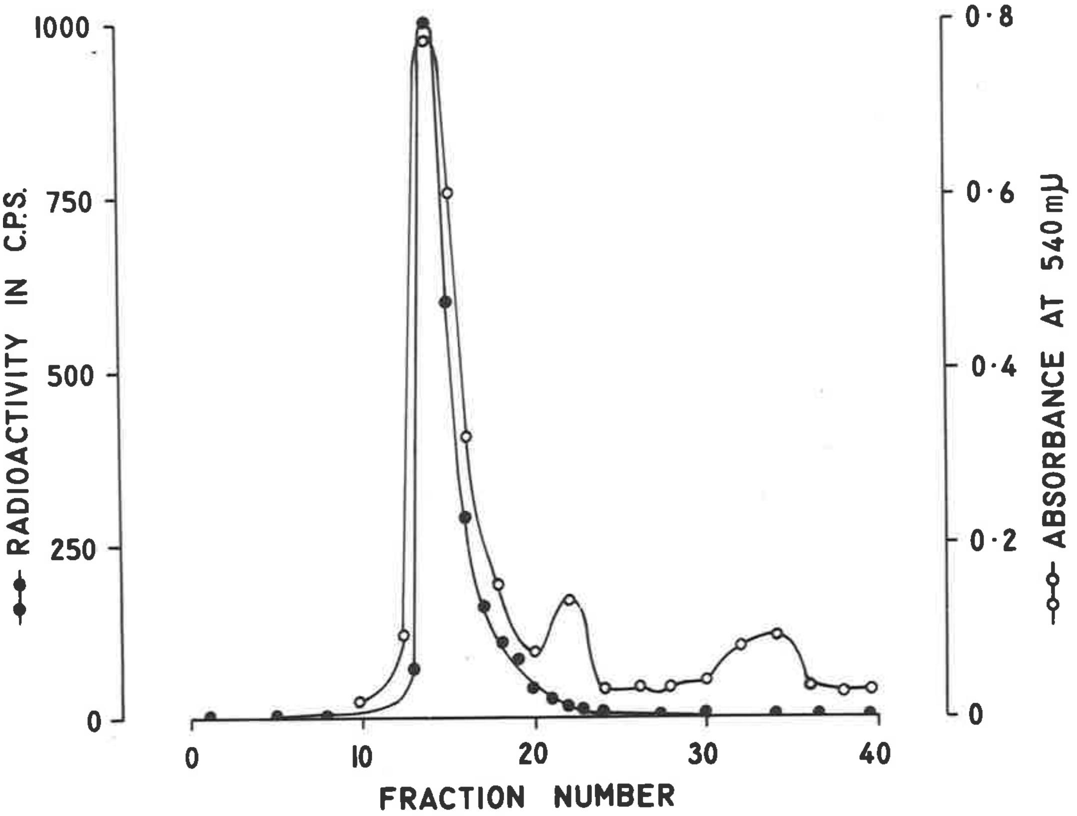


Figure 13B: Molecular sieving elution pattern of radioiron labelled partial hydrolysis mixture of 3% amylopectin. Sample volume = 0.5 ml. Column = 90 x 1.5 cm. Sephadex G-50 fine. Fraction volume = 3.75 ml. Carbohydrate content (orcinol reaction) is proportional to absorbance at 540 mμ. Bound iron measured in γ c.p.s. above background of fraction.

is found in saliva, but large amounts of the alpha amylase ptyalin are present. This enzyme has an optimum pH of 6.8 and is instantly inactivated and denatured when it mixes with gastric juice of low pH. The results of the experiments reported in this chapter suggest a new role for the salivary enzymes, a role which would be performed because of and not in spite of the short time available for this enzyme to act. This role is the interaction with dietary amylopectin to produce an iron chelating product which could serve the function of keeping dietary iron in solution in the alkaline environment of the duodenum where it is absorbed.

CHAPTER VIII

CHELATING AGENTS IN DRUGS

I. SALICYLIC ACID

1. Purpose and Plan of the Investigation

Many of the drugs and antibiotics ingested by man are powerful chelators of metals including iron (Weinberg, 1960). Such a drug is salicylic acid, and its ability in this regard has been long recognised. Because of the widespread and often long-term use of this drug - especially as the acetyl derivative in the form of Aspirin - it was decided to investigate its effect on the gastrointestinal absorption of iron in man. A further reason for this investigation was the recognition that salicylates, usually in the form of aspirin, can cause iron deficiency anaemia (Summerskill and Alvarez, 1958; Beveridge, Bannerman, Evanson and Witts, 1965). Such salicylate anaemia ~~bleeding~~ is usually attributed to alimentary bleeding and there is frequently evidence for this association: patients with haematemesis or melaena frequently have a history of aspirin taking; and occult blood can be demonstrated in the stools of 50 to 70% of people taking aspirin (Scott, Porter, Lewis and Dixon, 1961). The demonstration reported earlier in this thesis that synthetic chelating agents such as EDTA and DTPA could profoundly depress iron absorption suggested another explanation for anaemia.

2. Materials and Methods

In vivo studies. 32 radioiron absorption tests were carried out on 16 haematologically normal young men. Each person had 2 absorption tests, one in which the labelled iron was given fasting and one in which it was given with salicylate. A dose of 2 µCi of Fe^{59} with 5 mg. of iron in 200 ml. of water was used. The test dose of sodium salicylate 730 mg. was such that the molar ratio of salicylate:iron was 50:1, such a molar excess of chelating agent to iron having previously been shown necessary if a chelating agent is to affect iron absorption (Chapter III).

Because salicylic acid binds ferric iron more strongly than ferrous iron, an equal number of studies were carried out with 5 mg. of ferric sulphate and with 5 mg. of ferrous sulphate to which was added 50 mg. of ascorbic acid to maintain reducing conditions in the stomach.

Absorption of Fe^{59} was measured in a whole body gamma spectrometer after allowing 14 days for the unabsorbed radioactivity to be excreted. The method used was that described previously (Chapter III).

