"THE DESIGN OF ORALLY ACTIVE IRON CHELATORS FOR THE TREATMENT OF THALASSAEMIA"

by

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A Thesis submitted to the University of Essex for the Degree of Doctor of Philosophy

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To my Father, my Mother

and all other Cypriot refugees

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PREFACE

Some of the work presented in this thesis is under patent arrangements. The first patent of the series is:

British Patent Specification Number 8208608

"Pharmaceutical Compositions" by

G. Kontoghiorghes
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ABSTRACT

"The Design of Orally Active Iron Chelators for the Treatment of Thalassaemia"

G. Kontoghiorghes

The object of this study was to design orally active and cheap iron chelators for the treatment of transfusional iron overload in patients suffering from β thalassaemia. Although hundreds of compounds have been screened both *in vitro* and *in vivo* for this purpose, they failed because they were no more effective than the drug desferrioxamine in present use. This drug, however, is expensive and has to be given by injection.

Several pyridine derivatives belonging to two distinct classes have been synthesised in this project. One class of compounds posseses an α-keto hydroxy and the other a hydroxamic acid binding site. When different substituents were introduced onto the pyridine ring, changes in the charge and the iron affinity of the ligand at physiological pH, and the lipid/water solubility of their iron complexes were observed. The following compounds were most intensively studied for their properties in relation to iron: 1,2-dimethyl-3-hydroxy-pyrid-4-one (L_1), 1-methyl-3-hydroxy-pyrid-2-one (L_2), 2,4-dihydroxy-pyridine-1-oxide (L_3), 2-hydroxy-pyridine-1-oxide (L_4), 2-methyl-3-hydroxy-pyr-4-one (L_5), 2-hydroxy

4-methoxy-pyridine-1-oxide (L_6) and 2-hydroxy-4-oxy (2'-methoxy-ethyl)-pyridine-1-oxide (L_7).

A protocol for screening these compounds iron chelating properties was developed which included inorganic iron (III) reactions, cell membrane permeability studies, iron mobilisation from transferrin and ferritin in vitro as well as iron mobilisation from iron overloaded mice in vivo.

In the reactions of the ligands with transferrin biphasic kinetic processes were observed, which are consistent with the hypothesis that the two iron binding sites of transferrin are functionally distinct. Furthermore, it was shown that ligands varied in their ability to mobilise iron from this protein, with L_1, L_3, L_6 and L_7 being more efficient than L_2, L_5 and other known chelators.

The reaction of the ligands with ferritin was much slower compared to that with transferrin but the efficacy amongst the ligands in the iron mobilisation process was again L_1, L_3, L_6 and $L_7 > L_2$ and L_5.

In a preliminary toxicological study L_4 and L_6 were found to be toxic when injected to mice (500mg kg^{-1}). The ligands L_1, L_3 and to a lesser extent L_2, caused high iron excretion from iron overloaded mice both when given intraperitoneally or orally (300mg kg^{-1})

It is clear that the ligands described will be of use in the study of many aspects of iron metabolism. But more important the results indicate that the compounds or their analogs may be developed for possible use in the treatment of transfusional iron overload in β thalassaemia.

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LIST OF CHEMICALS

FISONS Chemical Company: Loughborough, U.K.

- Iodomethane
- n-Octanol
- Iron dextran

BDH: Poole, Dorset, U.K.

- Copper sulphate
- Zinc sulphate
- Calcium sulphate
- Magnesium sulphate
- Sodium hydrogen carbonate
- Ferrous ammonium sulphate

SIGMA Chemical Company: London, U.K.

- Tris
- Ferritin (Type I, equine spleen)
- Transferrin (Human)

BEHRING Diagnostics (HOECHST, U.K.): Middlesex, U.K.

- Transferrin (Human)

OXOID: Basingstoke, Hampshire, U.K.

- PBS

RADIOCHEMICAL Centre: Amersham, U.K.

- $^{59}FeCl_3$

CIBA Laboratories: Horsham, U.K.

- Desferrioxamine B mesylate

ALDRICH Chemical Company: Gillingham, Dorset, U.K.

- All other chemicals

ABBREVIATIONS

DFB	: Desferrioxamine
DHB	: 2,3-Dihydroxy benzoic acid
EDTA	: Ethylene diamine tetra-acetic acid
DTPA	: Diethylene-triamine- penta-acetic acid
RA	: Rhodotorulic acid
EDHPA	: Ethylene diamine-N,N'-bis(2-hydroxy phenyl acetic acid)
PIH	: Pyridoxal isonicotinoyl hydrazone
CHA	: Cholylhydroxamic acid
PBS	: Phosphate buffered saline
TRIS	: Tris (hydroxy methyl) amino ethane
DMSO	: Dimethyl sulfoxide
NTA	: Nitrilo triacetic acid
RBC	: Red blood cells
PAPHY	: Pyridine-2-aldehyde-2-pyridyl hydrazone
3,4 LICAMS	: 1,5,10-N,N',N"-tris (5-sulfo-2,3-dihydroxy benzoyl)triazadecane
$(Me)_3$MECAMS	: N,N',N"-trimethyl-N,N',N"-tris(2,3-dihydroxy-sulfobenzoyl)-1,3,5-triaminomethyl benzene

NOTE:

The reader is directed to the last page of this thesis, which when unfolded contains the chemical structures and abbreviations of all the compounds prepared and used extensively in this work.

CHAPTER ONE

GENERAL INTRODUCTION

The Role of Iron in Living Systems

Iron is an important component of many cellular processes and its redox properties are essential for both anaerobic and aerobic organisms. All living cells from the primitive monocellular to the more complex multicellular require iron for life, the single exception being the lactic acid bacteria.

Iron is abundant throughout the solar system including the earth's crust where it exists as insoluble oxides such as haematite (Fe_2O_3) and magnetide (Fe_3O_4) or sulfites such as pyrite (FeS_2). Its insolubility presents a nutritional problem to microorganisms and plants, and to a lesser extent, to multicellular animals.

Microorganisms respond to iron starvation by over-synthesising high affinity iron chelating agents called siderophores. The iron coordinating ability of these low molecular weight molecules is based on two prototypes: the hydroxamates and the catechols, which are usually synthesised by fungi and bacteria, respectively. Plants requirements for iron are indicated by the high iron content compared to that of animal on a dry weight basis. Thus "iron chlorosis" which is an iron deficiency state, is well documented. It is not yet known whether plants excrete siderophores or whether those of microbial origin are utilised in nutrition.[1]

In man iron deficiency anaemia is widespread among all populations, ages and sexes. There appears to be no specific protein mediated regulatory mechanism of iron excretion, iron being regulated by absorption. The rate of iron intake depends on the quality and quantity of dietary iron. Anaemia arising from iron deficiency is usually observed in young infants, but also during adolescence and pregnancy, due to increased iron demand, and in menstruating females due to iron loss.

Iron proteins are widespread in living systems performing a variety of functions such as oxygen transport, electron transport, biosynthesis and enzymatic degradation of metabolites and foreign substances. A general picture of the role of iron containing proteins is shown in Table 1.1.

This wide functional role of iron in proteins involved in energy transduction (cytochromes), biosynthesis and degradation of molecules (oxidases/reductases) and DNA synthesis (ribonucleotide reductase) is due to the existence and properties of two stable iron states (FeII, FeIII) within various protein moieties. This dependence on iron compelled many organisms to evolve specific iron sequestering agents in order to keep iron soluble for transport, storage and utilisation. The chelation of iron by those agents serves another important function, namely the protection against the toxic effects of free iron and the invasion by other organisms.

TABLE 1.1

Examples of Iron Proteins and their Function[36]

FUNCTION	PROTEINS	IRON MOIETY
Oxygen Transport	Haemoglobins	Haem
	Chlorocruorins	Haem
	Erythrocruorins [167]	Haem
	Haemerythrins	Non-haem
Oxygen Storage	Myoglobin	Haem
Electron Transfer	Cytochromes	Haem
	Rubredoxins	Fe,S
	Ferredoxins	Fe,S
Enzymatic Function	Peroxidases	Haem
	Catalase	Haem
	Cytochrome b_2	Haem
	Tryptophan Hydroxylase	Haem
(Catecholamine Metabolism) →	Tyrosine Hydroxylase	Haem
	NADH-Ubiquinone-Reductase.	Fe,S
	Nitrite Reductase	Fe,S
	Hydrogenase	
	Pyrazon Dioxygenase	Fe,S
	Lipoxygenase	Non-haem
Drug detoxification	CytP_{450} and b_5	Haem
DNA synthesis	Ribonucleotide Reductase	Non-haem
Collagen Synthesis	Proline Hydroxylase	Non-haem
Tricarboxylic Acid Cycle	Aconitase	Non-haem
Iron Transport	Transferrin	Non-haem
Iron Storage	Ferritin	Inorganic Fe
	Haemosiderin	

Iron Toxicity

Iron toxicity is believed to be the result of the oxidation of iron (II) to iron (III) in the presence of O_2. This electron transfer may produce radicals which react mainly with the fatty acids of the cell membrane causing lipid peroxidation and eventual membrane damage. An illustration of the radical reaction which could take place *in vivo* is summarised in Scheme 1.1.[2]

Scheme 1

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{-\cdot}$$

$$O_2^{-\cdot} + O_2^{-\cdot} \xrightarrow{2H^+} H_2O_2 + O_2$$

$$O_2^{-\cdot} + N: \xrightarrow{2H^+} H_2O_2 + N^{\cdot +}$$

$$O_2^{-\cdot} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2$$

$$OH^{\cdot} + RH \longrightarrow R^{\cdot} + H_2O$$

$$OH^{\cdot} + RCH = CHR \longrightarrow R^{\cdot}CH\text{-}CH(OH)R$$

In the first step a superoxide radical is formed which generates hydrogen peroxide when it reacts with itself or a nucleophile (N:). The reaction of superoxide with hydrogen peroxide yields the hydroxyl radical ($OH^{\cdot}$) which is highly reactive towards both saturated and unsaturated molecules including proteins, nucleic acids and lipids. The radicals formed in Scheme 1 are capable of initiating

cascades and eventual widespread tissue damage. Such events are normally controlled by superoxide dismutase and catalase. Vitamin E and other molecules such as 2,3 dihydroxybenzoic acid are also capable of trapping radicals and thus in principle, can also minimise tissue damage.

Iron Transport and Storage

It was mentioned above that iron has to be complexed effectively if its toxic effects are to be avoided. This role together with the function of making iron available to cells for normal physiological function are fulfilled in vertebrates by transferrin, an iron transport protein and ferritin, an iron storage protein.

At the present time there are three main types of vertebrate transferrins all sharing the property of having two iron (III) binding sites within a single polypeptide chain of molecular weight of about 80000. These are serum transferrin, lactoferrin and ovotransferrin (or conalbumin). The first is mainly found in serum, the second in leucocytes together with external secretions such as milk and tears, and the third in avian eggwhite.

Transferrin is responsible for the normal distribution of iron in the various tissues as depicted in Table 1.2. Thus the major function of transferrin is to distribute iron throughout the body between the sites of absorption, utilisation, storage and haemoglobin degradation.

TABLE 1.2

Distribution of Iron in the Body of a 70kg man[3]

PROTEIN	TISSUE	TOTAL IRON (g)	%
Haemoglobin	Red blood cells	2.6	57.6
Myoglobin	Muscle	0.4	8.9
Mitochondrial cytochromes		0.017	0.4
Catalase		0.005	0.1
Other cytochromes and haem-proteins		?	
Non-haem iron	Liver	0.35	7.8
	Spleen	0.02	0.4
	Muscle	0.86	19.0
	Bone marrow	0.26	5.8
	Other tissues	?	
Transferrin	Plasma	0.004	0.1

In particular, transferrin is highly active in the donation of iron to bone marrow for the synthesis of haemoglobin. For this reason, the daily turnove of plasma iron is relatively large (35mg day^{-1}) and thus transferrin is charged and discharged with iron approximately ten times per day. Serum levels of transferrin are 2-4gL^{-1}, and usually about 30% of the iron binding sites are saturated with iron.

Iron is stored in the body by the water soluble protein ferritin and by the insoluble haemosiderin which is probably the lytic product of ferritin.[4] Ferritin has a high molecular weight (450000) and a spherical shape consisting of 24 subunits capable of incorporating up to 4500 inorganic iron (III) atoms in its central cacity in the form of a micelle. Iron is thought to move in and out of the central cavity through six channels perforating the protein shell.

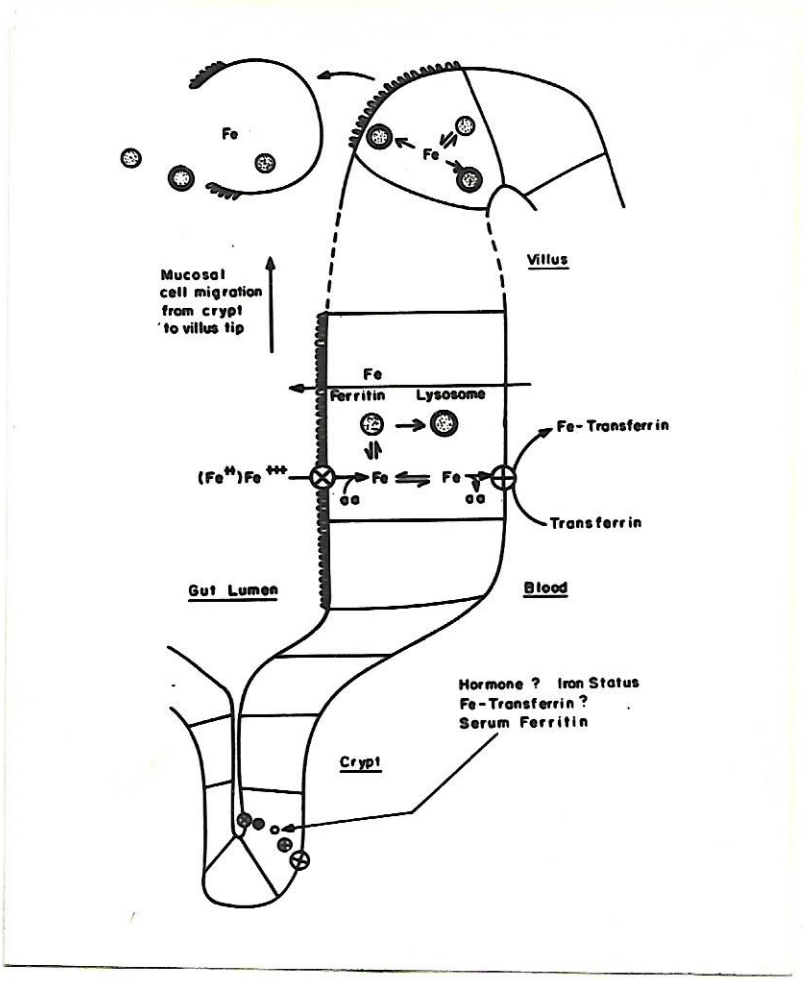
In mammals ferritin is found mainly in the liver, spleen and bone marrow where, as in the case of transferrin, it provides iron for haemoglobin synthesis. Its wide distribution in other tissues is probably for protection against the toxic effects of free iron.

The Regulation of Iron Body Levels

The lack of a regulatory excretion mechanism for iron in the body strongly implies that body levels are controlled by gut absorption. Two main factors are thought to determine iron absorption, namely the state of the iron stores

and the rate of erythropoiesis. A proposal for the mechanism of iron regulation as described by Linder and Munro[5] is depicted in Fig. 1.1.

FIG. 1.1



Mechanism and regulation of iron absorption in the intestinal mucosa, according to Linder and Munro[5]

It was suggested that iron adsorbs to specific receptors on the mucosal cells in the intestine. This bound iron is subsequently transferred to the cytoplasm by an energy dependent process. Iron in the cytoplasm is thought to be bound to either ferritin or small molecular weight chelators, all of which are in equilibrium with each other.

The amount of iron taken by transferrin on the serosal surface of the cell is thought to be dependent on the number of receptors present. This in turn is believed to be regulated by iron levels. Iron retained in the cell is returned to the lumen of the gut by exfolliation at the tip of the villus. Iron can also pass in the opposite direction mainly from the plasma to the mucosal cell and then into the lumen. Again this is thought to be an active process in the lower part of the small intestine. However there are certain observations which are not accounted for in this model.[6]

(a) the amount of iron absorbed is dependent upon the quantity of iron present in the intestinal lumen;
(b) iron(II) salts are better absorbed than iron(III) salts;
(c) iron associated with animal protein is better absorbed than iron associated with a vegetable diet;
(d) the enhancement by chelators and reducing agents on iron absorption.
(e) erythropoiesis.

Several questions remain unresolved in relation to the mechanism of iron transport from extracellular transferrin to the intracellular sites of storage and utilisation. Furthermore, the reverse phenomena, namely iron release and transport from ferritin and haem breakdown to sites of intracellular utilisation, or to transferrin, are also unexplained.

In an attempt to solve this problem, it has been suggested[7,8,9] that between the sites of iron transport, storage and utilisation there is a transit (or labile) iron pool which consists of low molecular weight iron complex. Several ligands have been implicated such as ascorbic acid, ATP, citrate, glucose, fructose, glycine, glutathione and others. This pool is particularly evident in reticuloendothelial cells, epithelial cells of the small intenstine, erythrocyte precursors, liver parenchymal cells and cultured Chang cells. It has been suggested that this low molecular weight complex is not only in equilibrium with the metabolic processes requiring iron but is also involved in iron movement between the different organs of the body and furthermore in the regulation of iron absorption.

Diseases of Iron Overload

Iron overload can be the result of two major processes, increased absorption from the gut, and multiple transfusions of red blood cells. Increased iron absorption from the gut could be caused by excess dietary iron, by the

utilisation of iron cooking utensils[10] or, furthermore, the result of a physiological abnormality (Idiopathic haemochromatosis). Multiple transfusion programs are presently used for the treatment of:

(a) aplastic anaemia which is a functional inability of the bone marrow to deliver cells to the peripheral blood, and

(b) β thalassaemia.

β thalassaemia

The thalassaemias are a heterogeneous group of inherited diseases resulting from a defect in the rate of synthesis of one or more of the peptide chains of haemoglobin.[11] The inherited disease occurs mainly in the mediterranean countries, Middle East, parts of Africa and South East Asia. This geographic distribution of the thalassaemias and indeed that of sickle cell anaemia, is thought to be related to areas which previously had high frequency of malaria. In such places, people with the thalassaemia trait were apparently more resistant to malaria than normal individuals and so increased in numbers through the process of natural selection.[12] Consequently, the incidence of thalassaemia major increased in these regions. It is estimated, for example, that one in seven Cypriots is a carrier of β thalassaemia.

In β thalassaemia major, the depression of β-chain synthesis can be total (β°) or partial (β^{+}). In most cases this leads first to excessive α-chain production which precipitate and causes destruction of the red cell precursors and in the absence of treatment, to gross anaemia and other secondary abnormalities and finally death at an early age. Most of the pathological conditions can only be avoided by using regular transfusions of red blood cells in order to maintain near normal haemoglobin levels (>9g/dl).[13]

Transfusional Iron Overload

Repeated blood transfusions result in iron overload which causes mainly hepatic, cardiac and endocrine damage. T.C. Iancu, et.al.[14] made ultrastructural observations of liver biopsies of patients suffering from thalassaemia major. The results suggested that at early stages of iron overload, haemosiderin and ferritin form membrane bound arrays which are associated with lysosomes. At later stages, however, there is a gradual increase in the number of iron laden lysosomes and in advanced cases, haemosiderin aggregates are located in or around disrupted lysosomes. In such cases, evidence of cell injury could be observed. Collagen deposition in the liver of β-thalassaemia patients is a further factor promoting liver cell damage. The mechanism for this phenomenon is unknown. In the patients large amounts of stored iron are also present in the reticuloendothelial cells of both the spleen and the liver where red blood cells are broken down. Another organ

highly affected is the heart which is apparently more vulnerable to iron overload than the liver and heart failure is known to be the major cause of the death in thalassaemia major.[15]

Another observation at the molecular level is that in severe iron overload serum transferrin becomes saturated and excess iron is thought to be circulating in the plasma in a loosely bound form which is highly toxic.[16]

Treatment of Iron Overload

The only treatment of transfusional iron overload currently in use is long term iron chelation therapy. After prolonged experimentation and clinical trials net iron loss from transfused thalassaemia patients has been achieved using the drug desferrioxamine (DFB). Recent adaptations of this therapy involve the administration of DFB subcutaneously 12h per day using a small pump strapped to the patient's abdomen. Iron excretion is further enhanced by the administration of ascorbic acid (500mg/day).[17] Drinking tea is also part of the therapy programme because such activity decreases iron absorption from the gut.[18]

Efforts have been made to decrease iron overload by transfusing at longer time intervals using young red blood cells, isolated in a cell separator. However, the process is both tedious and expensive. Other attempts to improve the present treatment included administration of DFB entrapped in RBCs[19] or liposomes.[20,21]

The main disadvantages of using DFB are that it has to be injected and the high cost. Carriage of the syringe cause detrimental psychological effects to the patients but also the cost of the drug, currently about £2,500 per patient per year, makes this treatment impossible in the Third World countries. These two reasons have prompted the search for new, orally effective, iron chelators.