In vitro studies. A spectrophotometric technique was used to estimate the relative binding strength of salicylate and human gastric juice for iron. The absorption spectrum for iron salicylate has a maximum at 520 mμ and the stability of this chelate was tested against gastric juice in the following way. Mixtures were prepared which were 0.0005 M. with respect to sodium salicylate and 0.0005 M. with respect to $FeCl_3$. The absorbance of the reaction products at pH ~~3~~ 2.5 was measured at 520 mμ. Measurements were then made on mixtures of these amounts of salicylate and iron but containing 5% v/v gastric juice at pH 2.5 Samples of gastric juice obtained from 6 normal persons in the fasting state were tested in this fashion.

3. Results

Salicylate and iron absorption. Absorption of the iron-salicylate chelate did not differ significantly from that of iron alone, either in the ferrous form (Table 16) or in the ferric (Table 17). The normal variation between two tests performed under identical conditions in this laboratory infrequently exceeds 12%. In only 2 of 16 subjects (no. 32, 41) did sodium salicylate reduce absorption by more than this amount.

Salicylate and gastric juice. Figure 14 shows that the absorption maximum of the iron-salicylate complex occurs at 520 mμ, at which wavelength neither $FeCl_3$ nor gastric juice has any measurable absorbance in the concentrations used.

Table 16

Effect of Orally Administered Sodium Salicylate on Ferrous Iron Absorption (5 mg. Iron, 50 mg. Ascorbic Acid)

Subject		Fe^{59} Absorption (%)	
		Control	Na salicylate
31		47*	48
32		46*	16
33		18	14*
34		18	7*
35		16	18*
36		12	11*
37		10*	10
38		8*	10
	Mean	21.9%	16.8%

*Administered first.

Table 17

Effect of Orally Administered Sodium Salicylate on Ferric Iron Absorption (5 mg. Iron)

Subject		Fe^{59} Absorption (%)	
		Control	Na salicylate
39		43*	41
40		23*	14
41		19	5*
42		18*	10
43		16	21*
44		15	15*
45		10*	8
46		4	4*
	Mean	18.5%	14.8%

*Administered first.

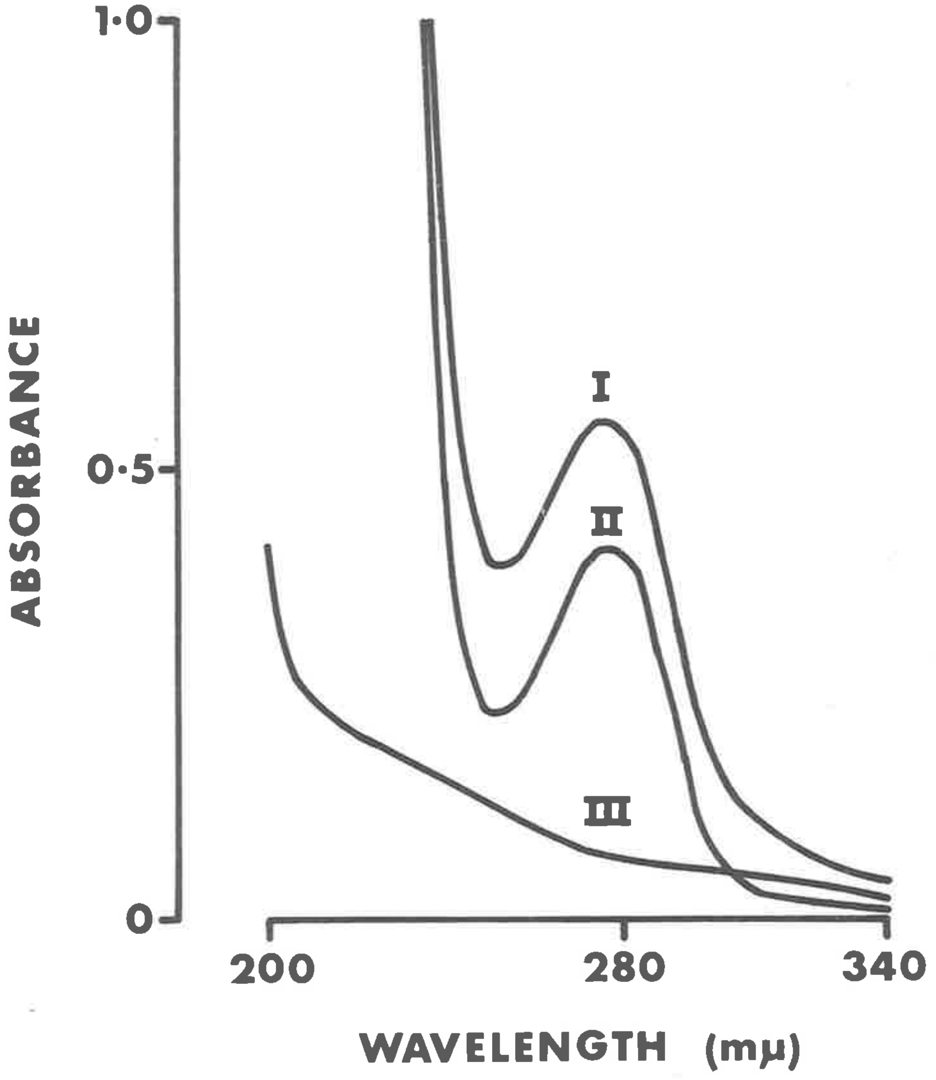


Figure 14: Spectral transmittance curves of gastric juice.
I. 10 ml. gastric juice + 1 ml. 0.001 M. $FeCl_3$ + 9 ml. H_2O at pH 2.5.
II. 10 ml. gastric juice + 10 ml. H_2O at pH 2.5.
III. 0.00005 M. $FeCl_3$ at pH 2.5.

However, the iron-salicylate complex was not formed when gastric juice was added to the salicylic acid and ferric iron, indicating the presence in gastric juice of a component which has a greater avidity for iron than does salicylic acid.

4. Comment

These results show that chelating iron by salicylic acid does not affect the extent of its alimentary absorption. The explanation for this behaviour was shown to be in the iron-binding property of gastric juice, which was such that iron was bound more strongly, and therefore preferentially, to gastric juice. These observations illustrate one of the dangers of extrapolating studies of a defined chemical system to the complex situation which exists in the gut. In this environment the net absorption will be affected not only by the substances under study but of the products of digestion and the secretions of the gut itself.

CHAPTER IX

CHELATING AGENTS IN DRUGS

II. "ALLOPURINOL"

1. Purpose and Plan of the Investigation

The combination of a metal ion with a chelating or complexing agent almost always increases its tendency to exist in a higher valence state (Rubin, 1960). For example, desferrioxamine has been shown not to react with ferrous iron (Goodwin and Whitten, 1965) unless under conditions which permit or facilitate prior oxidation to the ferric state.

The enzyme xanthine oxidase is widely distributed throughout the body and performs a variety of metabolic functions besides the principal one of catalysing the oxidation of hypoxanthine to xanthine and xanthine to uric acid (Elion, Taylor and Hitchings, 1964). Inhibition of xanthine oxidase might affect iron metabolism because the ferritin-xanthine oxidase system has been proposed as a mechanism for the mobilisation of iron from the liver and intestinal mucosa (Mazur, Green, Saha and Carleton, 1958). Inhibition of the enzyme in the gut mucosa might result in the accumulation of ferric ferritin which would be unavailable for transfer across the cell membrane to the plasma. The absorption of iron would be decreased when the cell is exfoliated at the end of its lifespan. Thus, on this reasoning, inhibition of xanthine oxidase might exert a paradoxical effect on iron metabolism - diminished absorption from the intestine with increased stores in the liver. Because of the effect of this enzyme on the valency state of intracellular iron, it was decided to investigate the effect of the potent inhibitor of xanthine oxidase "Allopurinol" (4-hydroxypyrazolo (3,4-d) pyrimidine).

2. Materials and Methods

56 radioiron absorption studies were carried out on 28 healthy volunteers. Iron absorption was measured using 3 μCi of Fe^{59} in 200 ml. of water in which there was a carrier of 5 mg. of iron as ferrous sulphate and 50 mg. of ascorbic acid. Each person had two absorption tests, one in which the labelled iron was given fasting and one in which it was given during the administration of allopurinol (Burroughs Wellcome). The method of testing was randomised, and the initial study was the control test in 15 persons and the allopurinol treatment in 13. Three dose schedules of allopurinol were used.

3-day maintenance dose. 8 subjects were given 300 mg. of allopurinol daily for 2 days before and on the day of the iron absorption study. The drug was given orally in 3 divided doses daily. This amount of allopurinol is the usual maintenance dose required in gouty subjects to keep the serum level of uric acid within the physiological range.

3-day high dose. 8 subjects were given 800 mg. of allopurinol daily in 4 divided doses for 3 days. A maximum effect on purine metabolism is usually achieved with this daily schedule.

9-day high dose. 12 subjects were given 800 mg. of allopurinol daily for 4 days before the absorption test, the day of the test and for 4 days after the test. This schedule was adopted in an attempt to influence xanthine oxidase activity in the intestinal mucosa during the life-span of the cells.

Iron absorption was measured in a whole body counter as previously described (Chapter III). The subjects were fasted for 12 hours before and 2 hours after the test dose of Fe^{59}, and absorption was estimated 14 days after the oral radioactivity had been given.

3. Results

Allopurinol did not alter the extent of absorption of radioiron. The mean absorption in 28 control studies was 15% and in a similar number of studies after allopurinol 13%. This difference is not statistically significant. The normal variation between two tests performed under identical circumstances in this laboratory has not exceeded 12%, and only 3 of the 28 subjects exceeded this amount in this study. Two subjects showed reduced absorption with allopurinol of this degree and one increased absorption.

In the studies when allopurinol was given in the dosage of 300 mg. daily for 3 days the mean absorption was 13% compared with the mean absorption in the control study of 15% (Table 18). With the dosage of 800 mg. daily for 3 days the mean was 14% after allopurinol and 18% in the fasting study (Table 19). When allopurinol was given for 9 days in a dosage of 800 mg. a day the mean absorption was 12%, an identical mean to the control study (Table 20). None of these differences was statistically significant.

4. Conclusion

These results show that the xanthine-oxidase inhibitor "allopurinol" is not associated with a significant change in iron absorption in man. The study also provides indirect evidence that the ferritin-xanthine oxidase system is not a major physiological mechanism in the control of iron absorption at the mucosal level. It does not, however, contribute information about the effect of the drug on the mobilisation of iron from the liver, a point which is in dispute at the moment (Ayvazian, 1964; Powell and Emmerson, 1966).

Table 18

Effect of 3-day Maintenance Dosage of Allopurinol on Radioiron Absorption (300 mg. Allopurinol/day)

Subject	Sex	% Absorption of Fe^{59}	
		Without allopurinol	With allopurinol
47	M	7	10
48	M	10	0
49	M	11	12
59	M	14	10
51	F	15	35
52	M	20	16
53	M	20	10
54	M	23	9
Mean absorption (%)		15	13

Table 19

Effect of 3-day High Dosage of Allopurinol on Radioiron Absorption (800 mg. Allopurinol/day)

Subject	Sex	% Absorption of Fe^{59}	
		Without allopurinol	With allopurinol
55	M	3	6
56	M	5	9
57	M	11	4
58	M	13	10
59	M	16	19
60	M	18	11
61	M	33	20
62	M	44	33
Mean absorption (%)		18	14

Table 20

Effect of 9-day High Dosage of Allopurinol on Radioiron Absorption (800 mg. Allopurinol/day)

Subject	Sex	% Absorption of Fe^{59} Without allopurinol	% Absorption of Fe^{59} With allopurinol
63	M	2	8
64	M	2	9
65	M	7	14
66	M	9	9
67	M	12	13
68	M	13	13
69	M	15	7
70	M	16	9
71	M	16	4
72	F	18	13
73	M	18	27
74	M	20	13
Mean absorption (%)		12	12

CHAPTER X

CHELATING AGENTS IN BODY SECRETIONS

1. Purpose and Plan of the Study

Saliva, gastric juice, intestinal juice, bile and pancreatic juice are all secreted into the gut lumen and therefore must be considered as possible sources of endogenous chelating agents. A consideration of the chemical requirements for metal chelate formation - especially in the case of iron - suggests that gastric juice is likely to be the most important of these substances, although saliva could also play a part. Gastric juice seems likely to be important because dietary iron is released from food conjugates in the acid/peptic digestion in the stomach (Sanford, 1960) and rapid formation of iron chelates usually requires an initially low pH such as that found in the stomach.