Iron Chelators for Clinical Use

Most chelators tested *in vivo* are based on three prototype structures, namely the hydroxamic acids and the catechols, both of which are naturally occurring microbial siderophores, and EDTA, a much less specific chelator. All three classes contain oxygen in their iron binding sites, an indication of the preference of the iron(III) to bind to this element. The following chelators have been extensively studied as compared to the rest:

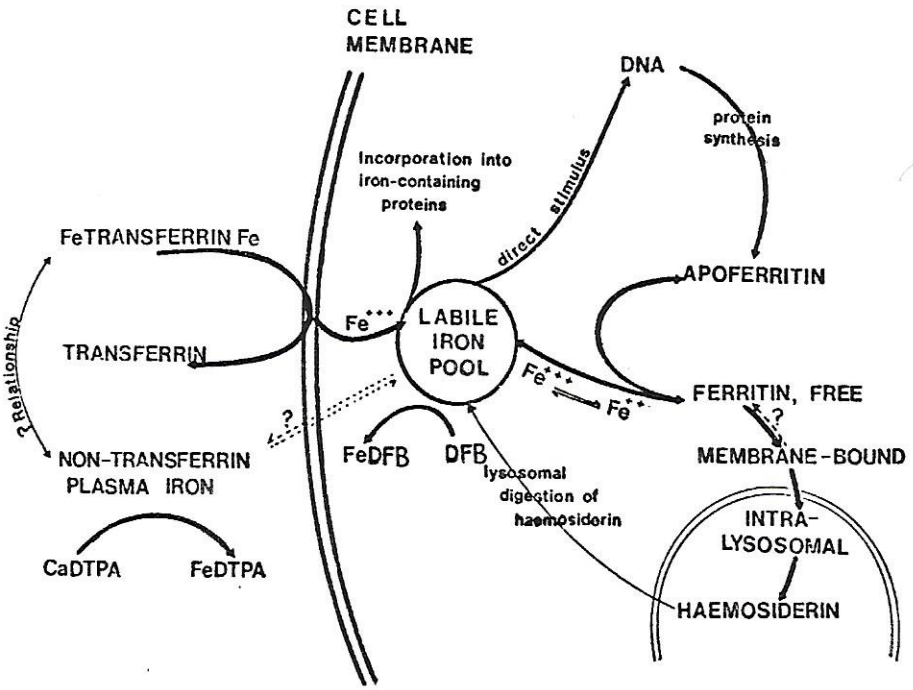
(a) Desferrioxamine (DFB) is a hydroxamic acid produced by *Actinomyces*, it is highly water soluble and quite rapidly excreted. Iron mobilisation *in vivo* by DFB is thought to be mainly from:[22]

(i) intracellular chelation in parenchymal cells of the liver and reticuloendothelial cells, and

(ii) extracellular chelation of iron not co-ordinated to transferrin and iron bound at the cell surface.

On the molecular level most of the intracellular iron is thought to be mobilised from the transit or

FIG. 1.2



Mode of action of DFB and DTPA in iron mobilisation[23]

labile iron pool.[23] (Fig. 1.2) Ferrioxamine, the iron complex of DFB, cannot enter cells but it can diffuse out, since from studies with Chang cells it was shown[24] to mobilise intracellular iron from an iron labile (transit) pool. Ascorbic acid acting synergestically with DFB increases iron excretion probably by enlarging the labile iron pool in cells. However, this loosely bound iron may enhance the toxicity of cells and may be one of the causes leading to cardiac failure.[25,26]

(b) 2,3-Dihydroxy-benzoic-acid (DHB)

2,3-Dihydroxy-benzoic acid[27] is a catechol type ligand which causes increased urinary excretion of iron in rats when it is applied orally. Unfortunately, it proved ineffective in humans.[28]

(c) Rhodotorulic Acid (RA) is a hydroxamate, which is insoluble in water, and thus is ineffective orally. However, it increases iron excretion when administered intraperitoneally to rats.[29] Administration to humans[28] caused inflammation and pain at the injection site.

(d) Diethylene-triamine -penta-acetic acid (DTPA) is an EDTA analogue which was found to remove extracellular iron, not bound to transferrin in rats.[30] However, like EDTA it possesses a high affinity for Ca(II) Zn(II) and Mg(II) and thus produces toxic side effects. Consequently its use was not continued.[28]

(e) Ethylene diamine-N,N'-bis-(2-hydroxy phenyl acetic acid) (EDHPA) is a drug which was found to be more effective intraperitoneally than DFB in mice and also effective orally.[31] However, in man it did not increase iron excretion substantially when applied orally but some of its derivatives are being further evaluated.[28] One would predict that this compound possesses a relatively high affinity for Ca(II), Mg(II) and Zn(II).

(f) Pyridoxal isonicotinoyl hydrazone (PIH). PIH was shown to increase faecal iron excretion up to 8-9 times above the normal level when it was applied orally to rats.[32,33] However, little is known at present about the toxicity of this compound and its effects on other metals.

(g) Cholylhydroxamic acid (CHA) is a hydroxamate designed to mimic bile acids. Thus by being absorbed and transported in the enterohepatic circulation, while accomplishing at the same time chelation and excretion of parenchymal iron through the bile, this drug is thought to be recyled to the circulation for sequestering more iron. When it was applied orally to rats it caused high iron excretion. It behaved similarly in man, with the only apparent toxic effect being transient diarrhoea in the latter.[34,28] Further studies are in progress for the synthesis of analogues of this compound and for more toxicity studies.

(h) Other Chelators

Other chelators are currently being evaluated for the treatment of iron overload including mainly conjugates of DHB and spermidine as well as enterobactin and tropolone analogues.[28]

Aim of this Work

The primary aim of this work is to synthesise cheap and orally effective iron chelators for the treatment of iron overload in β thalassaemia. A secondary aim is to establish a simple inexpensive screening procedure for iron chelators, based on the reaction of the ligands with iron(III) *in vitro* and with iron overloaded mice *in vivo*.

CHAPTER TWO

SYNTHESIS OF IRON CHELATORS

2.1 Chelate Specificity

The main objective of this work was the search for iron chelators able to remove iron from patients suffering from iron overload. These chelators should be cheap, orally effective and non-toxic. Before discussing the underlying concepts of this work, some basic aspects regarding chelators in general will be introduced.

A chelator is a molecule capable of forming a heterocyclic ring with a metal atom as the closing member. It must possess at least two functional groups, the donor atoms of which can donate a pair of electrons for the formation of a bond with the metal. The major donor atoms are N, O and S which can function either as members of an acidic group, e.g. -COOH, -OH (enolic, phenolic), -SH,  , in which case, the proton is displaced by the metal, or as lone pair donors (Lewis bases), e.g. C = O, $-NH_2$, -O-R, -OH (alcoholic), -S-thioether.

Bidentate ligands can be classified according to:

(a) the nature of their two functional groups; acidic (e.g. oxalic acid), basic (e.g. ethylene diamine), or one acid and the other basic (e.g. glycine);

(b) the rigidity of the ligand skeleton; for instance, catechol is rigid whereas ethylene diamine is flexible;

(c) the donor atoms, e.g. O,O; N,N; N,O.

In the case of chelation the electrons of the base are used in the formation of a covalent bond. Those bases containing N, O, or F as the donor atom are stronger (hard bases) than those containing P, S or I (soft bases). The acids which prefer hard bases are called "hard acids", for example, iron(III) is a "hard" metal because of its high charge density, and therefore it prefers to form complexes with hard bases, especially O.

Further factors which influence complex formation are

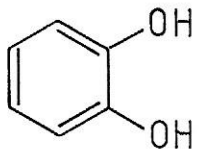
(a) the ring size of the chelates. Five and six membered rings are much more stable than four, seven or eight membered rings;

(b) the basic strength of the coordinating atoms. For a series of closely related chelating agents the greater the basic strength, i.e. higher pK_a, the greater the stability of the complex.

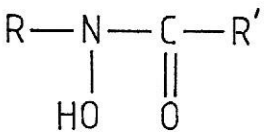
(c) The degree of substitution of the coordinating atoms. Substitution can alter the basic strength of the chelating molecule and often more importantly it can hinder chelation due to steric effects.

There are many chelators which bind iron(III), the most selective being those which are naturally occurring. These are of several classes, many of which are microbial chelators which possess a high affinity for iron(III). The

two major groups are based on either catechol or hydroxamic acid.



CATECHOL



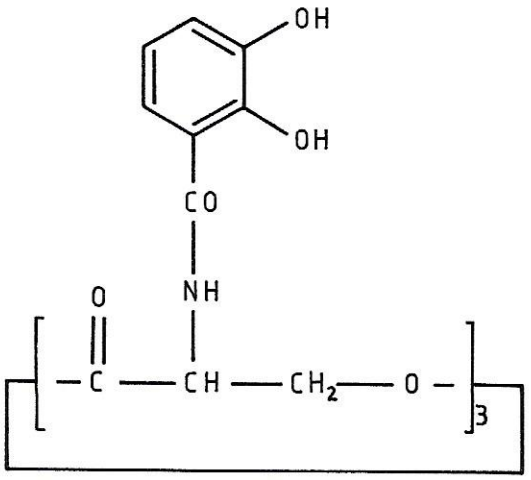
HYDROXAMIC ACID

2.1.1 Catechol Type Siderophores[35]

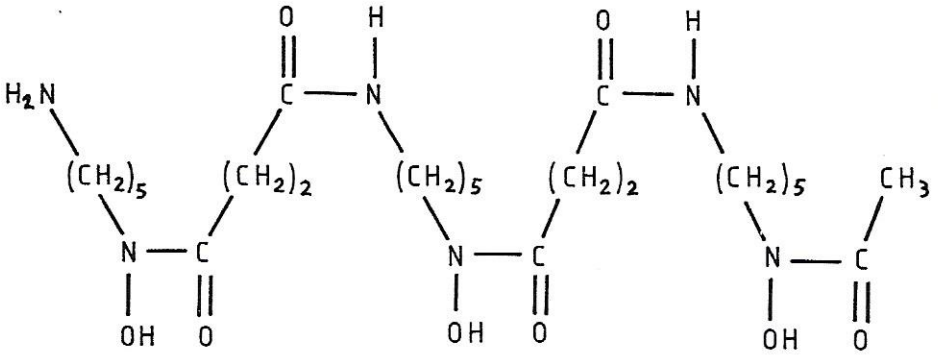
These are mainly found in bacteria cultures. When the iron concentration in the surrounding medium is low they are secreted in order to sequester iron. Enterobactin (Fig. 2.1) is one of the major siderophores found in enteric bacteria. The hydrolysis product of enterobactin, the bidentate 2,3dihydroxy-N-benzoyl-serine has a markedly lower affinity for iron(III). 2,3Dihydroxy-N-benzoyl derivatives of threonine, glycine, lysine and spermidine have also been characterised in low iron cultures of a wide range of bacteria.

In considering their possible use in the treatment of iron overload diseases the catechol chelator types have the advantage of being acid stable and also possessing high specificity for iron(III) (see Chapter 3). However, catechol type ligands oxidise very easily and thus they are unstable, they also have very high pKa's in which case protons interfere in their binding properties (see Chapter 3), and, furthermore, they form charged complexes with

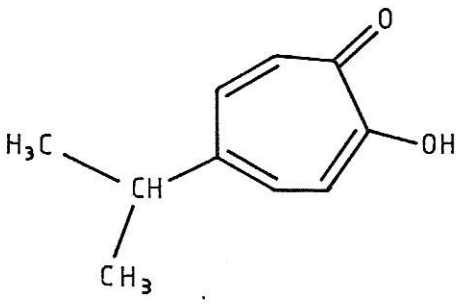
FIG. 2.1



ENTEROBACTIN



DESFERRIOXAMINE B



3-ISOPROPYL-TROPOLONE (γ-THUJAPLICIN)

iron(III) at physiological pH, rendering non-facilitated transfer across membranes unlikely.

2.1.2 Hydroxamate Type Siderophores[35]

While catechol-type siderophores are found mainly in bacteria, hydroxamates are mainly found in fungi. There are many classes in the hydroxamate group, most of which are forming hexadentate complexes with iron(III), the most important being the following:

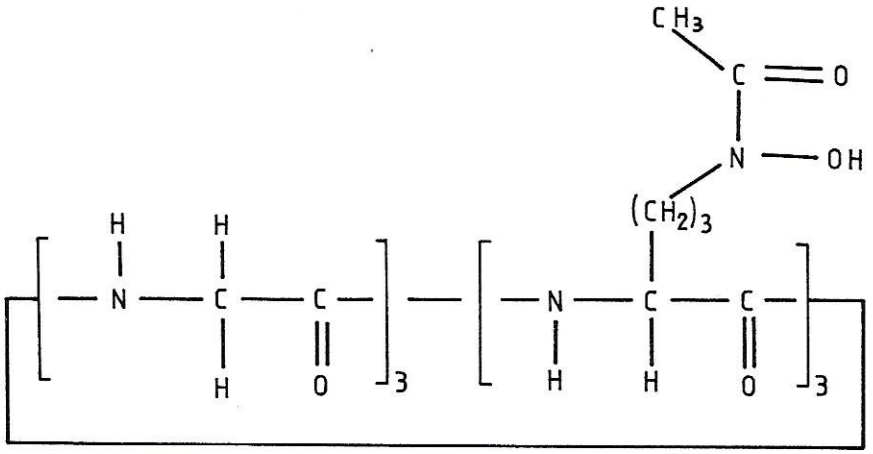
(a) The ferrichrome family. These are cyclic hexapeptides containing a constant trimeric sequence of δ-N-hydroxy-ornithine which is involved in the complex formation and three other amino acids (Fig.2.2)

(b) The rhodotorulic acid family. Rhodotorulic acid is a quadridentate ligand. δ-N-acetyl-δ-N-hydroxy ornithine is a common dipeptide found in this group (Fig. 2.2)

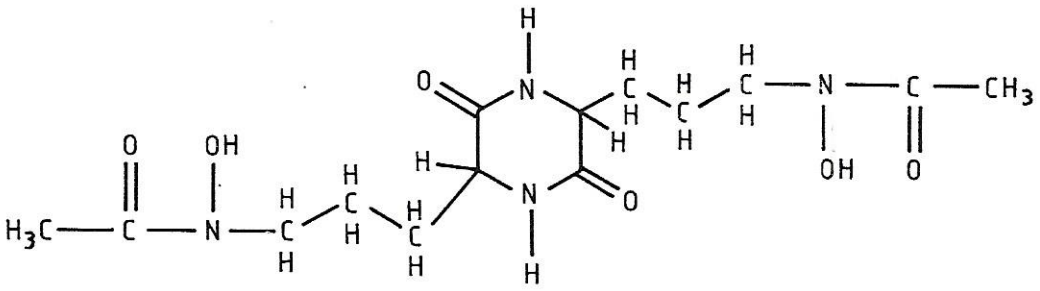
(c) The citrate hydroxamate family. The compounds in this group are amide derivatives of citric acid, containing two aliphatic chains bearing one hydroxamic acid each. (Fig. 2.2)

(d) The mycobactin family. These and their iron(III) complexes are insoluble in aqueous media and are membrane bound. There are three different functional groups involved in iron binding, a cyclic hydroxamate, an open chain hydroxamate and a substituted oxazoline (Fig. 2.3)

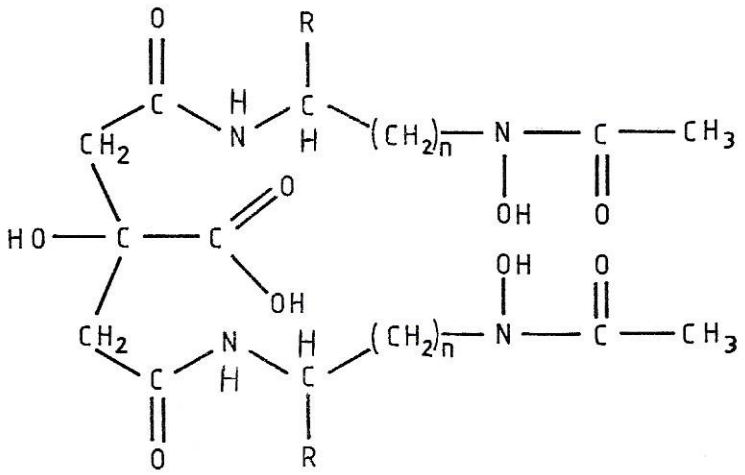
FIG. 2.2



FERRICHROME FAMILY



RHODOTORULIC ACID FAMILY



CITRATE HYDROXAMATE FAMILY

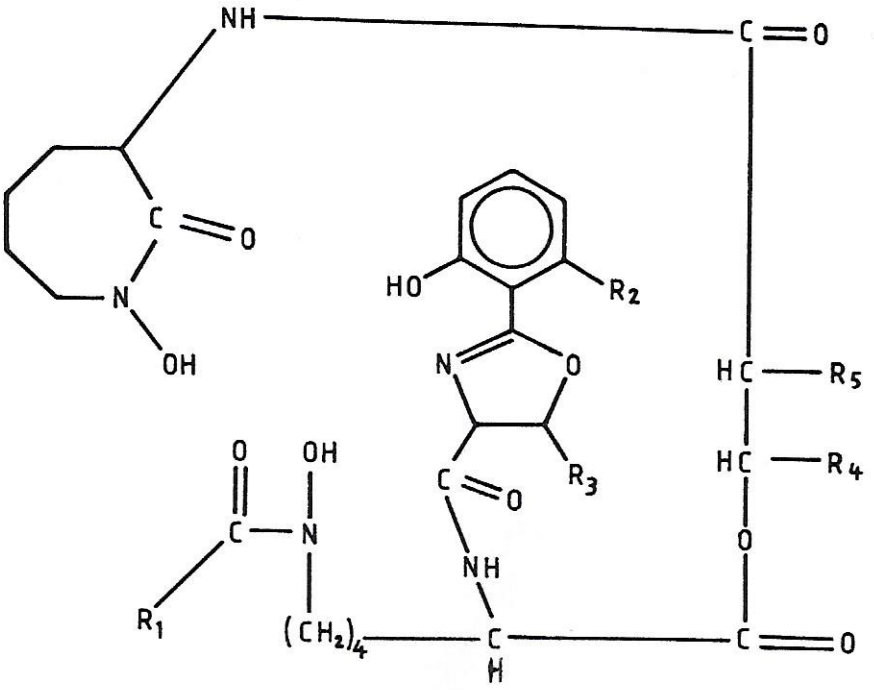
(e) The ferrioxamine family. This family includes desferal which is the mesylate salt of desferrioxamine B. It consists of 1-amino-ω-hydroxy-amino alkane, succinic acid and acetic acid units. It is a hexadentate ligand possessing three hydroxamic acid groups (Fig. 2.1)

In general the hydroxamates have a lower affinity for iron(III) than catechol based ligands (Fig.2.1) but because of their lower pKa there is minimal competition from protons in their iron binding properties at physiological pH values (see Chapter 3). These ligands could form neutral complexes and thus in theory might be expected to permeate cell membranes. However,they are rather hydrophilic and do not readily partition into membranes. Another disadvantage of this class of molecules is that the hydroxamate function is labile in acid solution and thus is unlikely to survive oral application.

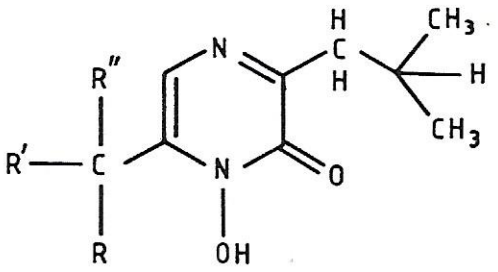
2.1.3 Aspergillic acid Antibiotics[37]

This is a group of pyrazine cyclic hydroxamic acids which are mainly thought of as antibiotics rather than siderophores. They are bidentate ligands of aromatic hydroxamate character and are theoretically expected to be acid stable and form neutral complexes with iron(III). However, these molecules are toxic probably because of their hydrophobic character (Fig. 2.3).

FIG. 2.3



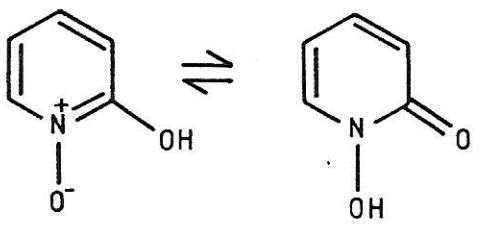
MYCOBACTIN FAMILY



ASPERGILLIC ACID FAMILY

R = H R′ = C_2H_5 R″ = CH_3 Aspergillic Acid

N-OXIDE N-HYDROXY



Tautomeric Forms

2-Hydroxy-pyridine-1-oxide

2.1.4 Thujaplicins[38]

These compounds are potent fungicides occurring in the heartwood of western red cedar, where they are believed to play a major role in the high natural durability of this plant. There are three classes, the α-, β- and γ-thujaplicins all being derivatives of tropolone. Recently these compounds and other α-hydroxyketones including maltol and N-methyl-3-hydroxy-pyrid-4-one were found to act as siderophores of mutant strains of Salmonella typhimurium which were unable to synthesise enterobactin, their normal siderophore.

2.2 Design of Iron(III) Specific Ligands with Properties Suitable for Clinical Use

The object of this work was to synthesise compounds which retained the advantages of the previously described natural ligands without their associated disadvantages. The properties which will fulfil the basic requirements of an orally effective iron chelator should be:

(a) high specificity for iron(III);

(b) relative low affinity for other biologically important metals, e.g. Cu, Zn, Ca;

(c) pKa $\leqslant$8.0, thus minimising competition with protons at physiological pH (see Chapter 3);

(d) high stability towards oxidation;

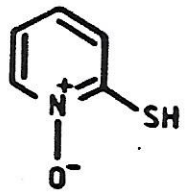
(e) high stability in acidic environments, e.g. in stomach;

(f) both the free ligand and the iron(III) complex should be sufficiently hydrophilic and so possess good water solubility;

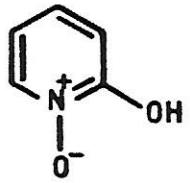
(g) both the ligand and the complex should be neutral and possess a partition coefficient such that they will permeate membranes;

(h) non-toxic.

Bearing in mind these basic properties of the ideal iron chelator and the properties theoretically attributed to aspergillic acid, it was originally thought that 2-hydroxy-pyridine-1-oxide (L_4), Fig. 2.3, another aromatic hydroxamic acid, could fulfil these requirements. However its iron(III) complex was found to be insoluble. L_4 has been previously studied for antimicrobial activity[39] and it was found to be comparable to aspergillic acid. It has been studied also for its ability to enter metal complex formation[40] with metals other than iron(III) and was found to bind Cu ($\log K_1 = 7.3$) and Zn ($\log K_1 = 5.1$). 2-Mercapto-pyridine-1-oxide which is closely related to 2-hydroxy-pyridine-1-oxide (L_4) has had enormous industrial application as a biocidal agent. Its trade name is "Omadine" and its biocidal activity[41] is thought to be related to its chelation properties. These classes of compounds exist as a mixture of two tautomers (Fig. 2.3), the N-hydroxy form predominating.



2-Mercapto-pyridine-1-oxide



2-Hydroxy-pyridine-1-oxide (L_4)

In the early stages of this work other related compounds, i.e.:

(a) 2-hydroxy-5-nitro-pyridine-1-oxide

(b) 2-hydroxy-4-nitro-pyridine-1-oxide

(c) 2-hydroxy-3,5-dinitro-pyridine-1-oxide

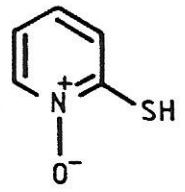
were prepared. These were also disappointing, possessing a lower affinity for iron(III) (see Chapter 3). Their failure was attributed to the lowering of the basicity (pK_a) of the hydroxyl group by the introduction of the nitro function. It was therefore decided to introduce electron releasing substituents which it was hoped would increase the basicity of the hydroxyl group and its affinity for iron(III). Since the pK_a of the nitro derivatives were less than 4, a substantial increase in the pK_a of other derivatives of 2-hydroxy-pyridine-1-oxide (L_4) were needed if the neutral ligand species were to predominate at physiological pH. The selected substituents were such that their introduction was not anticipated to change the overall neutral charge of the ligand and its iron(III) complex. Three other derivatives of the parent compound (L_4) were therefore prepared having the following substituents at the 4th position: $-OCH_3$(L_6), -OH (L_3); $-OCH_2CH_2OCH_3$(L_7) (Fig. 2.5). L_6 is a known compound and it has been previously shown to possess antibacterial activity.[42] However, its iron co-ordinating properties have never previously been examined. Introduction of the $-OCH_3$ and $-OCH_2CH_2O\ CH_3$

were expected to increase the lipophilicity of these molecules. In contrast the introduction of the hydroxyl function in L_3 was expected to increase the hydrophilicity of the ligand and its iron(III) complex, while leaving the overall complex uncharged. 2,4-Dihydroxypyridine-1-oxide (L_3) and 2-hydroxy-4-oxy(2'-methoxy ethyl)-pyridine-1-oxide (L_7) are new compounds. During the progress of this synthetic work it was realised from a literature survey that other compound types are able to fulfil the theoretical criteria of the ideal iron chelator outlined previously. These compounds were maltol (L_5) and N-substituted-3-hydroxy-pyrid-2-ones and the same -4-ones. Their chelation site is similar to tropolone a known chelator (see Chapter 3). Maltol is a well-characterised compound and its iron binding properties have been widely studied under both in vitro and in vivo conditions. 1,2-Dimethyl-3-hydroxy-pyrid-4-one (L_1) and 1-methyl-3-hydroxy-pyrid-2-one (L_2) are known compounds but their metal chelation properties especially those for iron(III) have not previously been studied.

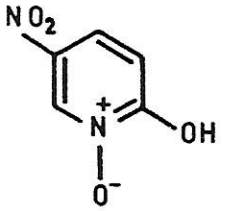
The synthesis of the above compounds will now be described.

FIG. 2.4

Compounds Tested for their Iron Chelation Properties

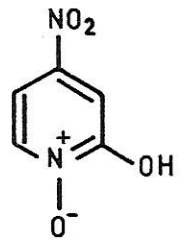


2-Mercapto-pyridine-1-oxide

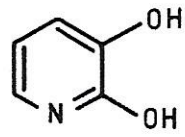


2-Hydroxy-5-nitro-pyridine-1-oxide

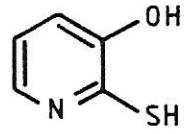
2-Hydroxy-3,5-dinitro-pyridine-1-oxide



2-Hydroxy-4-nitro-pyridine-1-oxide



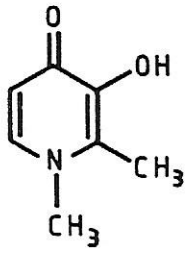
2,3-Dihydroxy-pyridine



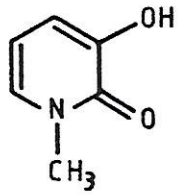
2-Mercapto-3-hydroxy-pyridine

FIG. 2.5

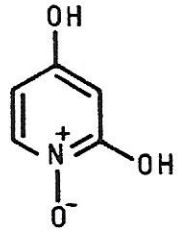
Compounds Tested for their Iron Chelation Properties



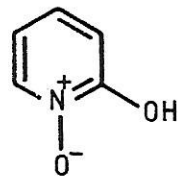
1,2-Dimethyl-3-hydroxy-pyrid-4-one (L_1)



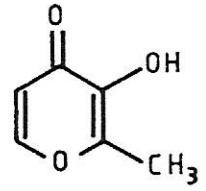
1-Methyl-3-hydroxy-pyrid-2-one (L_2)



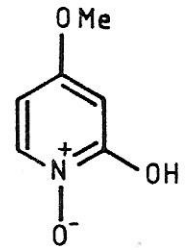
2,4-Dihydroxy-pyridine-1-oxide (L_3)



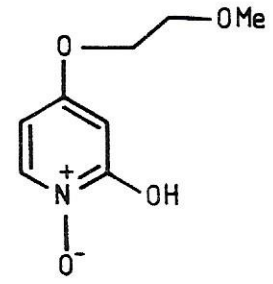
2-Hydroxy-pyridine-1-oxide (L_4)



1-Methyl-3-hydroxy-pyr-4-one (L_5)



2-Hydroxy-4-methoxy-pyridine-1-oxide (L_6)



2-Hydroxy-4-oxy(2-methoxy ethyl) pyridine-1-oxide (L_7)

2.3 Synthetic Approach

2.3.1 2-Hydroxy-5-nitropyridine-1-oxide

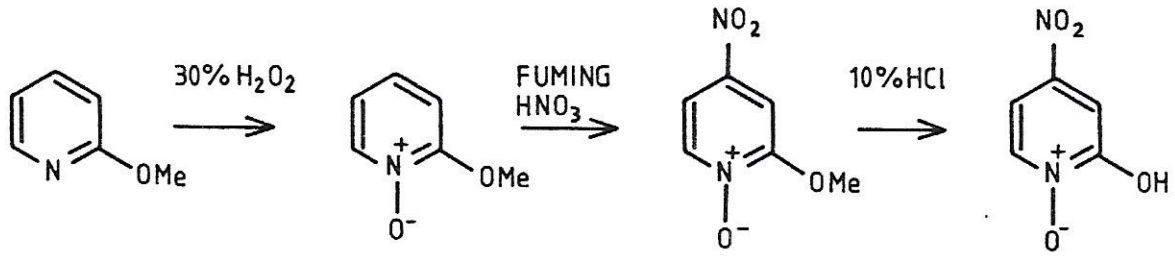
2-Hydroxy-5-nitropyridine-1-oxide was prepared according to the method of Lott, W.A. et.al.[39] 2-Hydroxy-pyridine-1-Oxide (3.1g) was dissolved in glacial acetic acid (17ml) by stirring and low heating for 1 h. Nitric acid (2ml, sp.gr.1.42) was added slowly with cooling. Yellow crystals were formed which were recrystallised from glacial acetic acid yielding 2.5g (60%) MP 185-188°C (lit 198-199°C).

2.3.2 2-Hydroxy-3,5-dinitro-pyridine-1-oxide

The dinitro derivative was prepared according to the method of Den Hertog, H.J. et.al[43] Hydrogen bromide (5.4ml, 48-50%) was mixed with distilled water (100ml) and 2-hydroxy-pyridine-oxide (5g) was added. The solution was evaporated in vacuo to dryness. The resulting white solid, 2-hydroxy-pyridine-1-oxide hydrobromide was re-crystallised from ethyl alcohol and diethyl ether as colour-less crystals. To these crystals (1.88g) acetic acid (9.4ml) was added followed by the slow addition of nitric acid (3.15ml, sp.gr.15) maintaining the temperature below 35°C. On leaving the brown solution overnight, yellow crystals of 2-hydroxy - 3,5-dinitro-pyridine-1-oxide formed (1.3g, 52%) MP 175-178°C (lit. 193.5-194.5°C).

2.3.3 2-Hydroxy-4-nitro-pyridine-1-oxide

This is a three step reaction involving known procedures.

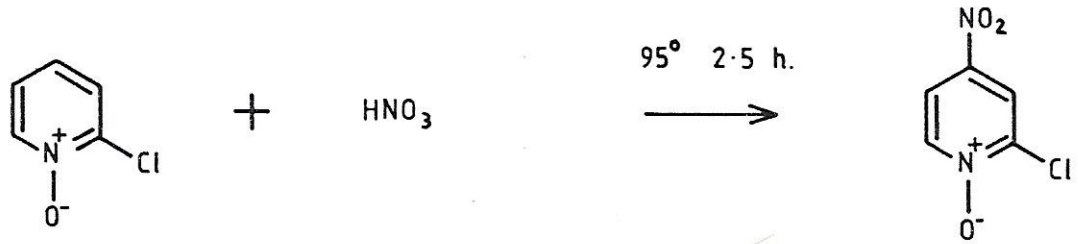


The first two steps of the synthesis are similar to those used by Den Hertog et.al[44] for the preparation of 2-ethoxy-4-nitro-pyridine-1-oxide. To 2-methoxy-pyridine (4g), H_2O_2 (30ml.30%) and then glacial acetic acid (30ml) were added. The mixture was left at 60°C for seven days and then evaoprated in vacuo giving a residue which was nitrated by adding a mixture of fuming nitric acid (8ml) and concentrated sulphuric acid (12ml) and then heating at 80°C for 2.5h. The pH of the solution was raised to 7 by adding ammonia solution while cooling in an ice bath. After leaving overnight the inorganic salt was filtered off and the yellow solution was extracted continuously for several days with chloroform. The chloroform layer was dried over anhydrous sodium sulphate, evaporated in vacuo and allowed to cool overnight, thus forming crystals which were filtered off and washed withligroin and left to dry in vacuo yielding 2-methoxy-4-nitro-pyridine-1-oxide.

2-Methoxy-4-nitro-pyridine-1-oxide[45] was placed in HCl (7.15ml, 10%), heated on a steam bath for one hour and concentrated to 2.8ml under reduce pressure. Yellow crystals 2-hydroxy-4-nitro-pyridine-1-oxide were formed, which were recrystallised from water to yield yellow needles (0.27g, 30%) mp 168°C (lit. 170-171°C).

2.3.4 2-Chloro-4-nitro-pyridine-1-oxide

The above compound is the starting material for the synthesis of three homologues of 2-hydroxy-pyridine-1-oxide with substituents at the fourth position of the pyridine ring. Basically 2-chloro-4-nitro-pyridine-1-oxide is prepared by the nitration of 2-chloro-pyridine-1-oxide as previously described by Brown, V.E.[46] and by Finger, C.G., et.al[47] The latter procedure was found to be more reliable.

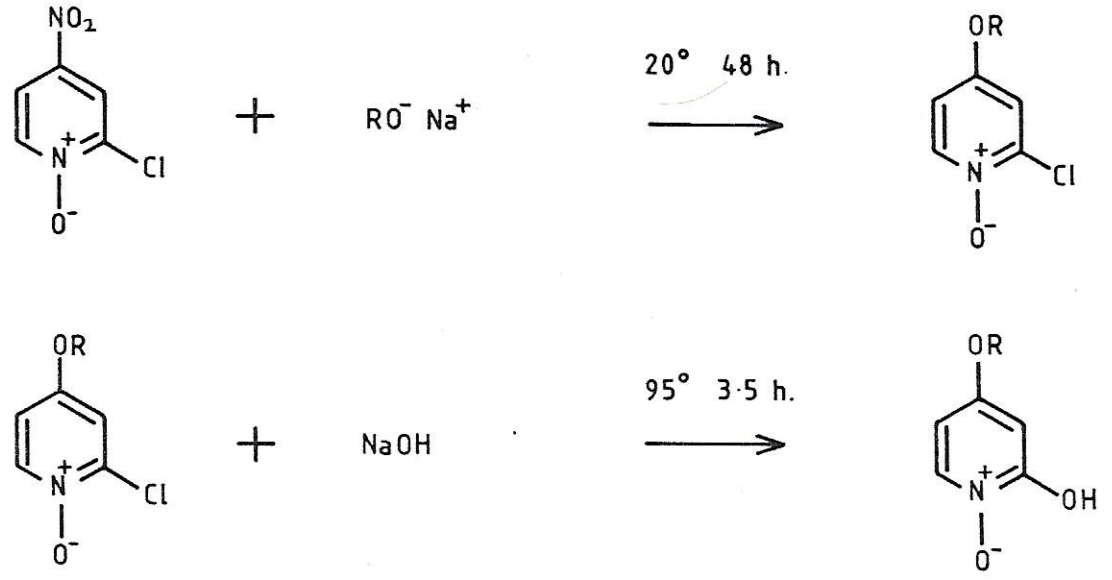


To 2-chloro-pyridine-1-oxide (10g) in an ice bath, H_2SO_4 concentrated (15ml) was first added, followed by the dropwise addition of a mixture of H_2SO_4 concentrated

(15ml) and HNO_3 fuming (27ml, sp.gr.1.5) over a 70 minute period. The acidic solution was heated in a steam bath for 2.5h, allowed to reach room temperature, poured onto ice water (600 ml) and stirred until all the ice had melted. A solid was formed which was filtered off, dissolved in hot chloroform, dried and the solvent evaporated in vacuo leaving a yellow solid. The filtrate was neutralised with saturated Na_2CO_3 and extracted continuously with chloroform, dried and evaporated in vacuo, yellow solid, 2-chloro-4-nitro-pyridine-1-oxide. (Yield 7.46g, 56%). NMR ($CDCl_3$): H(3), δ = 8.32 ppm singlet; H(5), δ = 8ppm quartet; H(6), δ = 8.4 ppm doublet.

2.3.5 2-Hydroxy-4-methoxy-pyridine-1-oxide (L_6)

This preparation is performed in a two-step reaction involving first the substitution of the nitro group of 2-chloro-4-nitro-pyridine-1-oxide by the methoxide anion, and second, the displacement of the chloro substituent by the hydroxy anion.[48,49]



(L_6) R = Me

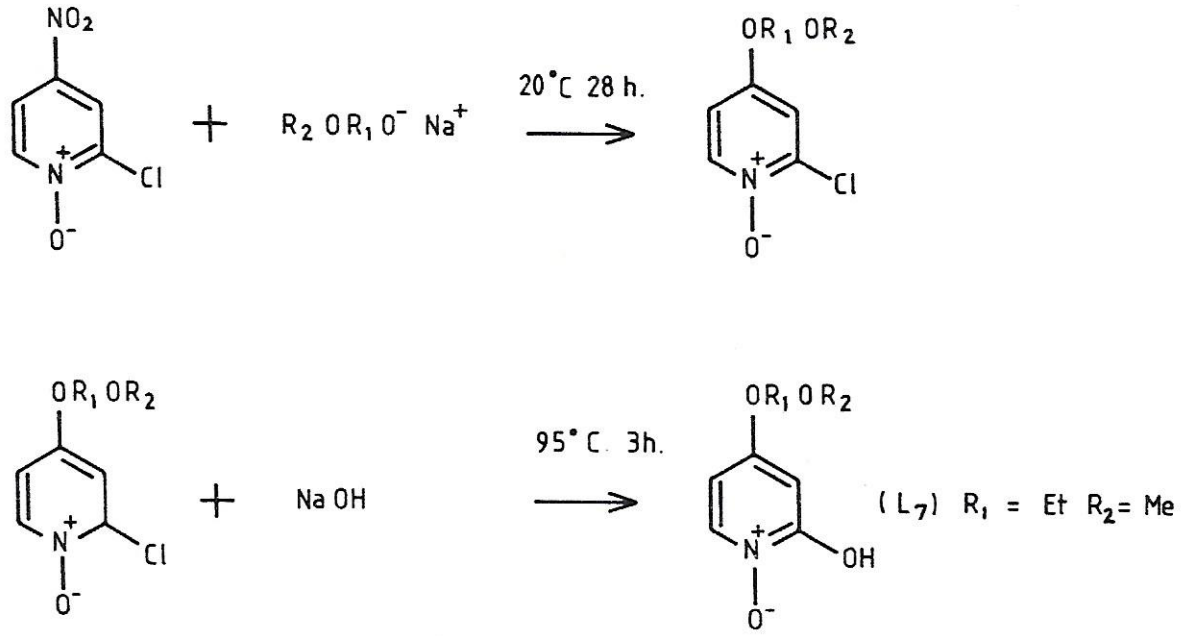
In the first step sodium methoxide is prepared by dissolving Na (0.46g) in absolute methanol (50ml). The reaction was exothermic and when it reached 20°C it was filtered to remove the remaining suspension. The filtrate was added to 2-chloro-4-nitro-pyridine-1-oxide (3.5g) in methanol (10ml), dissolved and stirred for two days. The solvent was then evaporated in vacuo leaving an orange solid, 2-chloro-4-methoxy-pyridine-1-oxide, which was stored at -20°C.

NMR (D_2O): H(3),δ = 7.16 ppm doublet; H(6),δ = 8.05ppm doublet; H(5),δ = 6.85 ppm quartet; Me,δ = 3.7 ppm singlet.

2-Chloro-4-methoxy-pyridine-1-oxide (3.3g) in NaOH (33ml, 10%) was placed on a steam bath for 3.5h. The mixture was then acidifed with concentrated HCl to pH 2.5. A white solid was isolated which was susceptible towards oxidation. mp 165-170° (lit 175-176°), 0.73g, 22% . Although it was anticipated to obtaining colourless crystals on recrystallisation from water, the brown impurities remained. However, the NMR spectra of this material failed to indicate any gross contamination. NMR (D_2O): H_3, δ = 5.88 ppm singlet; H(5), δ = 5.95ppm quartet; H(6), δ = 7.52 ppm doublet.