The present study was designed to determine whether chelating substances were normally present in human gastric juice, and if so to learn something of their nature, amount and role.

2. Materials and Methods

Subjects studied. The iron chelating ability of gastric juice was studied in 30 normal subjects. Specifically, the subjects studied had no disturbance of their haematological or gastrointestinal systems and all demonstrated normal gastric hydrochloric acid production.

<u>Collection of gastric juice</u>. Gastric juice was obtained from all subjects after a 12 hour fast. A radio-opaque stomach tube was introduced under radiological control so that its tip lay at the centre of the greater curvature. With the subject in the semi-left lateral position the stomach was emptied and this material discarded. Fasting gastric juice was then collected by intermittent suction for a period of 30 minutes. Particular attention was paid to the exclusion of nasopharyngeal secretions and saliva during the collection.

<u>Measurement of iron chelating ability</u>. Chelating ability was assessed by the radioiron solubility test described in Chapter V. 2 ml. of 20% v/v gastric juice were used for these tests and the results were expressed as mg. of ferric iron bound per ml. of gastric juice.

<u>Molecular sieving</u>. The various protein components of gastric juice were separated on the basis of molecular size by upward passage through a 2.5 x 40 cm. "Sephadex G-200" column (Figure 15). The eluting buffer used was physiological saline buffered to pH 8.0 with 0.02 M. NH_4Cl/NH_4OH. The effluent from the column was collected in fractions which

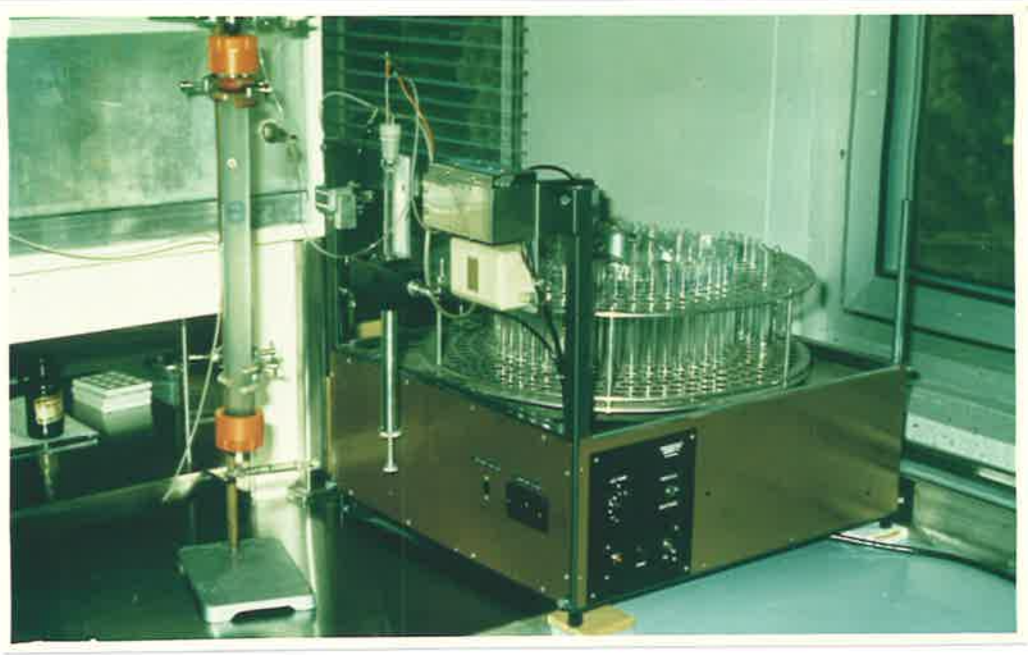


Figure 15: Chromatography column and fraction collector used for Sephadex Gel filtration.

were assayed for relative protein content by measurement of ultraviolet light absorption at 280 mμ. The component responsible for binding iron was identified by prior labelling of the specimen with radioiron and also by carrying out radioiron solubility tests on the eluent fractions of unlabelled specimens.

Polyacrylamide disc gel electrophoresis. Separation of the gastric juice components was also achieved by using the technique of polyacrylamide disc gel electrophoresis whereby the fractions are separated on the basis of molecular charge as well as size (Davis and Ornstein, 1961). The gel columns were formed in 60 mm. lengths of glass tubing of 5 mm. internal diameter. Electrophoretic concentration of the sample material took place in a short zone of a large pore gel after which separation of the components was achieved in a gel of small pore size using a current of 4 milliamps per tube. The gels were stained and fixed with Amido Schwartz in 7% acetic acid after which electrophoretic destaining removed any unbound pigment. The apparatus used is illustrated in Figure 16.

3. Results

Marked iron chelating ability was found in the gastric juice of all 30 patients studied. The results which are

Figure 16: Apparatus used for polyacrylamide disc gel electrophoresis.

presented in Table 21 show that the iron-binding capacity ranged from 0.04 to 0.22 mg. Fe^{+++}/ml. of gastric juice with a mean of 0.14 mg. Fe^{+++}.

When gastric juice of the subjects studied was resolved by molecular sieving, a consistent elution pattern was found (Figure 17). The radio-iron was found to be associated with the totally excluded high molecular weight fraction. In order to ensure that these results were not caused by a modification of the gastric juice protein caused by an in vitro reaction with ferric iron, iron solubility tests were carried out on the eluted fractions of unlabelled gastric juice. Once again only the first high molecular weight fraction demonstrated iron-binding ability.

In the electrophoretic studies of gastric juice radioiron was also located in a specific band (Figure 18).

Further control experiments carried out with blood plasma and with purified transferrin showed that iron-binding peak in molecular sieving and the iron-binding band in the disc gels did not correspond in position to transferrin, the normal iron binding protein of serum.