2.3.6 2-Hydroxy-4-oxy(2'-methoxy-ethyl)-pyridine-1-oxide (L_7)

The method of preparation adopted for 2-hydroxy-4-oxy (2'-methoxy— ethyl)-pyridine-1-oxide is similar to that of 2-hydroxy-4-methoxy-pyridine-1-oxide.



Sodium metal (0.23g) was dissolved in redistilled methoxy ethanol (30ml). The resulting solution was added to 2-chloro-4-nitro-pyridine-1-oxide (1.75g) and stirred for 28h at 20°C. Methoxy-ethanol was removed by distillation under reduce pressure leaving an oily brown solid, which was washed with diethyl ether (25ml) and then dissolved in water (25ml). The aqueous solution was extracted into chloroform (3x25 ml) then evaporated in vacuo leaving a yellow solid on cooling, 2-chloro-4-oxy-(2'-methoxy-ethyl) pyridine-1-oxide.

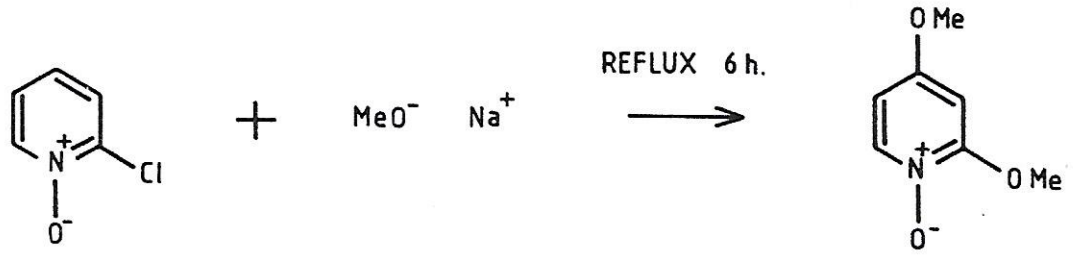
NMR ($CDCl_3$): H(3), δ = 6.05 ppm doublet; H(5), δ = 6.05ppm quartet; H(6), δ = 7.62ppm triplet; Me, δ = 3.42 ppm singlet;

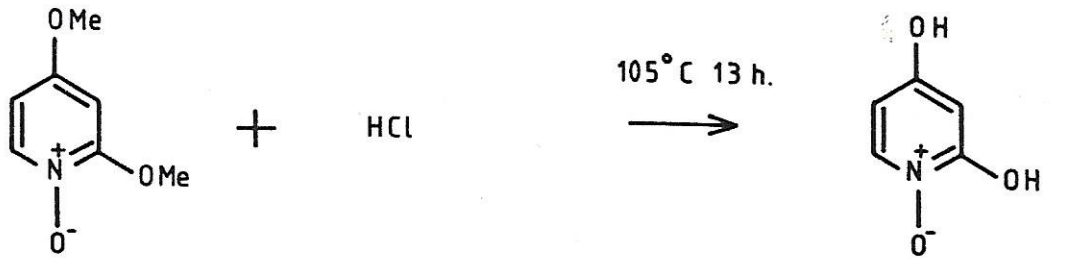
$CH_2(2')$,δ = 4.08 ppm triplet; $CH_2(1')$,δ = 3.7ppm triplet.

NaOH (10%) was added to this solid, 2-chloro-4-oxy(2'-methoxy-ethyl) -pyridine-1-oxide and left in a steam bath for 3 hours. The solution was acidified to pH 2 using concentrated HCl, reduced in volume by evaporating in vacuo and left to crystallise. The white solid was recrystallised from ethanol, 2-hydroxy-4-oxy(2'-methoxy-ethyl)-pyridine 1-oxide. mp 134°, (0.58g, 29%) NMR ($CDCl_3$):
H(3),δ = 6.05 ppm doublet; H(5),δ = 6.05ppm quartet;
H(6),δ = 7.62 ppm triplet; Me,δ = 3.42 ppm singlet;
$CH_2(2')$,δ = 4.08 ppm triplet; $CH_2(1')$,δ = 3.7 ppm triplet.

2.3.7 2,4-Dihydroxy-pyridine-1-oxide (L_3)

2,4-Dihydroxy-pyridine-1-oxide was prepared in a two-step reaction involving the substitution of both the nitro and the chloro groups of 2-chloro-4-nitro-pyridine-1-oxide by the methoxide anion, forming 2,4-dimethoxy-pyridine-1-oxide according to the method of Talic, Z,[50] followed by acidic hydrolysis resulting in the formation of 2,4-dihydroxy-pyridine-1-oxide.



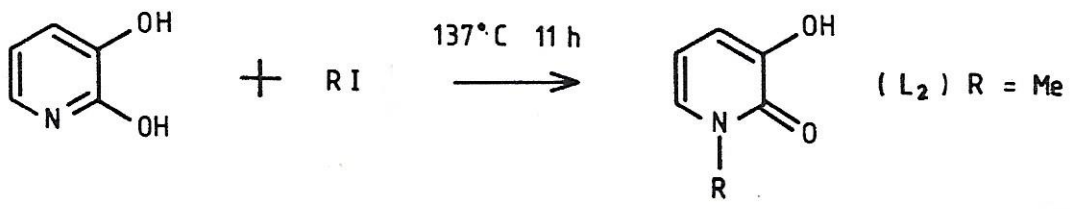


Sodium methoxide was prepared by dissolving Na metal (0.66g)in methanol (33ml). This was mixed with 2-chloro-4-nitro-pyridine-1-oxide (2.3g) in methanol (20ml); placed under reflux for 6 h, filtered and the solvent evaporated in vacuo. The solid obtained was extracted with chloroform, the chloroform solution was then reduced in volume and left to crystallise.
NMR (D_2O): H(3),δ = 6.55 ppm singlet; H(5),δ = 6.58ppm doublet; H(6),δ = 7.85 ppm triplet; Me(2),δ = 3.92 ppm singlet; Me(4),δ = 3.75ppm singlet.

Attempts to hydrolyse the methyl ether bond using 10% HCl in a steam bath for 13h failed. However, the hydrolysis succeeded when more extreme conditions were used, namely that of 20% HCl boiled at 105°C for 13h. An orange-white solid was formed on cooling, 2,4-dihydroxy-pyridine-1-oxide (0.42g, 30%). NMR (dDMSO + trace of D_2O): H(3),δ = 6.08ppm singlet; H(5),δ = 6.12ppm quartet; H(6),δ = 7.88ppm doublet.

2.3.8 1-Methyl-3-hydroxy-pyrid-2-one (L_2)

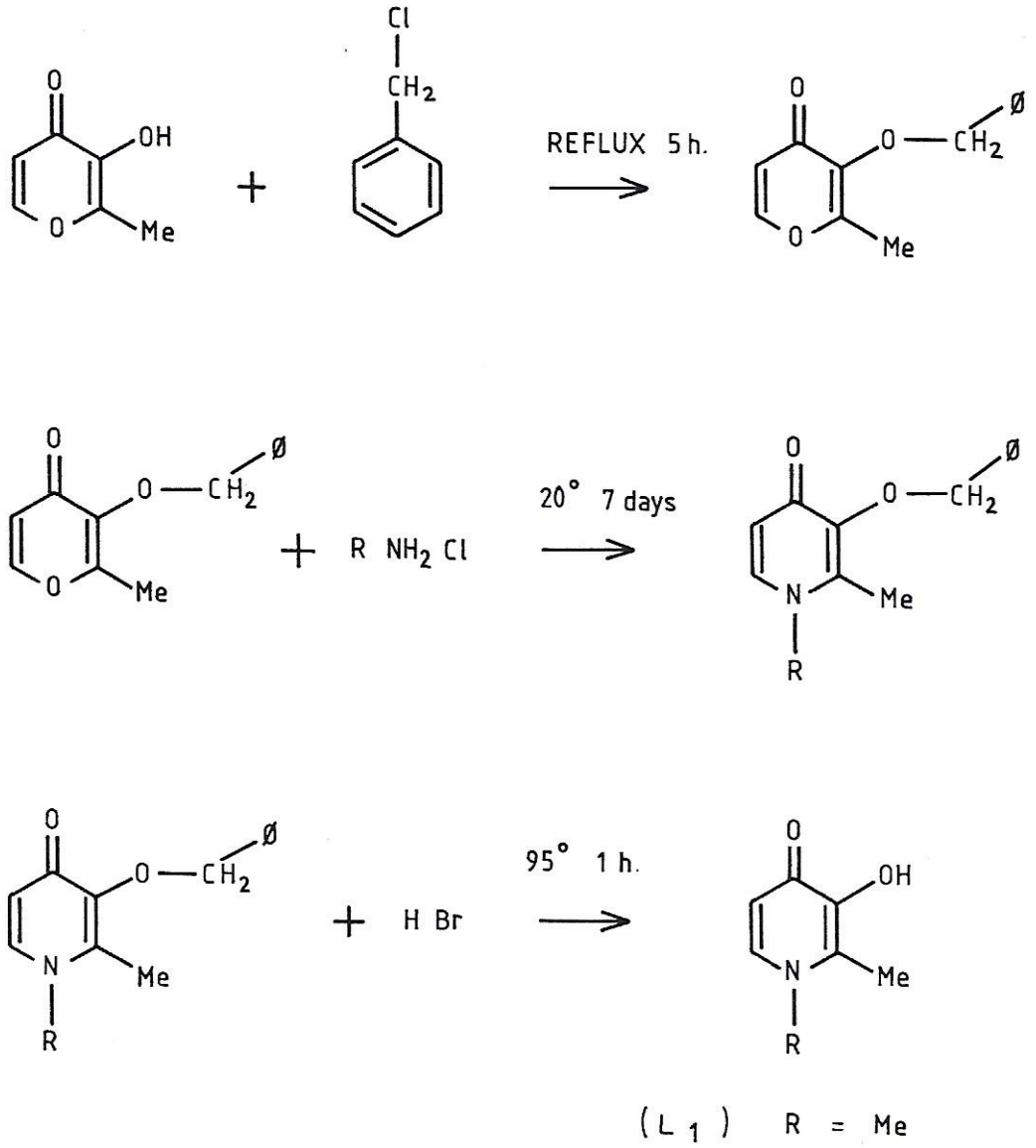
1-Methyl-3-hydroxy-pyrid-2-one was prepared according to the method of Mohrle H. and Weber, H.[51]



2,3-Dihydroxypyridine was recrystallised from water forming needle-like crystals and (2.78g) was placed in a tube cooled to -70°C. Methyl iodide (10ml) was added, the tube sealed and shaken overnight at room temperature, placed in a steel bomb, transferred into a thermostatic oven and heated at 137°C for 10.5h. After cooling to room temperature, the enclosed tube was removed from the steel bomb, cooled to -70°C and opened to yield a brown resinous product floating on methyliodide. The methyl-iodide solution was carefully removed and the resinous product dissolved in distilled water (50ml). Fresh H_2SO_3 (25ml) was prepared by bubbling SO_2 in water for five minutes and then mixed with the aqueous solution of the crude product. The pH was adjusted to 6.5 using a saturated solution of Na_2CO_3. The solution was filtered and extracted continuously with chloroform for two days. The chloroform solution was then dried over Na_2SO_4, evaporated in vacuo giving a white solid, 1-methyl-3-hydroxy-pyrid-2-one. mp: 126-128° (lit. 129-131°), (1.36g, 55%). NMR ($CDCl_3$): Me,δ = 3.68ppm singlet; H(4),δ = 6.93ppm singlet; H(5),δ = 6.83 ppm singlet; H(6),δ = 6.2 ppm triplet.

2.3.9 1,2-Dimethyl-3-hydroxy-pyrid-4-one (L_1)

The method of preparation is based on the method of Harris, R.L.N..[52] 2-Methyl-3-hydroxy-pyr-4-one (3.56g) was dissolved in methanol (36ml) at 60°C. NaOH (30%, 4ml) was added yielding a green solution, followed by benzyl chloride (4.08g). This mixture was refluxed for 5h, forming an orange solution, and then left to cool



(L 1) R = Me

SCHEME 2.1

The Synthetic Pathway for the Preparation of L_1

overnight. The volume was reduced to one-third, by evaporation in vacuo. Water (8ml) was added and the product extracted into methylene chloride (2x4ml). The extracts were combined, washed with NaOH (5%, 2x4ml), water (2x4ml) and finally dried over Na_2SO_4 yielding 2-methyl-3-benzyloxy-pyr-4-one.

2-Methyl-3-benzyloxy-pyr-4-one (4.8g) and methyl ammonium chloride (1.6g) was dissolved in a mixture of water (200ml), ethanol (100ml), NaOH (2g) and the solution stirred at 20°C for one week. After this period the solution was acidified to pH 2.5 using HCl and evaporated in vacuo to dryness giving a yellow solid. Water (40ml) and HBr (20ml sp.gr. 1.46-1.49) were added and the resulting mixture was placed on a steam bath. The solid dissolved within 1h forming an orange solution, which was evaporated in vacuo. When crystals formed it was allowed to stand at 4°C to facilitate further crystallisation, 1,2-dimethyl-3-hydroxy-pyrid-4-one (2.58g, 63%).

NMR (D_2O): Me(1),δ = 3.76ppm singlet; Me(2),δ = 2.33ppm singlet; H(5),δ = 6.83ppm doublet; H(6),δ = 7.78ppm doublet.

2.4 The Structure-Function Relationship of the Ligands

The rationale behind the synthesis of ligands was not a quantitative synthetic approach for screening purposes but a qualitative one based on molecules containing certain well-defined structural features. The chemical characteristics of the synthesised ligands (L_1 - L_7) could be summarised as follows:

(a) all the ligands possess a heteroaromatic ring, either pyridine or pyrone;
(b) all the ligands are bidentate;
(c) all the ligands coordinate iron via two oxygen atoms which are bound directly to the heteroaromatic ring.

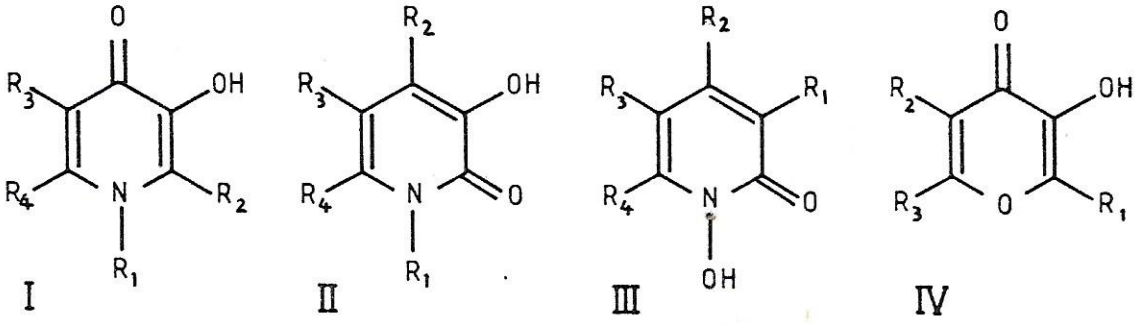
There are two major classes. The first type is that of an α-hydroxy-ketone (e.g. L_1, L_2, L_5) and the second, that of hydroxamic acid based on 2-hydroxy-pyridine-1-oxide (e.g. L_3, L_4, L_6, L_7). Each ligand, when binding to iron(III) is expected to lose a proton from the hydroxyl group of the coordinating site thus forming a 3:1 (ligand: iron(III)) neutral complex.

The substituents - CH_3, $-OCH_3$, -OH, $-OCH_2\ CH_2OCH_3$, were introduced in an attempt to increase the basicity of the ligand, to vary the lipophilicity (i.e. $-OCH_3$ and -OH are expected to increase and to decrease the lipophilicity of the ligand respectively), while preserving the status of the binding site and the neutral character of the ligand and its iron(III) complex.

The preparation procedures of L_1 and L_2 are comparatively easier than those of L_3, L_6 and L_7. The NMR spectra of all the ligands demonstrated that significant amounts of impurities were absent. However, in the case of L_6 and L_7 the surface of the crystals obtained during the preparation were dark brown, probably due to air oxidation. The yield was reasonably high in the syntheses of L_1 and L_2 but rather low in the other three ligands (L_3, L_6, L_7) probably due to the instability of intermediaries formed in the synthetic route. No systematic attempt was made to improve these yields.

The methods adopted in this chapter are suitable for the preparation of higher homologues. Basically all the compounds synthesised are derivatives of four prototype molecules, namely: N-substituted 3-hydroxy-pyrid-4-one (e.g. L_1), N-substituted 3-hydroxy-pyrid-2-one (e.g. L_2), 2-hydroxy-pyridine-1-oxide (e.g. L_4) and 3-hydroxy-pyr-4-one (e.g. L_5). Thus substituted prototypes could similarly be prepared from their starting materials.

These molecules can be represented as follows:



where R_x could be a hydrogen atom, an alkyl, substituted alkyl or any other suitable substituent. In the prototype molecules I and II the substituent R_1 will preferably be an alkyl, or substituent alkyl group. This will assist in stabilising the α-keto hydroxy binding site. Manipulation of the other substituents will influence other properites in the chelator molecule such as basicity, e.g. by electron releasing substituents, lipophilicity, etc.. Furthermore a search is needed for other feasible synthetic pathways in order to find the lowest possible cost for the production of these compounds and their analogs.

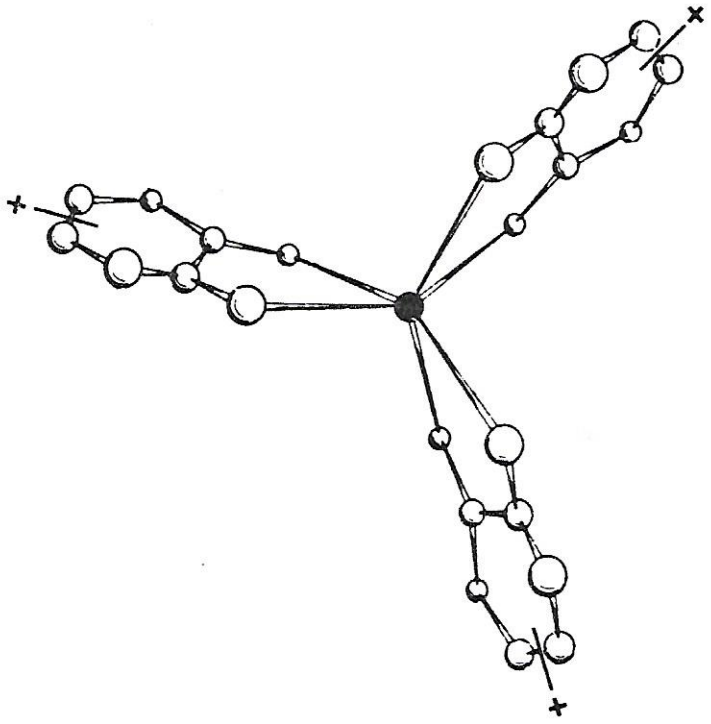


FIG. 3.1

A Diagrammatic Representation of the Ligand-iron(III) Complex

At physiological pH ranges iron(III), in the centre, is bound by six oxygen atoms, two from each of the three ligands. X is any other substituent in the six membered ligand not bound to iron(III).

CHAPTER THREE

INORGANIC IRON(III) CHELATION STUDIES

3.1.1 Chelation of Iron

The main interest in the chemical studies of the new ligand iron complexes was to establish whether these were water soluble, stable and electrically neutral at a physiological pH. Other important aspects which are monitored are the pK_a of the ligands, the stability constants of their iron(III) complexes at a near neutral pH and, briefly, their affinity for other metals.

Iron(III) is highly insoluble at a physiological pH, usually forming polynuclear complexes with very low solubility products ($Fe(OH)_3$ $Ksp = 10^{-39} M^3$). To solubilise this species of iron, one can lower the pH, reduce the iron, ferrous hydroxide being markedly more soluble ($Fe(OH)_2$ $K_{sp} = 10^{-15} M^2$), or use an iron(III) chelating agent.[53] *In vivo* only the last method is possible, and although a reducing agent (e.g. ascorbic acid) is of limited help, the current search for methods of iron mobilisation *in vivo* is based on the employment of iron(III) chelators.

Many iron chelators have been tested under both *in vitro* and *in vivo* and as a result the strategy behind the structure function relationships regarding effective iron chelators, under physiological conditions, is well documented and will be discussed briefly.[27,54,55,56,57,58,59]

In Chapter Two the structures of four different ligand types were introduced and their properties were discussed, iron chelation being the most important. In the reaction of iron(III) with a bidentate ligand (L) such as these, there is an equilibrium between iron(III), the ligand (L) and a series of complexes as follows:

$$Fe + L \overset{K_1}{\rightleftharpoons} FeL + L \overset{K_2}{\rightleftharpoons} FeL_2 + L \overset{K_3}{\rightleftharpoons} FeL_3$$

the formation constants K_1, K_2 and K_3 are given by

$$K_1 = \frac{[FeL]}{[Fe][L]} \qquad K_2 = \frac{[FeL_2]}{[FeL][L]} \qquad K_3 = \frac{[FeL_3]}{[FeL_2][L]}$$

and the stability products (β) by

$$\beta_1 = K_1, \ \beta_2 = K_1K_2, \ \beta_3 = K_1 \cdot K_2 \cdot K_3$$

$$\text{or } \beta_n = \frac{[FeL_n]}{[Fe][L]^n}$$

Stability products are usually reported for iron chelators, but although many ligands have much higher stability products than transferrin (Table 3.1), surprisingly they fail to mobilise iron(III) <u>in vivo</u>. There are two possible reasons for this in relation to chelation properties:

(a) protons (10^{-7}M) and Ca^{2+}(10^{-3}M) compete effectively with iron(III) for the ligand, and

(b) hydroxide ions (10^{-7}M) compete efficiently with the ligand for iron(III).

3.1.2 Affinity Constants with a Physiological Relevance

To account for the possible interference by other ions of iron chelation under physiological conditions, a new constant was developed by Schubert, J.[57] called the effective constant (K_{eff}) and defined by the equation:

$$K_{1eff} = \frac{[FeL']}{[Fe'][L']}$$

where [FeL'] is the sum of concentrations of all species containing Fe and L in the molar ratio of 1:1, e.g. [FeL'] = [FeL] + [FeHL] + [FeOHL] + ·····; similarly [Fe'] = [Fe] + [Fe(OH)] + FeA + ····· where [Fe'] is the sum of the concentrations of the free iron(III) and all other iron(III) which have reacted with other anion (A) and complexing ligands but not L; and [L'] = [L] + [LH] + [LCa^{2+}] + [LB] + ····· where [L'] is the sum of the concentrations of the free ligand and all other combinations of this ligand with cations such as Ca^{2+} and B, but not with iron(III).

To simplify the equation site reaction coefficients (α) are involved as follows:

$$\alpha_{FeL} = \alpha_{FeHL} + \alpha_{FeOHL} + ····· = \frac{[FeL']}{[FeL]}$$

$$\alpha_{Fe} = \alpha_{FeOH} + \alpha_{FeA} + ····· = \frac{[Fe']}{[Fe]}$$

$$\alpha_{L} = \alpha_{LH} + \alpha_{LCa^{2+}} + \alpha_{LB} + ····· = \frac{[L']}{[L]}$$

The most important α coefficients for in vivo conditions are likely to be those in equations (i) to (iv).

$$\alpha_{Fe(OH)} = 1 + [OH^-]\, K_{Fe(OH)} + [OH^-]^2\, K_{Fe(OH)_2} + [OH^-]^3\, K_{Fe(OH)_3} + \cdots\cdots \quad \text{(i)}$$

$$\alpha_{L(H)} = 1 + [H^+]\, K_{LH} + [H^+]^2\, K_{LH_2} + \cdots\cdots \quad \text{(ii)}$$

$$\alpha_{L(Ca^{2+})} = 1 + [Ca^{2+}]\, K_{LCa^{2+}} + \cdots\cdots \quad \text{(iii)}$$

$$\alpha_{Fe(HL)} = 1 + [H^+]\, K^H_{Fe(HL)} + \cdots\cdots \quad \text{(iv)}$$

Equation (i) takes account of the affinity of hydroxide anions for iron(III). Similarly, equation (ii) accounts for competition by protons, equation (iii) for competition by calcium ions, and equation (iv) takes account of the possible involvement of protonated iron complexes. Thus

$$K_{eff} = K_{(FeL')}\,(Fe')\,(L') = \frac{\alpha_{FeL}}{\alpha_{Fe}\,\alpha_L}\, K_{FeL}$$

In principle it is possible to maximise K_{eff} by decreasing α_{LH} and $\alpha_{LCa^{2+}}$, i.e. by decreasing the pK_a of the ligand and also by designing ligands of low affinity for Ca^{2+}. The first of these two variables is limited by the necessity to keep the ligand pK_a value ≥ 7.0 if a neutral ligand is desired under physiological conditions. There will be a further advantage if the protonated ligand (LH) can form

an iron(III) complex but this is unlikely. Another advantage can occur if Fe^{III} OH can bind to the ligand as FeOHL. These two possibilities would have the effect of increasing α_{FeHL} and α_{FeOHL} respectively and consequently increasing K_{eff}. A further major factor is K_{FeL} which also needs to be high, i.e. the ligands should have high affinity for iron(III).

The α_{FeOH} term in the equation does not depend on the ligand species, but on the state of iron(III) in solution. Iron(III) is thought to form mononuclear and polynuclear complexes depending on its concentration and pH. in vivo polynuclear iron(III) complexes are formed in ferritin and haemosiderin whereas mononuclear iron(III) complexes are formed if the concentration of iron is $< 10^{-3.7}$M, and in transferrin.

To account for the solubility of polynuclear iron deposits, such as ferritin iron, by a bidentate ligand and assuming that iron is in the form of $Fe(OH)_3$, a new term was introduced by Schubert, called the solubilising constant K_{sol} which is defined by the equation:

$$\log K_{sol} = \log \beta_{Fe(L)_n} + 21 - [pK_{sp} + n \log \alpha_{L(H)}]$$

assuming that there is no Ca^{2+} interference and $pK_{sp} = 39$ accounts for the solubility product of ferric hydroxide.

An extensive screening programme of different ligands has been undertaken in order to examine their iron chelation properties in vitro. Some of the results are presented in Table 3.1. It should be emphasised that solubilisation of insoluble iron deposits could be effectively achieved if log K_{sol} > 0, or if the log β of the iron chelate is >18 + n log α_{LH}, at physiological pH. Thus all the chelators depicted in Table 3.1 should be capable of solubilising insoluble iron deposits, such as those present in ferritin and haemosiderin, and, furthermore, loose iron not bound to transferrin. One would expect that chelators which have log K_{eff} values greater than that of transferrin should be able to mobilise iron from this protein. However, as it will be shown in Chapters 5 and 6 these stability constants are of no physiological significance if the rates of iron mobilisation from both transferrin and ferritin are very slow. In addition to the kinetic inaccessibility of the protein iron site, other factors can limit the efficacy of the chelator such as membrane permeability, solubility, toxicity and metabolism.

It is clear from Table 3.1 that the most promising class of chelators are the tropolones. Although 3-isopropyltropolone is a bidentate ligand, its log K_{eff} is sufficiently high to sequester iron from transferrin, mainly because proton and calcium interference are minor. It should be emphasised that the chelation site of tropolone closely resembles that of the chelators synthesised in this study.

TABLE 3.1

Stability Constants of Iron(III) Complexes

CHELATING AGENT	STABILITY CONSTANTS		
	log β	log K_{eff}	log K_{sol}
2,3-Dihydroxynaphthalene-6-sulfonic acid	44	26	8
8-Hydroxyquinoline	37	29	11
1,8-Dihydroxynaphthalene 3,6,disulfonic acid	37	25	7
Acetohydroxamic acid	28	22	4
3-Isopropyltropolone	32	30	14
Salicylic Acid	36	19	1
DTPA	27	20	2
DFB	31	24	6
Transferrin	36	24 20*	6

* According to Aisen, P. et al.[64]

3.1.3 Hexadentate versus Bidentate Ligands

Another important factor which contributes to higher stability constants is the "chelate effect". In comparing the reactions of a bidentate and the equivalent trimer hexadentate ligand with a metal cation there will be a greater increase in entropy in the latter case, whereas their enthalpy increases will be similar. Thus the overall formation constant with iron(III) for acetyl hydroxamine for example, is lower than that of DFB by 2.3 log units.[57] This superiority of a hexadentate ligand (L') over a bidentate one (L) is clearly demonstrated by the "dilution effect". If it is assumed that in the reactions (a) and (b) the formation constants K_A and K_B

$$Fe + 3L \overset{K_A}{\rightleftharpoons} FeL_3 \qquad (a)$$

$$Fe + L' \overset{K_B}{\rightleftharpoons} FeL' \qquad (b)$$

equal to 10^{30} and that these ligands are present in excess relative to iron, at a ligand concentration of 10^{-3} molar then considering the fraction of iron(III) bound by the bidentate ligand we have

$$\frac{[FeL_3]}{[Fe]} = 10^{30} \times (10^{-3})^3 = 10^{21}$$

and by the hexadentate we have

$$\frac{[FeL']}{[Fe]} = 10^{30} \times 10^{-3} = 10^{27}$$

Therefore the fraction bound by the hexadentate ligand is relatively independent of dilution compared to the bidentate one. It has been argued that the efficacy of polydentate over bidentate ligands is demonstrated by the polydentate nature of natural siderophores.[56] In principle this seems to be true when one considers these ligands competing in a culture medium low in iron. In iron overload, however, the efficacy of the ligands should be considered within the context of their ability to mobilise iron mainly from transferrin and ferritin, in which case, bidentate ligands may prove to be more effective. Other considerations one should have in mind in designing hexadentate ligands is that the higher the molecular weight of the ligands the lower their ability to:

(a) diffuse across membranes, and

(b) mobilise iron from ferritin, if their size is larger than the size of the channels in this protein.

It should also be emphasised that equilibrium conditions are unlikely to occur in living organisms and many factors can diminish the efficacy of a ligand <u>in vivo</u> such as metabolic transformations, inabsorption, etc..

3.1.4 Competition between Metals

In a complex between a cation and an anion or the negative end of a dipole, the electrostatic attraction and the stability of the complex will be greater when the species are small or highly charged. This model is appropriate for "hard" acids (e.g. iron(III)) and "hard" bases (e.g. oxygen containing bases), furthermore, for a given ligand the stability constant increases as:

(a) the oxidation state of a hard metal increases,

and (b) the charge increases for cations of the same ionic radius.

Furthermore, the stability constants follow the order of ionic potential and sizes in the Irving-Williams order of the first transition metal series, i.e. $Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$. Thus there is only a small probability for a ligand to have a high affinity for one metal while retaining small affinity for all other metals. This presents a serious problem for chelate facilitated removal of specific metals in the in vivo situation since many metals are essential for the normal physiological functions of the cell. There are many biologically important metals but in this preliminary study it was considered useful to briefly monitor the effect of the newly developed ligands on CaII, MgII, ZnII and CuII, the main interest being to what extent they form complexes at physiological pH.

3.1.5 The Influence of Ring Substituents on the pK_a, iron(III) Chelating Ability and Partition Coefficients

When designing new iron chelators several aspects including pK_a and lipophilicity have to be considered. In Chapter 2 it was emphasised that for a group of closely related chelating agents more basic co-ordinating groups produce more stable complexes. However, the higher the pK_a of the co-ordinating groups, the greater will be the competition from protons. The overall charge of the ligand and its iron complex are also important. Thus if the ligand is electrically neutral, depending on its partition coefficient, it will be able to permeate cell membranes, including the intestinal barrier. Thus the formation of a neutral complex will, in principle, facilitate mobilisation of intracellular iron.

These properties can be modified by the introduction of substituents in the aromatic ring. Electron releasing substituents would be expected to increase the basicity of the co-ordinating group and thus increase the stability of the complex.[65] The incorporation of long alkyl chains or a methoxy group will enhance lipophilicity which in turn will promote membrane permeability. However, there are limits to this trend as if they are too hydrophobic, their water solubility will be seriously diminished and they will tend to partition into the membranes and thus have toxic side effects (Chapter 4). The manipulation of these two major factors can be varied until a desirable balance for maximum biological activity is accomplished.

3.1.6 Short Term Objectives

In this project one of the main objectives was to find a useful and simple procedure for screening the efficacy of new synthesised ligands to bind iron(III) under physiological conditions. In the long term, a more detailed study of their iron chelation will be desirable. However, it was considered that in addition to thermodynamic studies, co-ordination properties under physiological conditions should be given strong weighting. Thus emphasis will be given to the following questions in the hope that the resulting observations will be of significance to the in vivo situation.

(a) Does the ligand form a water soluble iron complex?

(b) How many complex species exist and what are their charge at pH 7.4?

(c) What is the conditional formation constant ($\log K_{sol}$) at pH 7.4?

(d) What are the $\log K_{eff}$ and $\log \beta$ values?

3.2 Materials and Methods

3.2.1 Selection of Ligands

The ligands used for chelation studies are those synthesised as described in Chapter 2 and also some with related chemical structures which were commercially available (Table 3.2). In a preliminary spectrophotometric study of the ligand iron solutions, it was observed that all these ligands formed water soluble complexes under acid conditions but that some failed to do so at pH 7.4. It was decided to proceed to more detailed studies with the ligands L_1 - L_7.

3.2.2 Spectrophotometric Methodology

The reactions of the ligands with iron(III), protons and other metals were studied spectrophotometrically. All the solutions used were freshly prepared and the pH adjustments were carried out using HCl (16M) and NaOH (10M). The pH titrations of the ligand iron(III) complexes were carried out with the ligand in 3 or 4-fold molar excess and in the presence of $NaClO_4$ (0.1M), for maintaining constant ionic strength.

The stoichiometry of the ligand iron(III) complex (Job's plot[60]) and the conditional formation constants were determined at an acidic and a neutral pH using the method of continuous variation.[61] Thus two equimolar solutions, both 0.1M $NaClO_4$, one of iron(III) (prepared fresh from anhydrous $FeCl_3$) and the other of a ligand

TABLE 3.2

Ligands Tested for Iron Complexes

LIGANDS COMMERCIALLY AVAILABLE	PROPERTIES OF IRON COMPLEXES	
	pH 2	pH 7
2,3-dihydroxy-pyridine	soluble	insoluble
2-mercapto-3-hydroxy pyridine	soluble	insoluble
2-mercapto-pyridine-1-oxide	soluble	insoluble
2-hydroxy-pyridine-1-oxide (L_4)	soluble	precipitation on standing
2-methyl-3-hydroxy-pyr-4-one (L_5)	soluble	soluble
LIGANDS SYNTHESISED		
1,2-dimethyl-3-hydroxy-pyrid-4-one(L_1)	soluble	soluble
1-methyl-3-hydroxy-pyrid-2-one (L_2)	soluble	soluble
2,4-dihydroxy-pyridine-1-oxide (L_3)	soluble	soluble
2-hydroxy-4-methoxy-pyridine-1-oxide (L_6)	soluble	soluble
2-hydroxy-4-oxy(2'methoxy-ethyl) pyridine-1-oxide (L_7)	soluble	soluble
2-hydroxy-3,5-dinitro-pyridine-1-oxide [L_4-3,5(NO_2)$_2$]	soluble	no complex
2-hydroxy-5-nitro-pyridine-1-oxide [L_4-5(NO_2)]	soluble	no complex
2-hydroxy-4-nitro-pyridine-1-oxide [L_4-4(NO_2)]	soluble	no complex
(LIGAND CONCENTRATION 10^{-3} - 10^{-4} M)		

were mixed at different volume proportions to an equal final volume. For each mixture the pH was adjusted using HCl (16M) and NaOH (10M) and the absorption spectra were recorded. The absorbance at a selected wavelength usually the λ_{max} of the ligand iron(III) complex was measured and corrected for iron(III) or ligand absorption. This absorbance value (ΔA) was plotted against the molar ratio of the ligand (Job's plot) and the composition complex estimated. The co-ordinates of the Job's plot (ΔA, molar fraction of the ligand) measured in the physiological pH region were used, according to the computing method described by W. Likussar,[62] to estimate the most probable complex composition as well as the conditional stability constant K' for the complex formation.

The log β_3 and log K_{eff} of all the ligands were estimated from the conditional stability constant K' using the following formulae:

$$\log \beta_3 = \log K' - 21 + pK_{sp} + n \log \alpha_{L(H)}$$

$$\log K_{eff} = \log b_3 - \log \alpha_{Fe(OH)} - n \log \alpha_{L(H)}$$

(where n is the number of ligands chelating iron(III)) and under the following assumptions:

(a) that at pH 7.4 log K' is the same as log K_{sol}, i.e. the constant related to the solubility of polynuclear iron(III) complexes. The precipitation of $FeCl_3$ at pH 7.4 and the mobilisation of iron from ferritin (Chapter 6) provide evidence for the ability

of these ligands to solubilise polynuclear iron(III).

(b) that the iron(III) precipitate has a similar solubility product (K_{sp}) to that of $Fe(OH)_3$ ($K_{sp} = 10^{-39}$).

and (c) in the case of log K_{eff}, mononuclear iron complexes are formed with log $\alpha_{FeOH} = 8.4$, at pH 7.4.[54]

Unlike the iron(III) complexes which were coloured and thus studied in the visible absorption region, monitoring of the reaction with the other metals was studied spectrophotometrically in the uv region. The metal solutions were freshly prepared in the presence of concentrated HCl (16M) from the following metal salts: $CuSO_4 5.H_2O$, $ZnSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$; $MgSO_4 \cdot 7H_2O$. The ligands were dissolved in Tris. HCl (0.1M, pH 7.35) at the metal and ligand concentrations of 2×10^{-5}M and 6.6×10^{-5}M respectively. The pH was adjusted to 7.5 using NaOH (10M). At the end of the experiment an equal concentration of iron(III) was added to all the ligand metal mixtures.

pK_a determinations of the ligands was carried out by titrating the ligand in NaCl or $NaClO_4$ (0.1M) using HCl (16M) and NaOH (10M). The absorption spectra in the uv region were recorded at each pH and plots of the change in absorbance (ΔA), at a selected wavelength against pH, generated a sigmoidal curve from which the pK_a was determined.

3.3 The Reactions of the Ligands with Iron, Other Metals and Protons

Various ligand reactions with iron, other metals and protons were studied, placing greater emphasis on those which form iron complexes at physiological pH's, (Table 3.2).

3.3.1 pK_a Determination

The spectrophotometrically determined pK_a's of the different ligands show a wide variation. This is depicted in Table 3.3 together with the reported pK_a's of other ligands. A typical example of the sigmoidal titration curves is depicted in Fig. 3.1 for L_4. The pK_a's of L_4 and L_5 were previously reported.[40,63]

3.3.2 The Reaction of Ligands with Other Metals

A preliminary study of the reaction of the ligands with other metals was carried out by recording the absorption spectra in the uv region of the ligands themselves and those of the ligands plus Cu, Zn, Ca and Mg at pH 7.5. Under these conditions Cu and Zn formed complexes with all the ligands but Ca and Mg with none. When iron(III) was added to these metal ligand mixtures the characteristic iron(III) colour complex was formed.

Fig. 3.2

pH Titration of L_4

Absorption spectra profile.

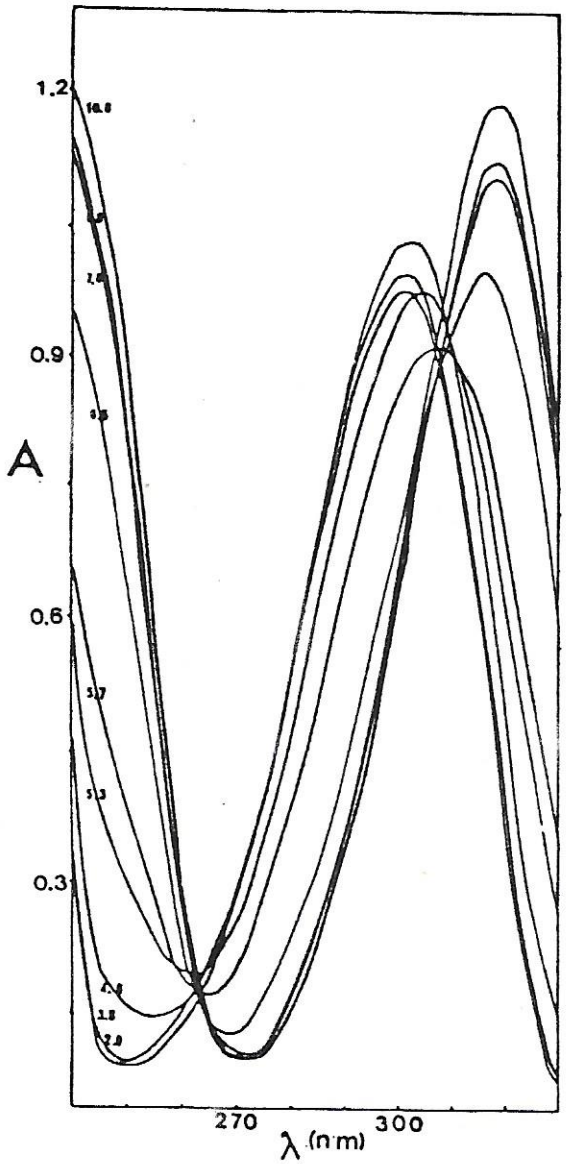
Vertical Axis: Absorbance.