4. Conclusion

The results presented in this chapter demonstrate the presence in normal human gastric juice of a high molecular

Table 21

Iron-Binding Capacity of Gastric Juice from Normal Human Subjects

Subject No.	Iron-binding capacity mg. Fe^{+++}/ml. gastric juice	Subject No.	Iron-binding capacity mg/Fe^{+++}/ml. gastric juice
1	0.126	16	0.149
2	0.132	17	0.127
3	0.102	18	0.116
4	0.190	19	0.105
5	0.165	20	0.182
6	0.143	21	0.187
7	0.220	22	0.198
8	0.115	23	0.149
9	0.220	24	0.149
10	0.094	25	0.056
11	0.088	26	0.061
12	0.176	27	0.171
13	0.217	28	0.179
14	0.219	29	0.058
15	0.162	30	0.057

Mean iron-binding capacity = 0.144 mg. Fe^{+++}/ml. gastric juice

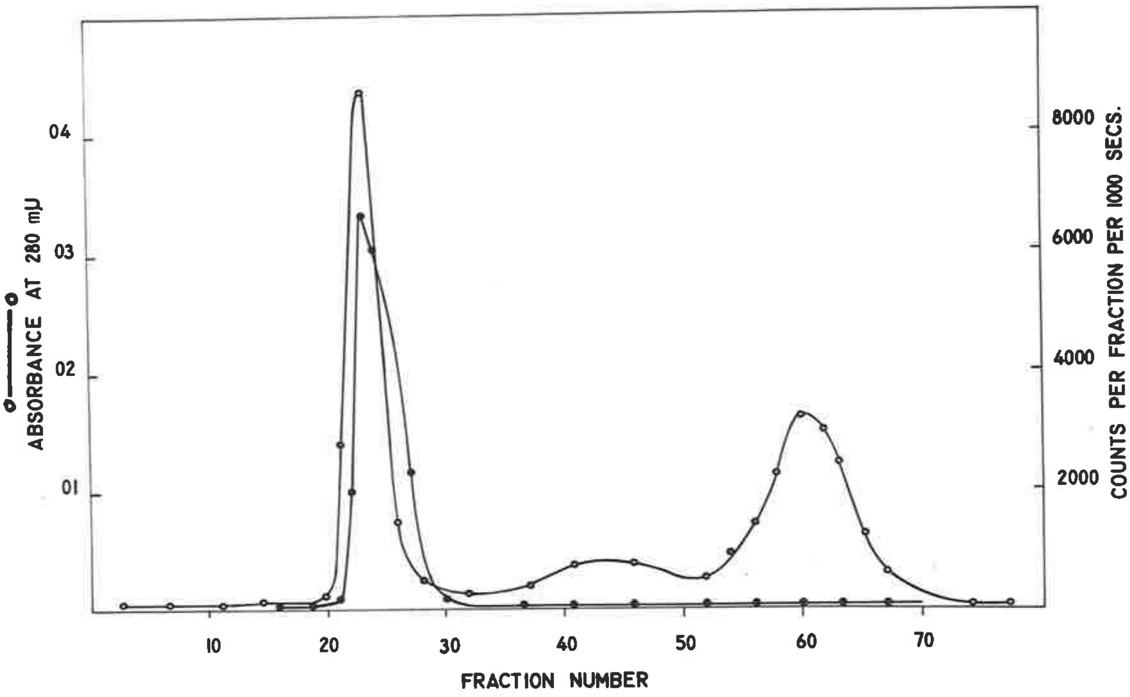


Figure 17: Sephadex G-200 elution pattern of normal human gastric juice labelled with radioiron. The considerable iron-binding ability is indicated by the high molecular weight peak at fraction 23.

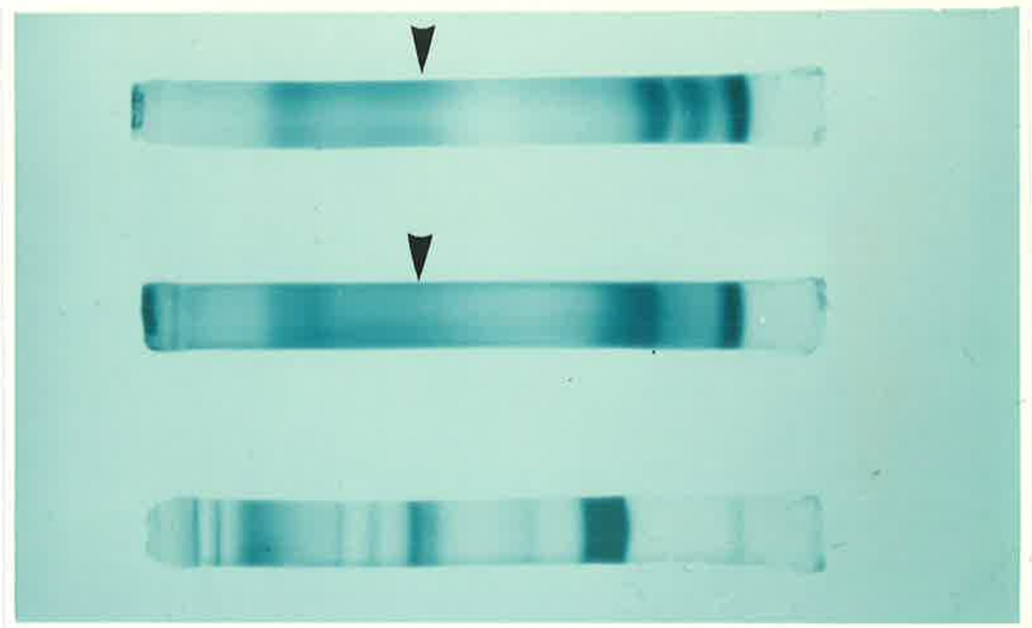


Figure 18: Polyacrylamide disc gel electrophoresis.
Top: Normal human gastric juice.
Centre: Normal human gastric juice.
Below: Normal human serum.
Sample applied at left.
Arrow indicates iron binding band.

weight iron-binding protein. The amount of this substance present in the gastric juice of the 30 normal volunteers studied was sufficient to bind the 30 mg. of iron present in a typical daily diet assuming a secretion of 2 to 3 litres of gastric juice/day. No evidence has yet been presented to indicate whether the effect this substance has on the absorption of iron is an enhancing or inhibiting one.

CHAPTER XI

DISCUSSION

The mechanism of gastrointestinal iron absorption in man has not been established with any certainty and the major advances in knowledge in this area in recent years have mainly concerned the mucosal regulating mechanisms. The work reported in this thesis has been concentrated on the possible role of dietary and luminal factors in iron absorption. Two reasons prompting this approach have been the realization that dietary iron is normally greatly in excess of the body's needs, and secondly, the puzzling fact that iron absorption occurs predominantly in the duodenum.