Horizontal Axis: Wavelength.

Each curve corresponds to the absorption spectra of L_4 (2×10^{-4}M) at different pHs, shown at the right hand side.

pKa estimation of L_4

The plot of the change in absorbance of L_4 at 320 nm against pH gave a sigmoidal curve from where L_4's pKa was estimated.


1.2
0.9
0.6
0.3
A
270
300
λ (nm)

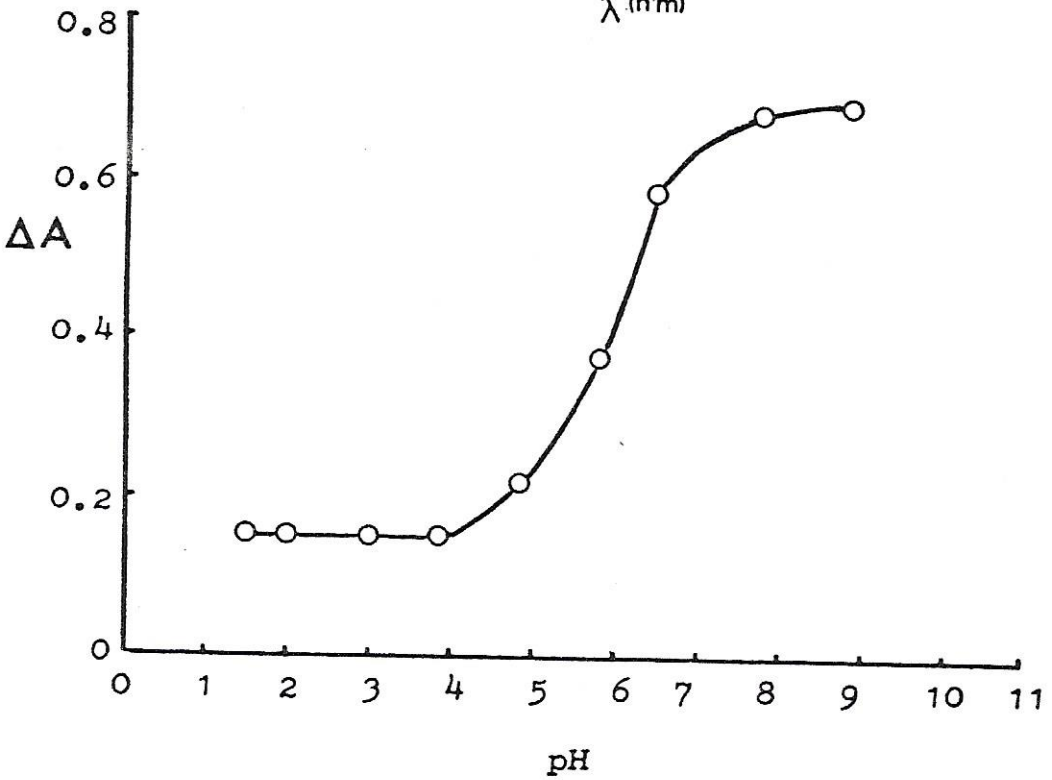

0.8
0.6
0.4
0.2
0
ΔA
0 1 2 3 4 5 6 7 8 9 10 11
pH

TABLE 3.3

The pK_a of the Ligands

LIGAND	pK_1	pK_2	% of neutral molecules at pH 7.4
L_1	9.7	3.3 (1-$\overset{+}{N}$H)	99.5
L_2	8.8	3.0 (1-$\overset{+}{N}$H)	96
L_3	6.7	8.4 (4-OH)	16.6
L_4	5.9		3
L_5	8.6		94
L_6	6.0		4
L_4- 4(NO_2)	3.6		0
L_4- 3,5(NO_2)$_2$	2.7		0
L_4- 5(NO_2)	3.7		0

3.3.3 The Reaction of Ligands with Iron(III)

The reaction of the ligands L_1 - L_7 with iron(III) were studied at acidic and neutral conditions. The results of these studies are presented in Tables 3.4 and 3.5, where the molar ratio of the ligand iron(III) complexes (L:Fe), the molar extinction coefficients (E) at certain wavelengths (λ), the colour and the binding constants of these complexes are reported.

L_1-iron(III) reactions

The pH titration of the L_1-iron(III) complex and its Job'splots at two different pH's are illustrated in Fig. 3.3. The pH titration indicates that there seems to be one complex species at pH 7.4, which also predominates over a wide region of acid/alkaline conditions. The Job's plot at pH 2 reveals a ligand to iron(III) molar ratio of 1:1 complex whereas that at pH 7.4 a 3:1 complex.

L_2-iron(III) reactions

L_2 is sensitive to light and air, forming purple colour solutions and crystals on standing. The colour of the soluble iron complex is the same as above at pH 7. The Figure (Fig. 3.4) illustrates the pH titration of the iron complex and the Job's plots at pH 2.7 and 7.0. At pH 2.7 the 1:1 L:Fe complex predominates and at pH 7.0 there seems to be a mixture of a 2:1 and a 3:1 L:M complex.

TABLE 3.4

Properties of the Ligand-Iron(III) Complexes

LIGAND	pH	log K'	L:Fe	$E^{(L-Fe)}_{M^{-1} cm^{-1}}$ [b]	λnm	Colour
L_1	2.0 7.4	9.56	1:1 3:1	3600	460	reddish purple
L_2	2.7 7.0	10.11	1:1 3:1 +(2:1)	3970	510	purple
L_3	3.25 7.4	9.86	2:1 3:1	4050	400	golden orange
L_4	3.35 7.0	7.65[a]	2:1 3:1[a]	2410[a]	400	greenish yellow
L_5		($\log \beta_3$) 29.7	3:1	4300	415	orange red
L_6	2.3 7.4	11.26	2:1 3:1	3350	420	reddish yellow
L_7	2.0		2:1	2510	450	reddish yellow

a = precipitation b = estimated error 5-10%

Fig 3.3

pH Titration of L_1-Fe.

Vertical Axis: Change in Absorbance[a] at 450 nm.

Horizontal Axis: pH.

L_1 = 2 x 10^{-3}[b], Fe = 0.5 x 10^{-3} M[b], NaCl = 0.1 M.

Job's plots of L_1-Fe at pH 2 (A) and at pH 7.4 (B).

A

Vertical Axis: Change in Absorbance[a] at 550 nm.

Horizontal Axis: Molar Fraction of ligand.

L_1 = 1 x 10^{-3} M[b], Fe = 1 x 10^{-3} M[b], NaCl = 0.1 M.

B

Vertical Axis: Change in Absorbance[a] at 460 nm.

Horizontal Axis: Molar Fraction of ligand.

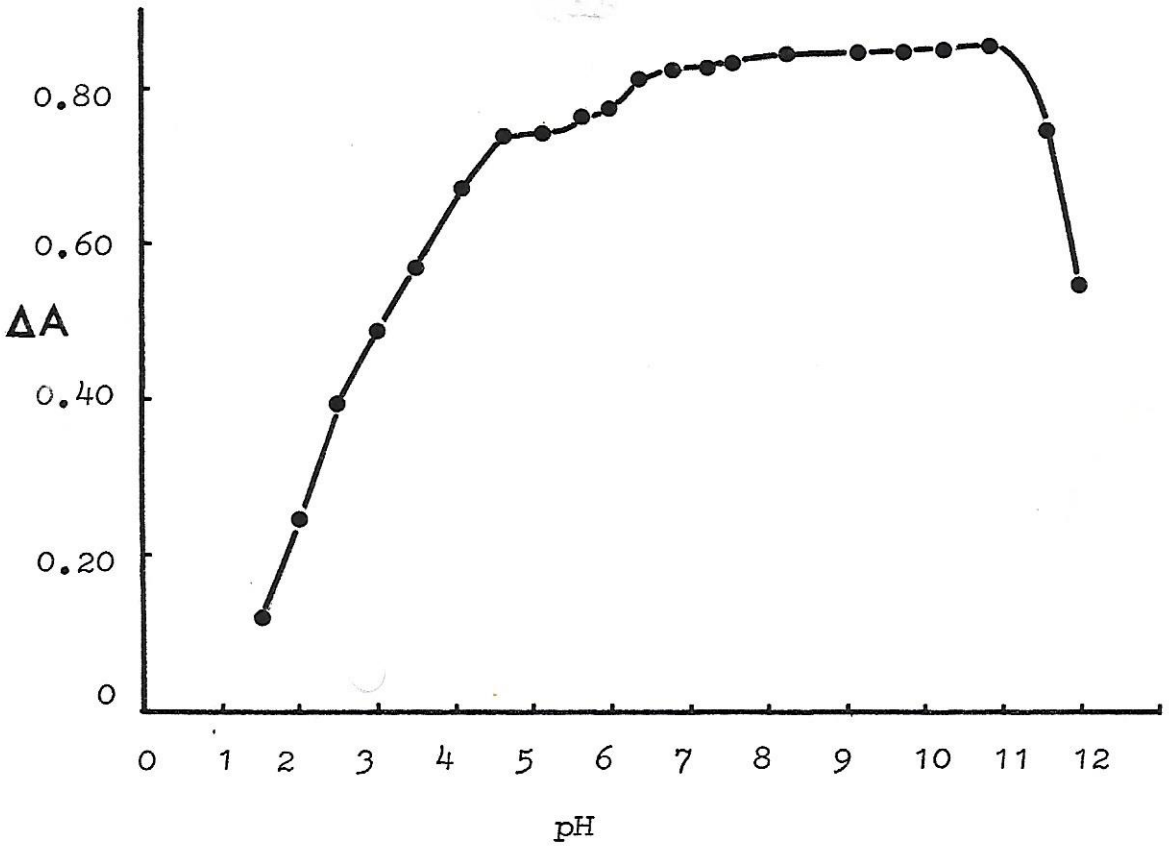
L_1 = 1 x 10^{-3} M[b], Fe = 1 x 10^{-3} M[b], $NaClO_4$ = 0.1M.

(□) The ligand iron mixture was left for 2.5 h and them the supernatant measured.

(○) The ligand iron mixture was left overnight and then the supernatant measured.

a = Arbitrary units.

b = Initial concentration.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ΔA
0.40
0.20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH

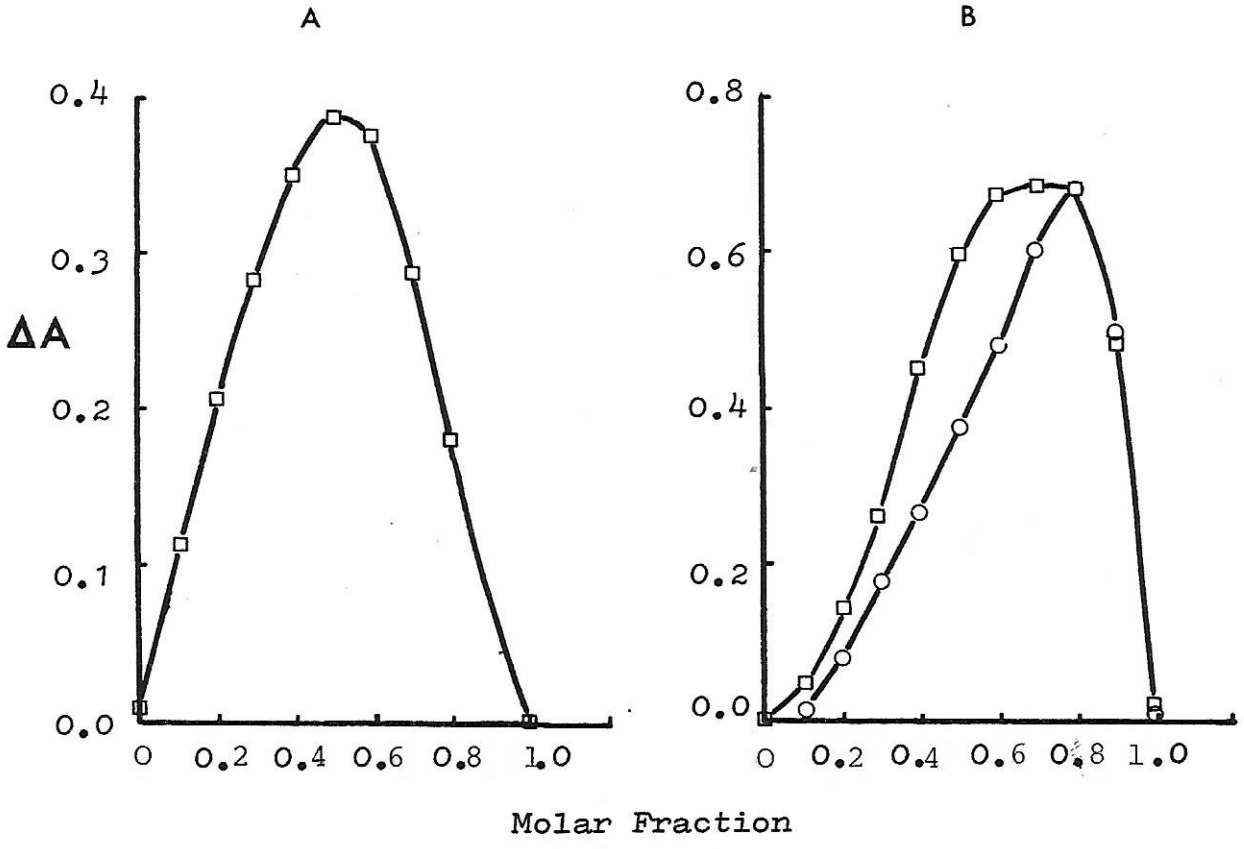

A
B
0.4
0.3
ΔA
0.2
0.1
0.0
0 0.2 0.4 0.6 0.8 1.0
0.8
0.6
0.4
0.2
0.0
0 0.2 0.4 0.6 0.8 1.0
Molar Fraction

Fig. 3.4

pH Titration of L_2-Fe.

Vertical Axis: Change in Absorbance[a] at 405 nm.

Horizontal Axis: pH.

$L_2 = 6 \times 10^{-4}$ M,[b] Fe = 1×10^{-4} M,[b] $NaClO_4$ = 0.1 M.

Jobisplots of L_2-Fe at pH 2.7 (A) and at pH 7.0 (B).

A

Vertical Axis: Change in Absorbance[a] at 610 nm.

Horizontal Axis: Molar fraction of ligand.

$L_2 = 6 \times 10^{-4}$ M,[b] Fe = 6×10^{-4} M,[b] $NaClO_4$ = 0.1 M.

B

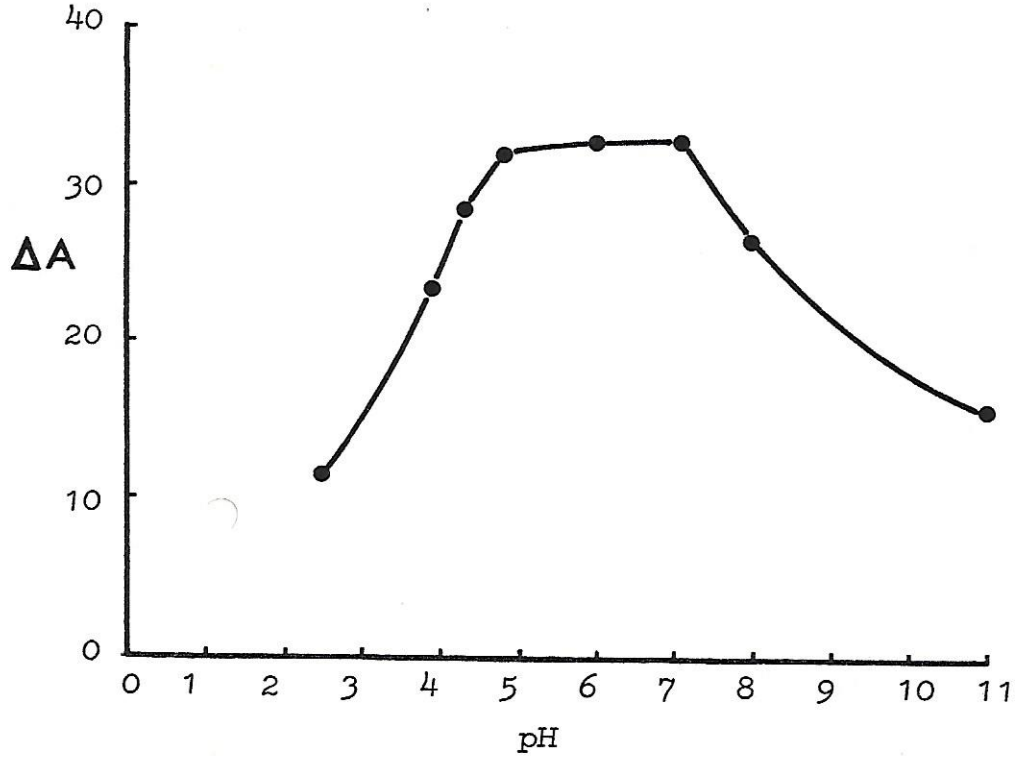
Vertical Axis: Change in Absorbance[a] at 510 nm.

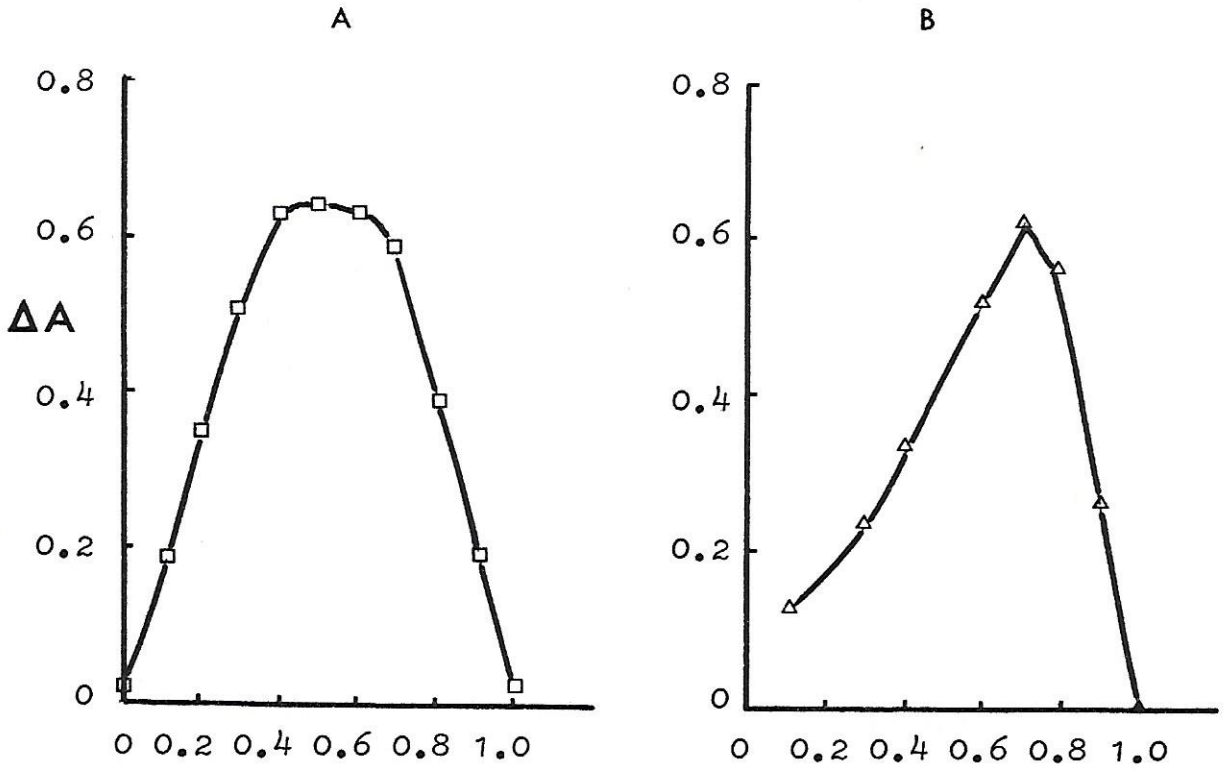
Horizontal Axis: Molar Fraction of ligand.

$L_2 = 6 \times 10^{-4}$ M,[b] Fe = 6×10^{-4} M,[b] $NaClO_4$ = 0.1 M.

a = Arbitrary units

b = Initial concentration.


40
30
ΔA
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
pH


A
B
0.8
0.6
ΔA
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1.0
Molar Fraction

L_3-iron(III) reactions

L_3 formed a soluble golden colour iron complex at pH 7.4 of predominantly 3:1 L:Fe ratio as illustrated in Fig. 3.5 . At pH 3.25 there is a mixture of 1:1 and 2:1 complexes, which can be identified from their difference in absorbance at 530nm and 470nm respectively (Fig. 3.5).