A typical human diet contains about 15 mg. of iron per day. However because of the unique property of iron that no mechanism exists for its excretion from the body, the daily requirement for the healthy adult male is only 0.6 mg. Even in the case of a pregnant woman the average daily iron requirement is increased only to 2 mg.

Two chemical properties of iron which cannot be ignored when considering the mechanism of its absorption are first the fact that the stable valency of iron in aqueous solution is the trivalent ferric state; at neutral pH ferrous iron in aqueous solution is instantly oxidised to the ferric state. Secondly, ferric iron is extremely insoluble at high pH when it precipitates as ferric hydroxide. The solubility product constant for ferric hydroxide is 1.1×10^{-36}. Nevertheless it has been well established that the major absorption of iron occurs in the duodenum, the most alkaline part of the gastrointestinal tract. Any satisfactory theory of the mechanism of iron absorption must therefore first of all explain how it remains in solution in the alkaline lumen of the duodenum so as to be absorbed by the mucosal cells.

An answer to the problem of the insolubility of inorganic ferric iron at high pH was suggested by the successful use of synthetic iron chelate compounds in the treatment of the iron deficiency states which frequently occur with plants growing on alkaline soils. One of the outstanding properties which chelation frequently confers on a metal ion is the ability to remain in solution in a situation where it would otherwise be quite insoluble. Accordingly the experiments described in Chapters III and IV were designed to answer the question of whether orally administered chelating agents could affect the extent of gastrointestinal iron absorption in man. In an attempt to explain conflicting results reported by other workers on the effect of EDTA on iron absorption, the effect of the molar ratio of chelating agent to iron was also studied. The results of this study demonstrated that chelation could profoundly affect the extent of gastrointestinal iron absorption, and furthermore could either enhance or depress it according to the nature of the chelating agent used. For this effect to be achieved it was necessary to administer a molar excess of chelating agent to iron. In the case of the synthetic chelating agents EDTA and DTPA the molar excess required to produce a depression of iron absorption was 10:1. In this study it was shown for the first time that fructose enhances iron absorption in man. For this effect to be produced the molar ratio of fructose to iron required is 50:1. The importance of a molar excess of chelating agent to iron does not appear to have been recognised hitherto, and lack of attention to this factor could well explain the conflicting results which have appeared in the literature.

That the enhancement of iron absorption by fructose is related to its ability to chelate iron is suggested by the experiments reported in Chapter IV where it was demonstrated that glucose given with iron in a 50:1 molar excess has no effect on iron absorption. The results obtained with fructose suggest that it would be a more effective haematinic than ferrous sulphate provided the requirement of the necessary molar ratio of chelating agent to iron was borne in mind. Such a preparation would be of acceptably low cost for widespread use. A further consideration which is being investigated is the possible relationship between the incidence of iron deficiency anaemia and the use of honey (fructose) and cane or beet sugars (sucrose). Witts (1966) has pointed out that "the great epidemic of chlorosis which filled the outpatient departments of Western Europe in the nineteenth century and which declined and disappeared in the first quarter of the present century was never adequately explained." The increase in world production of cane and beet sugar was a relatively late phenomenon in history and could be well related to the epidemiology of iron deficiency anaemia.

In order to be able to undertake the in vitro studies prompted by the results of these early investigations with EDTA, DTPA and fructose it became necessary to find methods for the detection and characterisation of chelating agents in biological samples. Preliminary studies had revealed that many of the methods usually employed by organic chemists were not quite suited to the study of biological samples of complex and often unknown composition. A quantitative test for chelating ability which was developed for this study was the radioiron solubility test. This test is extremely sensitive and is well suited to being carried out with large numbers of samples.

The in vitro studies reported in Chapter VI were undertaken to explain the ability of fructose but not glucose to form a stable chelate complex with iron. As a result of this investigation it was possible to ascribe the iron chelating ability of the open chain fructose molecule to the dihydroxy acetone structure which is found in it. This theory was tested by predicting that the sugars tagatose and sorbose, which had not previously been tested in this regard, would similarly be able to form stable chelate complexes with iron. Experiments confirmed the prediction and a theory was proposed and published which enables a prediction of chelating ability to be made for any sugar or polyol. It is hoped to be able to carry out studies with human volunteers to determine whether sorbose, tagatose and dihydroxy acetone enhance gastrointestinal iron absorption in man. If this turns out to be the case then powerful evidence would be provided to support the theory that the absorption promoting ability of fructose is related to its iron chelating ability and not to its reducing powers as is argued by Crosby and his co-workers (Pollack, Kaufman and Crosby, 1964; Pollack, Kaufman, Crosby and Butkiewicz, 1963).

Having established that chelation could affect the extent of gastrointestinal absorption of iron, it was then necessary to determine whether in fact man encountered in his diet, in food digestion products or in secretions into the gut lumen chelating agents which could form sufficiently stable chelates to keep dietary iron in solution in the alkaline duodenal lumen. To be of practical physiological importance any such substances would have to be present in appropriate quantities. In order to reduce the study to practicable proportions it was decided to exclude fats and to concentrate on egg albumin as a typical protein and on amylose and amylopectin as the two major components of all carbohydrate foods.

The major finding from this phase of the investigation was that amylopectin after being acted upon by saliva for as little as one minute acquired the ability to chelate ferric iron strongly. A most interesting aspect of this study was that action of saliva beyond about five minutes resulted in the loss of iron chelating ability by amylopectin. Quantitatively the amount of amylopectin in most human diets is such that the mechanism described here could be of great importance in keeping dietary iron in solution at high pH, permitting its absorption in the duodenum. The experiments described in Chapter VII suggest that the component in saliva responsible for this phenomenon is an enzyme. Further work will be needed to establish whether or not the enzyme is ptyalin. In any event, the phenomenon of saliva acting on amylopectin to produce a substance of marked iron chelating ability provides a possible role for salivary digestion which satisfactorily overcomes the difficulties associated with the traditional view of ptyalin as the initiator of starch digestion. The chelation role of ptyalin's action would not be in spite of but because of the short time this enzyme exists in contact with its substrate.