L_4-Iron(III) reactions

L_4 formed a soluble iron complex at pH 3.35 of 2:1 as shown in Fig. 3.6 but at pH 7.0 precipitation took place (green yellow). The pH profile of the iron complex in 50% dimethylsulfoxide (DMSO) is shown in Fig. 3.6 where a decreasing in absorbance is evident at 400nm in physiological pH, due probably to precipitation. Because of the precipitation problems it was difficult to monitor precisely the spectrophotometric studies associated with the iron(III) complex of this ligand.

L_5-iron(III) reactions

A very detailed study of the iron properties of L_5 has already been reported, but an experimental study of the pH titration and the Job'splot at pH 7.0 was carried out as shown in Fig. 3.7 , to find out whether similar properties can be detected in the uv region. The complex was orange red and indeed a L:M molar ratio of 3:1 can be shown as reported above. The pH titration indicates the existence of one complex species between pH 6-10.

Fig. 3.5

pH Titration of L_3-Fe.

Vertical Axis: Change in Absorbance[a] at 400 nm (■) and at 405 nm (●).

Horizontal Axis: pH.

L_3 = 7.5 x 10^{-4} M,[b] Fe = 2.5 x 10^{-4} M,[b] $NaClO_4$ = 0.1M (■)

L_3 = 8.0 x 10^{-4} M,[b] Fe = 2.0 x 10^{-4} M,[b] $NaClO_4$ = 0.1M (●)

Job's plots of L_3-Fe at pH 3.25 (A) and pH 7.4 (B).

Vertical Axis: Change in Absorbance[a] at 530 nm (○), 480 nm* (△) and 465 nm (□).

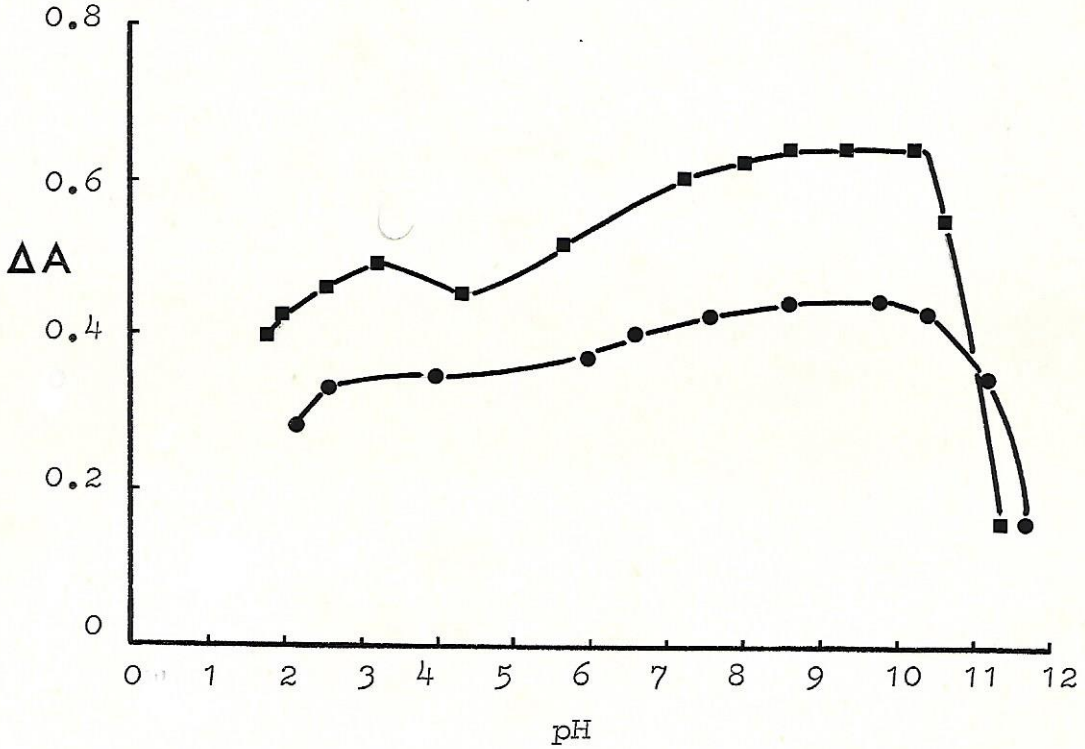
Horizontal Axis: Molar fraction of ligands.

L_3 = 1 x 10^{-3} M,[b] Fe = 1 x 10^{-3} M,[b] $NaClO_4$ = 0.1 M.

* Change in Absorbance was expanded (x2), in comparison to that at 530 nm.

a = Arbitrary units.

b = Initial concentration.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0.6
ΔA
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH

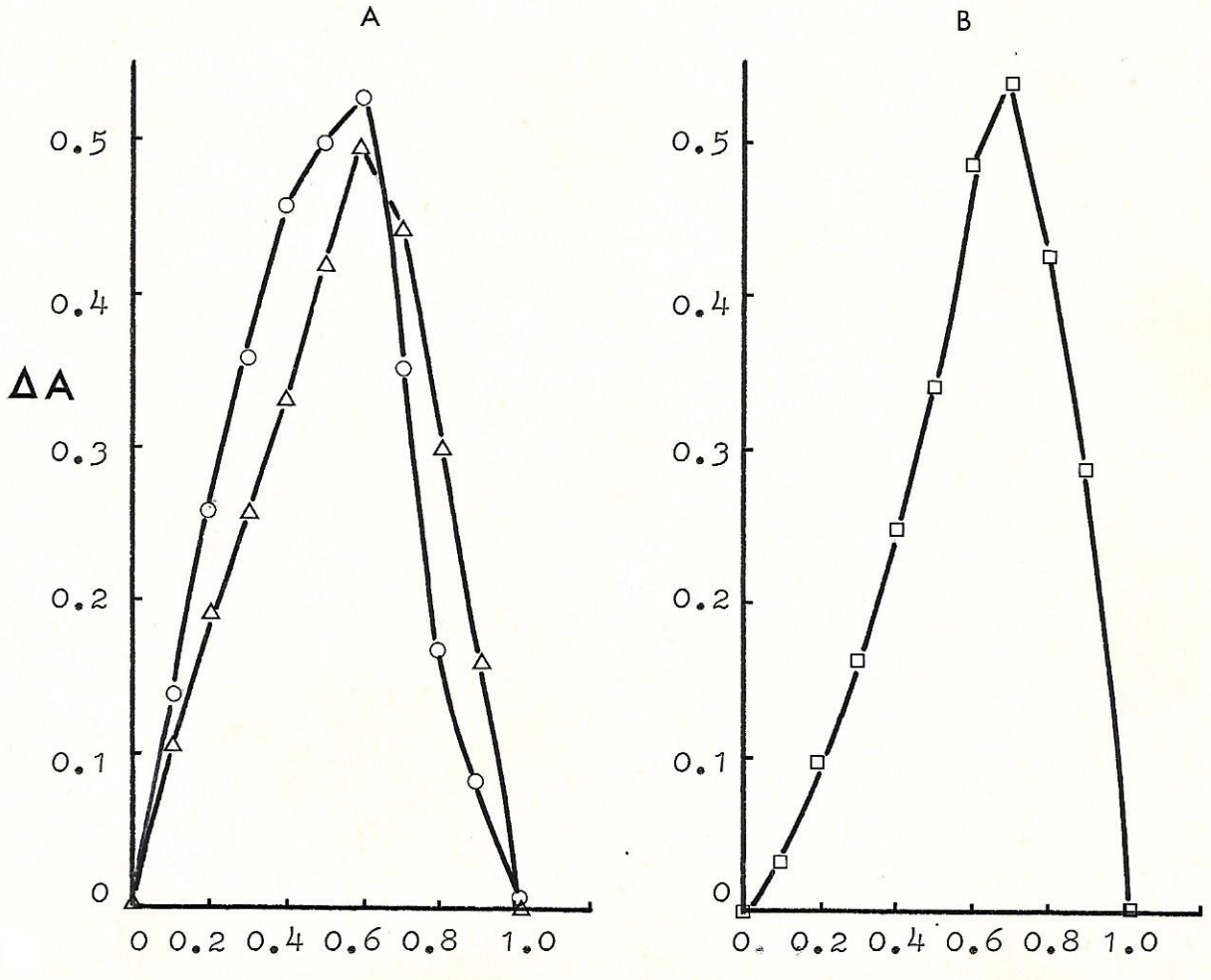

A
B
0.5
0.4
ΔA
0.3
0.2
0.1
0
0 0.2 0.4 0.6 0.8 1.0
0 0.2 0.4 0.6 0.8 1.0
Molar Fractions

Fig. 3.6

pH Titration of L_4-Fe*

Vertical Axis: Change in Absorbance[a] at 400 nm.

Horizontal Axis: pH.

L_4 : Fe = 3:1

* In 50% DMSO. Precipitation at neutral and alkaline pH.

Job's plots of L_4-Fe at pH 3.35 (A) and pH 7 (B).

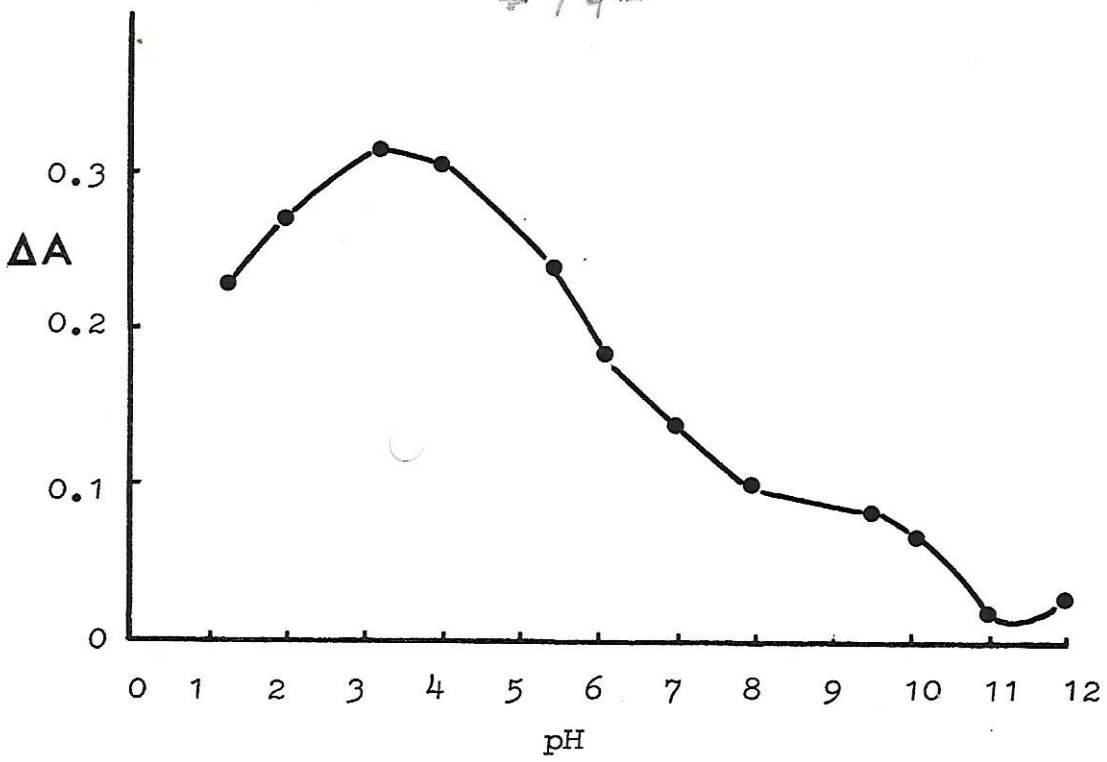
Vertical Axis: Change in Absorbance[a] at 445 nm (□), (△).

Horizontal Axis: Molar Fraction of ligand.

$L_4 = 6 \times 10^{-4}$ M,[b] Fe $= 6 \times 10^{-4}$ M.[b]

a = Arbitrary units.

b = Initial concentration.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0.2
0.1
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH

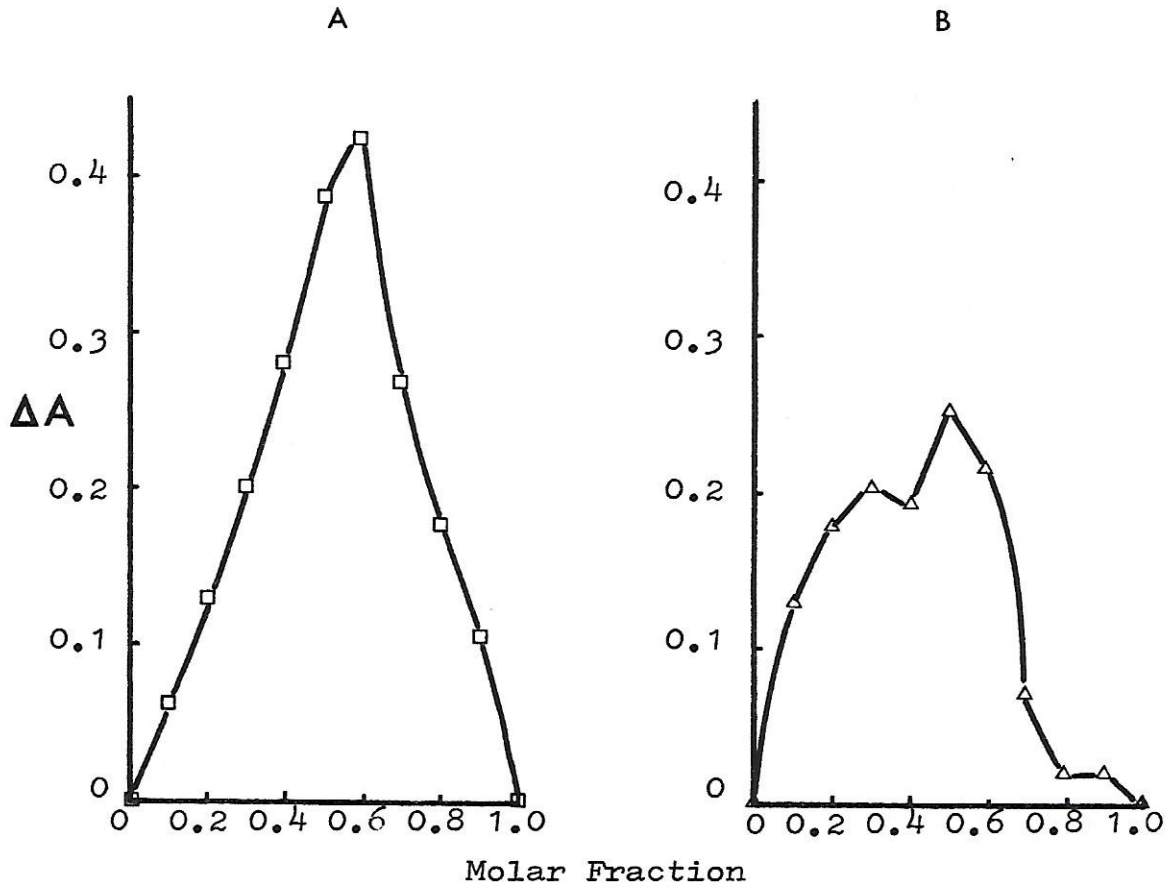

A
B
ΔA
0.4
0.3
0.2
0.1
0
0 0.2 0.4 0.6 0.8 1.0
Molar Fraction

Fig. 3.7

pH Titration of L_5-Fe

Vertical Axis: Change in Absorbance[a] at 320 nm (●) and 280 nm (■).

Horizontal Axis: pH.

$L_5 = 7.5 \times 10^{-4}$ M.[b] Fe $= 2.5 \times 10^{-4}$ M.[b]

Job's plot of L_5-Fe at pH 7.0.

Vertical Axis: Absorbance[*] at 220 nm.

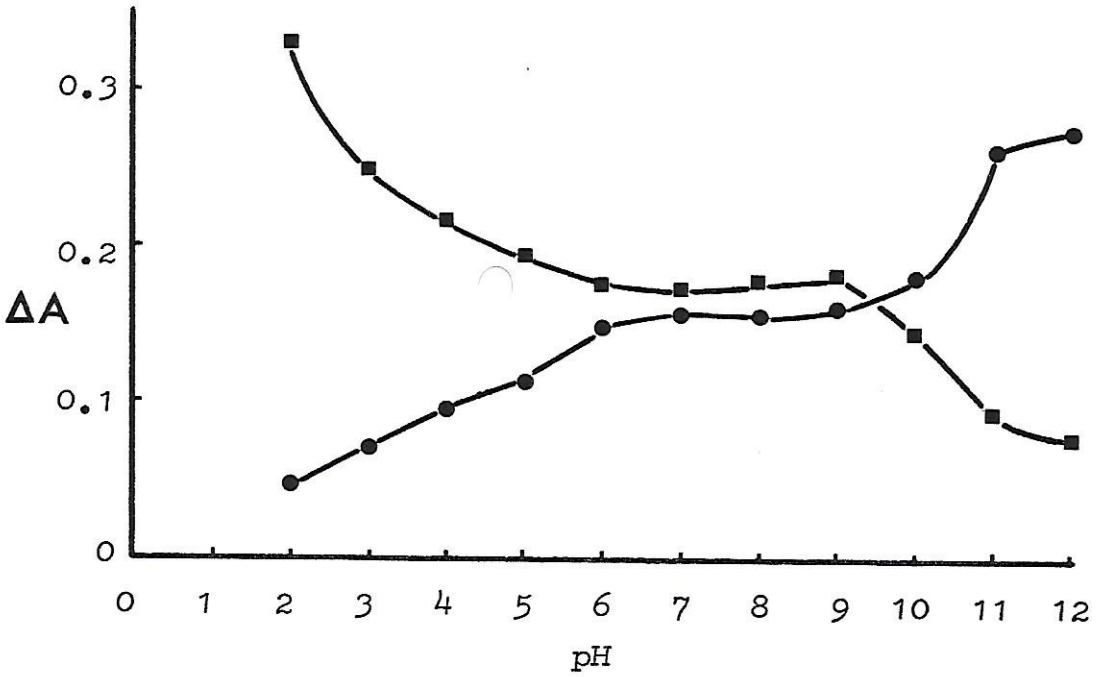
Horizontal Axis: Molar Fraction of ligand.

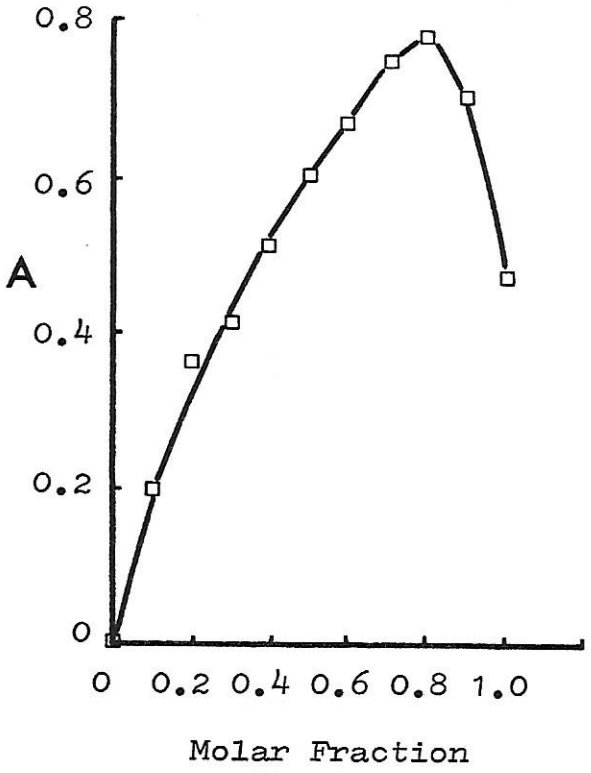
$L_5 = 1 \times 10^{-3}$ M,[b] Fe $= 1 \times 10^{-3}$ M.[b]

[*] 1 mM path length cuvette.

a = Arbitrary units.

b = Initial concentration


0.3
0.2
ΔA
0.1
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH


0.8
0.6
A
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1.0
Molar Fraction

L_6-iron(III) reactions

The pH titration and Job'splots of the L_6-iron(III) complex illustrated in Fig.3.8 indicate a L:Fe molar ratio of 3:1 over a wide range of pH (4-9). The Job's plot at pH 2.3 seems to be composed of two species of complexes of 2:1 and 3:1. There was some scattering in the absorption spectra studies.

L_7-iron(III) reaction

The pH titration of L_7-iron(III) complex is very similar to that of L_6. However although at acidic pH 2 a 2:1 complex seems to predominate, at pH 7.4 there is an unusual Job's plot probably of a mixture of 1:1 and 3:1 complexes. (Fig. 3.9)

3.4 Discussion of the Inorganic Reactions of the Ligands

In this chapter the ability of the various ligands (L_1 - L_7) to form iron complexes at a physiological pH range was demonstrated and furthermore their reaction with other metals of physiological importance and protons, were briefly examined.