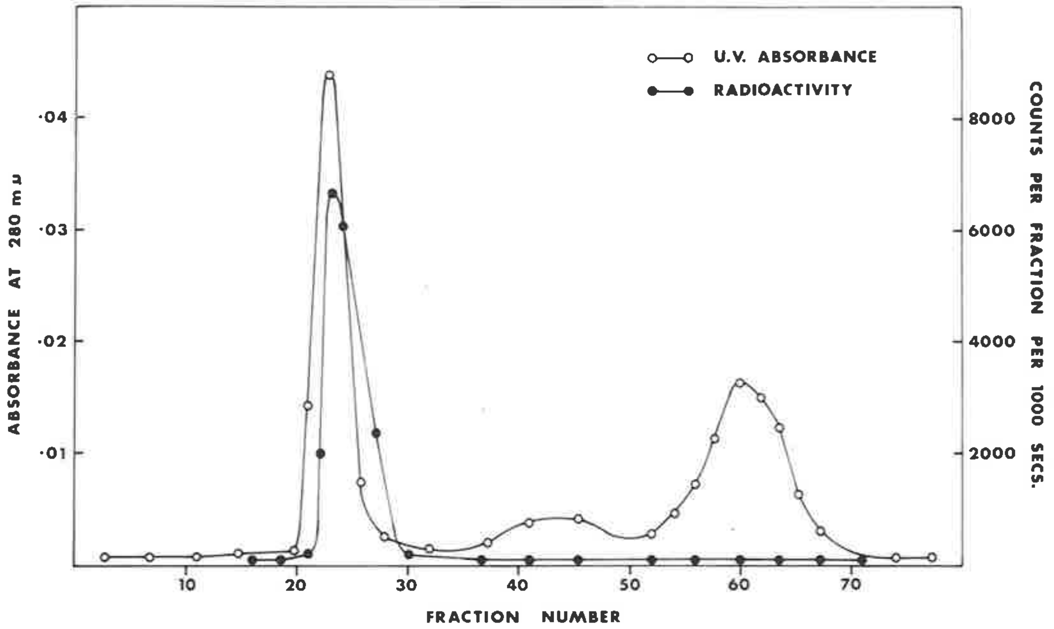
Two studies were carried out on drugs in the course of this investigation. The experiments with salicylic acid - a known iron chelator - were most instructive because although salicylic acid was not found to have any effect on iron absorption, the initially puzzling result prompted the study of gastric juice and gave the first clue to the presence of a powerful iron-binding substance in this secretion. The study on the xanthine-oxidase inhibitor "Allopurinol" was again negative but shed some light on the question of the role of this enzyme which occurs in intestinal mucosal cells as well as in liver. The results showed that "Allopurinol" is not associated with a significant change in iron absorption in man, and provided indirect evidence that the ferritin-xanthine oxidase redox system is not a major physiological mechanism in the control of iron absorption at the mucosal level.

The study of the substances secreted into the gut lumen was restricted to gastric juice as being likely to be of greatest importance. This study, reported in Chapter X, showed that the gastric juice of healthy human volunteers was capable of binding or chelating iron, and to a degree sufficient to keep in solution the amount of iron present in a normal diet. The component in gastric juice responsible for iron binding is a high molecular weight protein. Preliminary characterisation of the substance has been carried out using "Sephadex" gel filtration and polyacrylamide gel disc electrophoresis. That the role of this substance is to inhibit iron absorption is suggested by work carried out subsequently, but not reported in this thesis, which has shown that the iron binding protein of gastric juice is absent in subjects with idiopathic haemochromatosis (Davis, Deller and Luke, 1966). Sephadex gel elution pattern~~s~~ of normal and haemochromatotic radioiron labelled gastric juice was shown in Figure 19, and the position of the iron-binding band on a disc gel electrophoretogram is shown in Figure 18.

Much further work remains to be done to characterise the gastric juice iron-binding protein and to assess its physiological role. Studies at present being planned include preparation of fluorescent antibody which can be used to determine which mucosal cells are responsible for its synthesis; iron absorption experiments in animals and human volunteers to determine its effect on iron absorption; and quantitative estimations of the content of iron-binding protein in the gastric juice of subjects with disorders such as iron deficiency anaemia, transfusion siderosis, fibrocystic disease, cirrhosis of the liver and diabetes.

The techniques used in this study are also being adapted to determine whether there are metal binding proteins in gastric juice capable of binding other metals such as copper, zinc, molybdenum and cobalt. The first metal being examined in this

NORMAL



HAEMOCHROMATOSIS

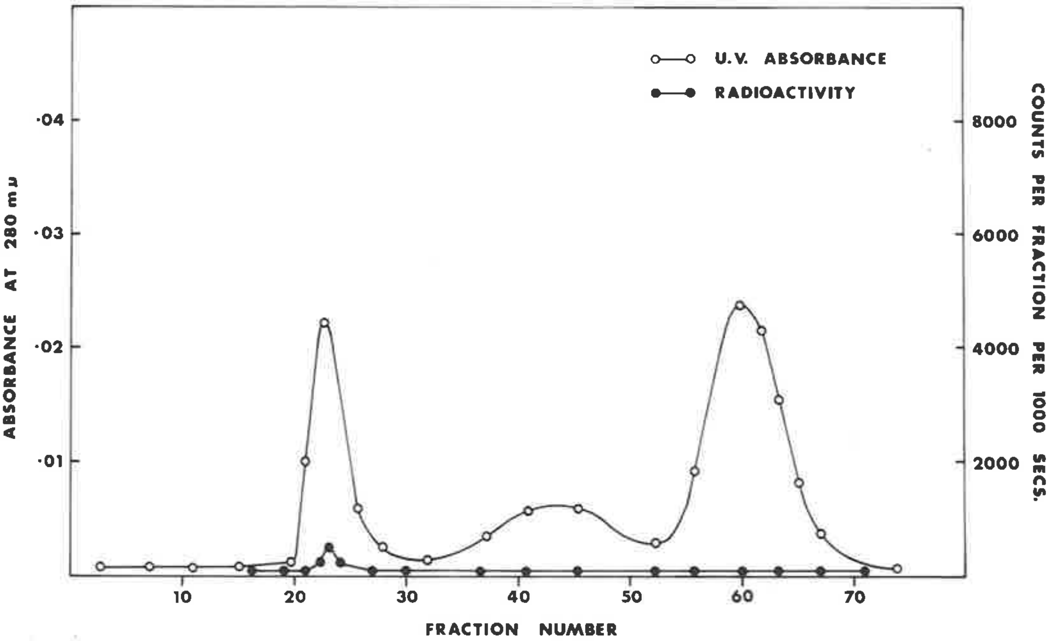


Figure 19: Sephadex G-200 elution pattern of Fe^{59} labelled normal gastric juice (top) and gastric juice from a patient with haemochromatosis (below). Note the considerable iron-binding ability as indicated by the high radioactivity of the first high molecular weight protein peak in the normal subject. This is negligible in the case of haemochromatosis.

regard is copper because of the existence of the copper overload condition, Wilson's disease.

The work presented in this thesis suggests that compounds which are able to react chemically with iron to form chelate compounds may be of physiological importance in two ways. Chelating agents of dietary origin are able to keep iron in solution in the alkaline environment of the duodenum and so permit iron to be absorbed by the mucosal cells. Direct evidence has been presented to show that one of these substances, fructose, enhances gastrointestinal iron absorption. However the endogenous chelating agents may play an opposite role in regulating the extent of iron absorption by inhibiting its absorption. At the luminal level therefore the net absorption of iron might be determined by the interaction of these two opposing mechanisms. If the amount of endogenous chelating agent secreted was controlled by the body's need to adjust iron intake, this would represent a negative feed-back mechanism controlling iron absorption operating at the luminal level.

Although the mechanism by which iron is transported across the gut mucosa has never been elucidated, a great deal of attention has been directed to this aspect. Hahn, Bale, Ross, Balfour and Whipple (1943) put forward the "mucosal block" theory of a self-regulating mucosal apparatus for the control of iron absorption. This theory was subsequently modified by Granick (1946) who suggested that apoferritin was continually being formed and broken down in the mucosal cell. When iron became available in the gastrointestinal tract this was believed to complex with apoferritin to form ferritin. Ferritin accumulated in the mucosal cell and prevented iron from entering the mucosa. The rate of release of iron from ferritin was postulated to depend upon oxygen tension. Subsequent work by numerous authors (Brown, Dubach and Moore, 1958; Bothwell, Pirzio-Biroli and Finch, 1958; Smith and Pannaccuilli, 1958) has shown that there is no absolute mucosal block to iron absorption.

A major finding which has been clearly established is that factors such as hypoxia, anaemia or acute blood loss all increase iron absorption. Iron absorption is in fact directly related to erythropoiesis. Workers who have established this relationship include Reynafarje and Ramos (1961), Bothwell, Pirzio-Biroli and Finch (1958), Moore, Roberts and Minnich (1941) and Ross and Chapin (1941). However the mechanism by which the message of the body's need is sent to the mucosa remains a mystery.

Recently further understanding of the role of the mucosa in iron absorption has come from the work of Crosby and co-workers

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who have demonstrated that although iron may be taken up by mucosal cells, such cells may be exfoliated and lost before their iron is passed on to plasma transferrin and made available for erythropoiesis (Crosby, 1963). However, the work presented in this thesis is not concerned primarily with the mucosal transport or subsequent utilization of iron and so does little to confirm or oppose the findings of other workers discussed above.

Among the important studies of the effect of luminal factors on the absorption of inorganic iron have been the demonstrations of the effect of valency state on the absorption of large doses of iron (Venkatachalam, Brading, George and Walsh, 1956; Brise and Hallberg, 1962) and the finding that certain organic acids are capable of enhancing iron (Brise and Hallberg, 1962). Such compounds as ascorbic acid and succinic acid as well as having reducing powers are also powerful iron chelators and it seems equally possible that their absorption promoting action is related to their iron chelating ability and not to their reducing action. Recently Saltman and co-workers have proposed a new theory of the mechanism of iron absorption which is based on the observation that fructose and some other sugars are able to form chelate complexes with iron (Charley, Sarkar, Stitt and Saltman, 1963a). These complexes are able to enhance the movement of iron across mucosal cells and facilitate the exchange of iron between cells and plasma (Stitt, Charley, Butt and Saltman, 1962). Saltman (1965) has proposed a chelation mechanism of iron absorption which is based on this ability of certain naturally occurring chelates to enhance iron absorption. These observations provided a most stimulating contribution to thought in this field and were the starting off point for the work reported in this thesis. The principal defect of the particular chelation theory proposed by Saltman is that it sheds no light on the means whereby the absorption of iron is limited to the body's needs. The work reported in this thesis has shown clearly that in man chelating agents can either enhance or depress iron absorption. As the absorption enhancing effect of dietary chelates is not directly related to the body's need for iron, the question that has been raised by the work presented here is whether an absorption inhibiting effect of endogenous chelates is a luminal mechanism for controlling the extent of gastrointestinal iron absorption in man.

The specific contributions to knowledge reported in this thesis have been the demonstration of the absorption inhibiting effect in man of the orally administered chelating agents EDTA and DTPA and the absorption enhancing effect of fructose; the demonstration of the importance of molar ratio of chelating agent to metal for either effect to be produced; the development of a radioiron solubility test for the in vitro study of chelation phenomena in biological samples; the elucidation of the ability of fructose but not glucose to form a stable chelate complex with iron resulting in a contribution to the understanding of the molecular structure of the iron-fructose molecule; development of a general rule enabling a prediction to be made of the iron chelating ability of sugars; the discovery that salivary digestion of amylopectin, if restricted to not more than a few minutes, results in the formation of a product which is a powerful iron chelator; the finding that salicylic acid, under the conditions used, does not affect the gastrointestinal absorption of ferrous or ferric iron in man and that this result can be explained by the successful competition of endogenous chelating agents for iron; the demonstration that the xanthine-oxidase inhibitor "Allopurinol" does not affect gastrointestinal iron absorption in man and that therefore the xanthine oxidase-ferritin system is probably not of major importance in controlling iron absorption in man; and the demonstration of a specific iron-binding high molecular weight protein component of normal human gastric juice.

The present study therefore, is simply the beginning of a new chapter in the long search for an understanding of the mechanism of gastrointestinal absorption of metals in man.

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