All the iron(III) complexes were coloured, stable in acidic, neutral and basic media, furthermore, they are water soluble except for that of L_4 which precipitated on standing. The Job's plots and pH titration profiles (Figs. 3.3 - 3.9) indicated the existence of a neutral 3:1 complex

Fig. 3.8

pH Titration of L_6-Fe

Vertical Axis: Change in Absorbance[a] 450 nm.

Horizontal Axis: pH

L_6 = 0.5 x 10^{-3} M,[b] Fe = 7.5 x 10^{-4} M.[b]

Job's plot of L_6-Fe at pH 7.4 (A) and pH 2.3 (B).

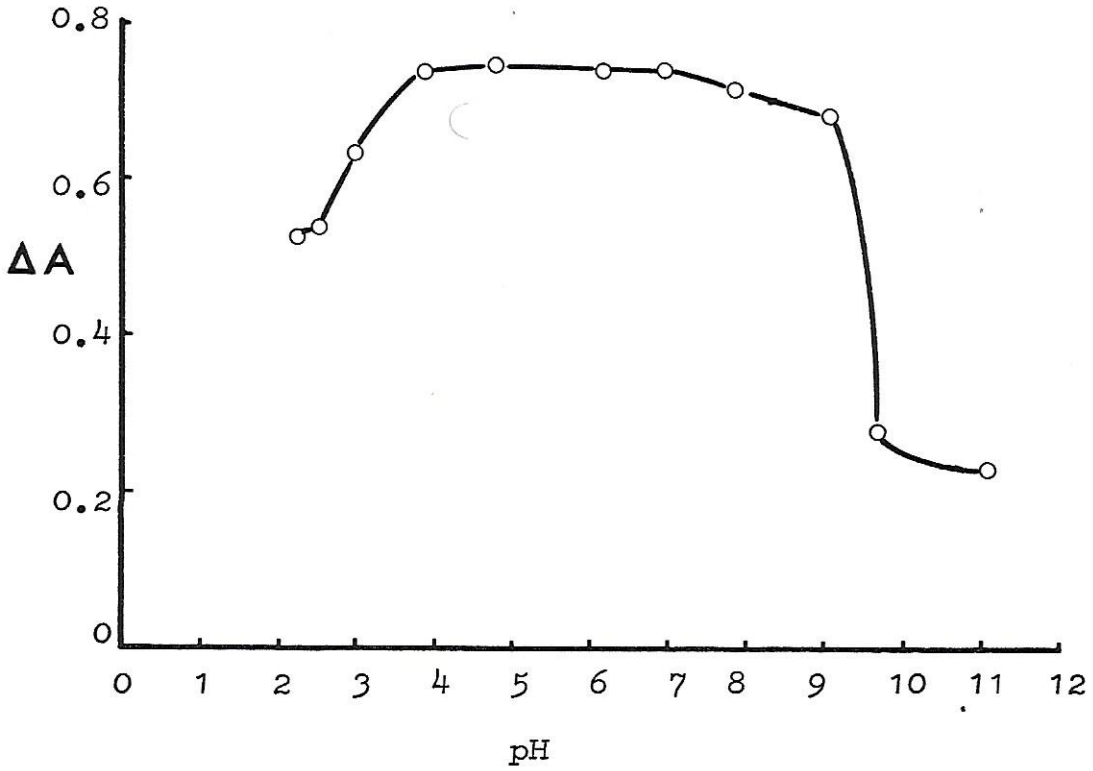
Vertical Axis: Change in Absorbance[a] at 420 nm (Δ) and 460 nm (□).

Horizontal Axis: Molar Fraction of ligand.

L_6 = 4 x 10^{-4} M,[b] Fe = 4 x 10^{-4} M,[b] $NaClO_4$ = 0.1 M.

a = Arbitrary units.

b = Initial concentration.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ΔA
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH

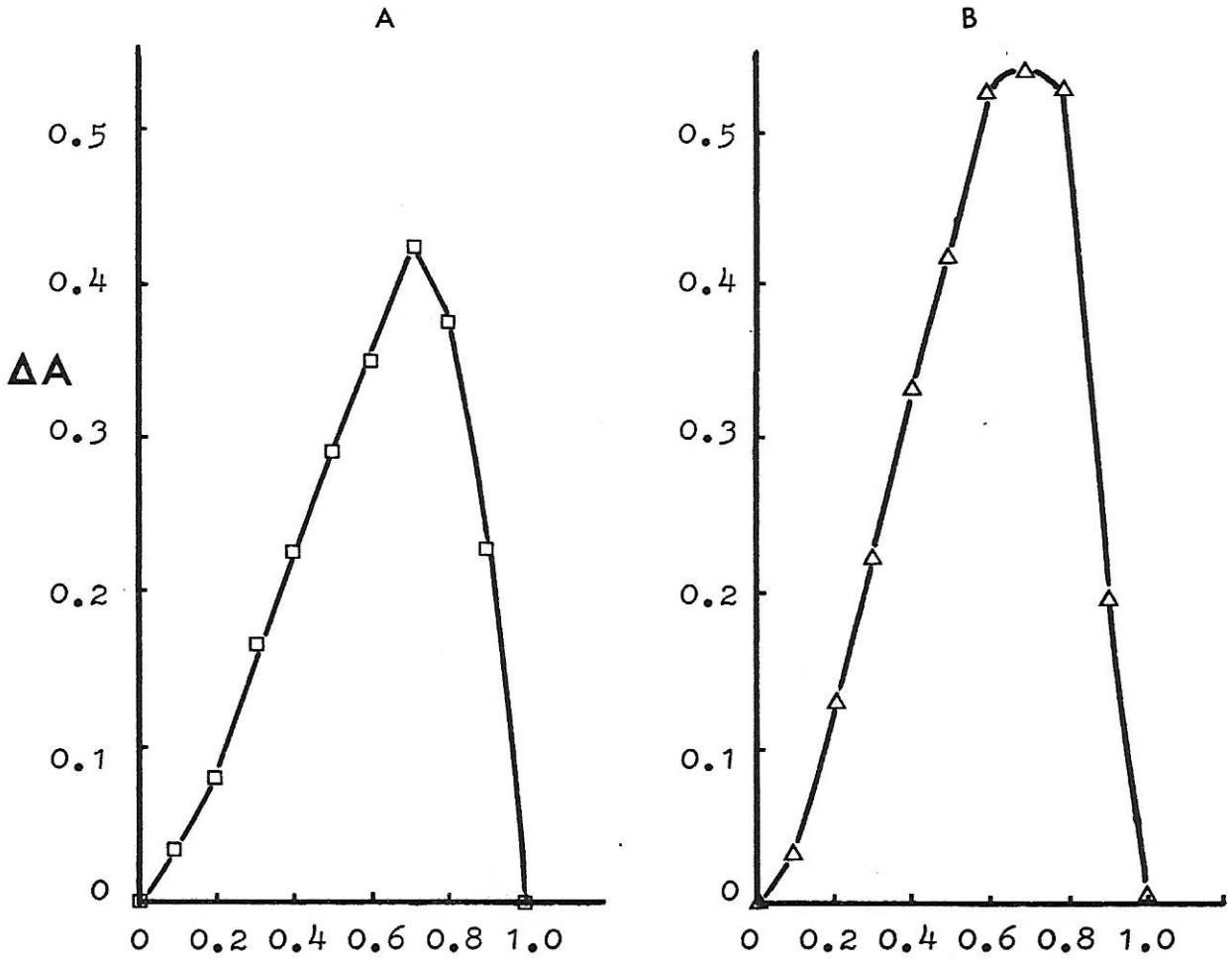

A
B
0.5
0.4
ΔA
0.3
0.2
0.1
0
0 0.2 0.4 0.6 0.8 1.0
Molar Fractions

Fig 3.9

pH Titration of L_7-Fe

Vertical Axis: Change in Absorbance[a] at 400 nm.

Horizontal Axis: pH.

L_7 : 8×10^{-4} M,[b] Fe = 2×10^{-4} M,[b] $NaClO_4$ = 0.1M

Job's plots of L_7-Fe at pH 7.4 (A) and pH 2.0 (B)

Vertical Axis: Change in Absorbance[a] at 450 nm* (□) and 530 nm (△).

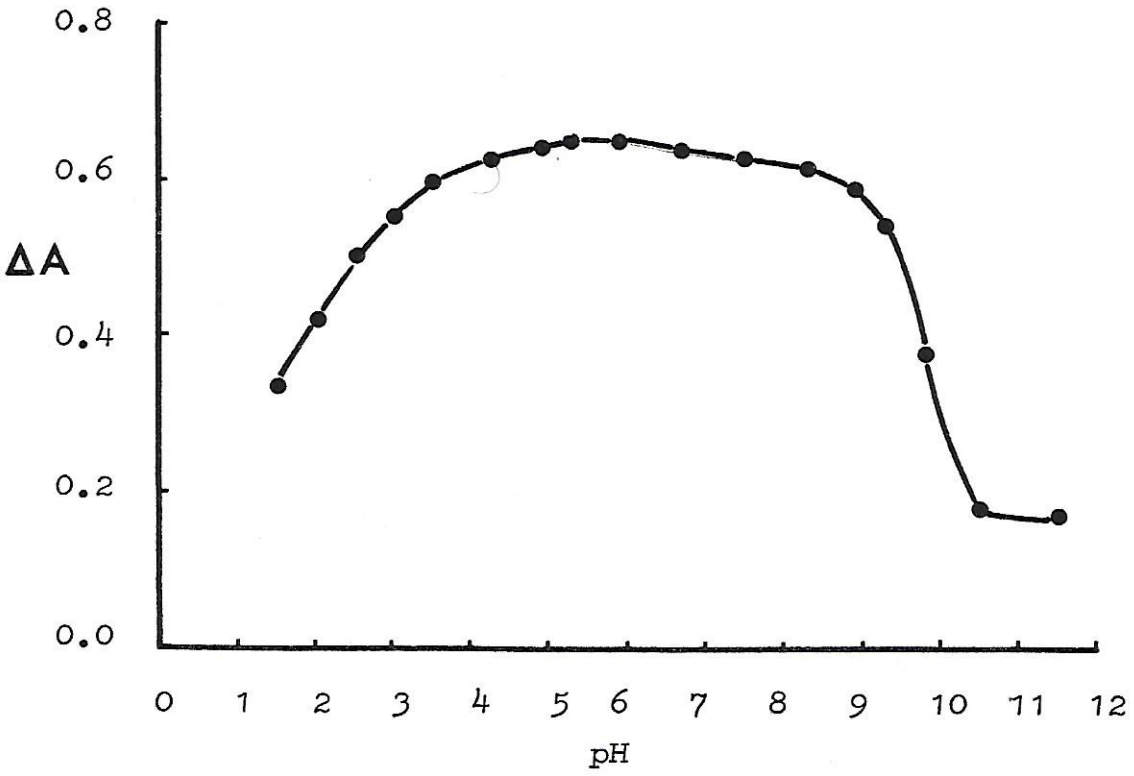
Horizontal Axis: Molar Fraction of ligand.

L_7 = 1×10^{-3} M,[b] Fe = 1×10^{-3} M,[b] $NaClO_4$ = 0.1M.

* The mixture was left for 25 h for suspensions to precipitate.

a = Arbitrary units.

b = Initial concentration.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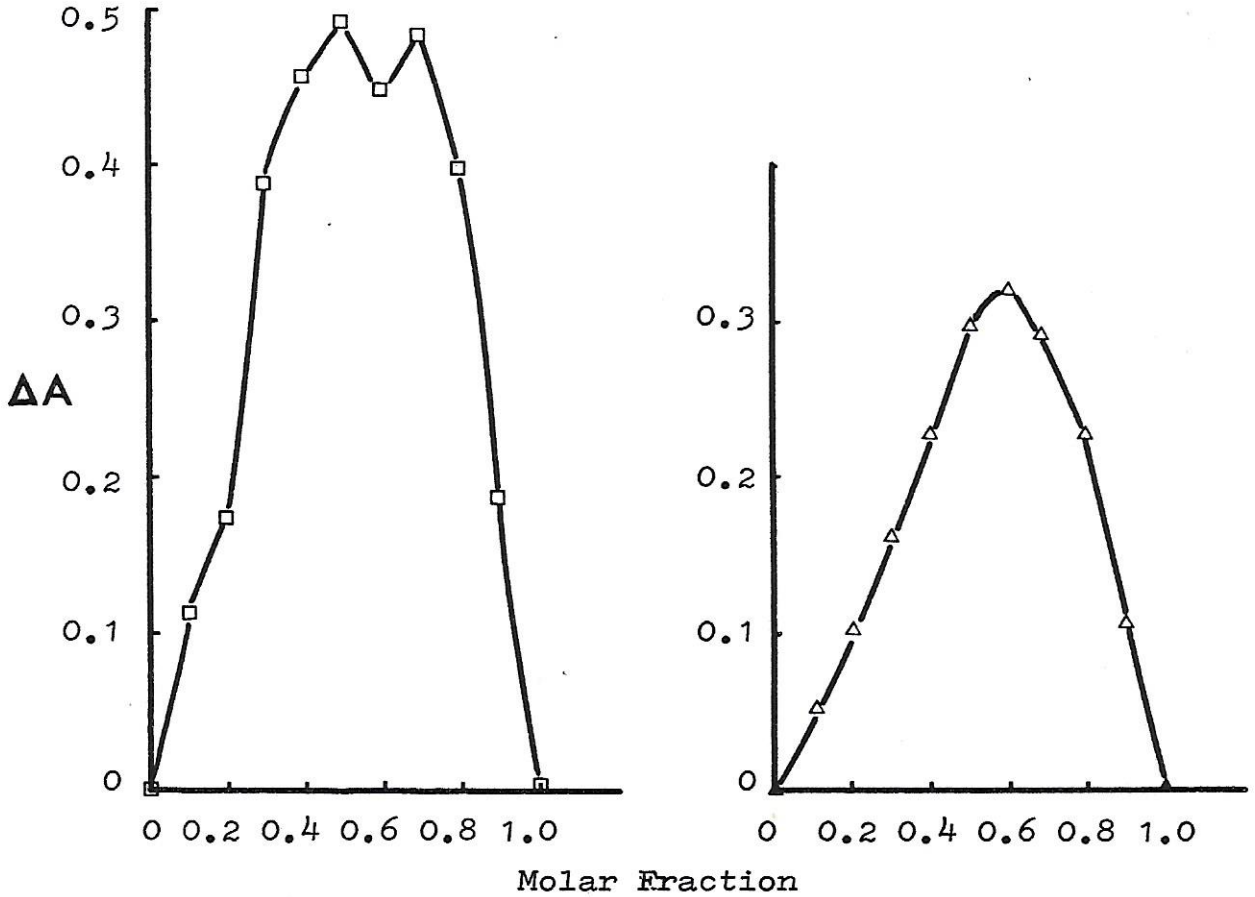
0.6
ΔA
0.4
0.2
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12
pH

A

B


0.5
0.4
0.3
ΔA
0.2
0.1
0
0 0.2 0.4 0.6 0.8 1.0
0.3
0.2
0.1
0
0 0.2 0.4 0.6 0.8 1.0
Molar Fraction

over a wide pH range including the physiological pH, with the single exception of L_2 which seemed to form a mixture of 3:1 and 2:1 complexes at this pH. The Job's plots at the acidic pH s studied, indicated the existence of charged iron(III) complexes, predominantly 1:1 with ligands L_1 and L_2 and 2:1 with the pyridine-1-oxide ligands (L_3, L_4, L_6 and L_7), as shown in Table 3.4.

The pK_a estimation of the hydroxyl co-ordinating group of the ligands revealed a general difference between the studied pyridine-1-oxide derivatives (L_3, L_4, L_6 and L_7) and the other pyridone ligands (L_1 and L_2). This difference arises presumably from the electron attracting effect of the nitrogen in the $-\overset{+}{N}-\bar{O}$ (1-oxide) group in the former. The electronic effects on the pK_a and particularly on the chelation properties of the ligands was also demonstrated by the substituent effect. Thus, whereas the electron withdrawing effect of the nitro group in the 2-hydroxy-pyridine-1-oxide derivatives caused substantial decrease in the pK_a and diminished the chelation properties of the binding site, the other substituents (-OMe and -OH) caused an increase in the pK_a and corresponding affinity for iron.

Other information arising from the pK_a studies is that L_1, L_2 and L_5 would be mostly neutral at physiological pH whereas the other ligands would be charged (Table 3.3). Thus the former ligands are expected to permeate membranes, depending on their lipophilicity (Chapter 4), but the latter ligands are not.

In the preliminary study of the reaction of the ligands with the other metals a complex formation was generally observed with Zn(II) and Cu(II) but not with Ca(II) and Mg(II). Thus unlike DTPA, minimal Ca(II) interference is expected in the iron(III) binding properties of the ligands *in vivo*. Although in iron overload, iron will be present in excess and thus it will be expected to be preferentially sequestered than other metals, the binding of Cu(II) and Zn(II) by these ligands, which is not an unexpected property, shows the need of a much deeper and detailed study of these and other metal reactions of physiological importance, with these new chelating agents.

The iron(III) binding constants of the ligands calculated under certain assumptions are large enough to predict, on thermodynamic grounds iron(III) removal from transferrin (except L_5) and ferritin (Table 3.5). It would be useful to examine the validity of these assumptions by estimating the binding constants under conditions favourable for complex formation, i.e. in the absence of interfering ions.

The entire method and approach of iron chelation *in vitro* at physiological pH together with that of the other metals, and the pK_a estimation carried out in this project, could be used as a preliminary screening programme for iron chelators *in vitro*. The technique is rapid, easy and cheap and would be a worthwhile preliminary exercise before the introduction of the relatively expensive protein and animal experiments.

TABLE 3.5

The Iron(III) Binding Constants of the Ligands

LIGAND	log β_3	log K_{eff}	log K_{sol}
L_1	34.5	19.2	9.6
L_2	33.5	20.0	10.1
L_3	29.9	21.5	9.9
L_5	29.7	17.7	8.0
L_6	29.3	20.9	11.3

CHAPTER FOUR

MEMBRANE PERMEABILITY TOWARDS IRON COMPLEXES

4.1.1 Introduction

One of the desired properties of the new chelators was the ability of both the ligand and its iron complex to permeate membranes. Thus in theory a neutral ligand could diffuse through a phospholipid membrane, form a neutral complex, with iron stored in the cell, and then diffuse out. This process will be repeated until an equal distribution of the iron complex inside and outside of the cell is observed, i.e. when equilibrium is established.

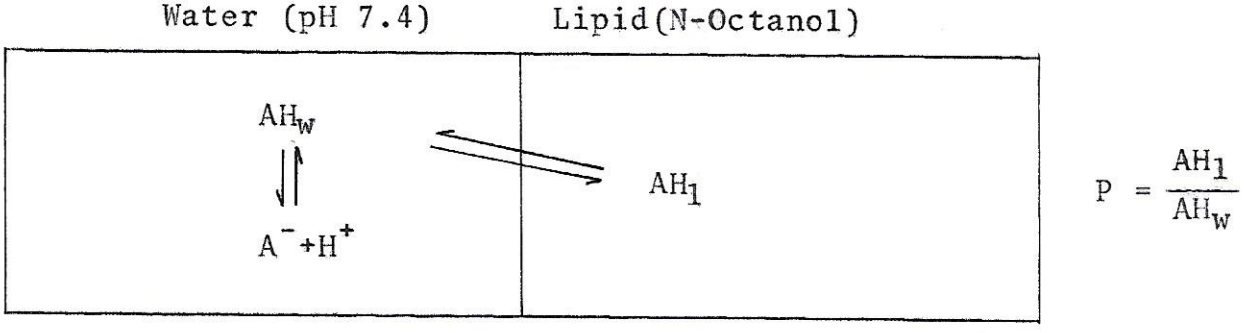
The ability of an iron(III) complex to permeate membranes is directly related to other aspects of iron metabolism, namely the physiology of iron absorption. Although this is not the subject of this study, the findings from such experiments could be of great relevance to the treatment of iron deficiency anaemia[66] by supplementation of iron(III) complexes of these ligands. Furthermore other ligands which chelate iron but their complex does not diffuse across the gut could be used for the treatment of acute iron poisoning of ingested iron and also for minimising increased iron absorption in cases such as idiopathic haemochromatosis and β thalassaemia intermediate.

4.1.2 Permeability of Red Blood Cells

A useful model for studying cell membrane permeability is the red blood cell (RBC) which is readily available, stable on centrifugation and damage is easily detected by the escape of haemoglobin.[67]
Although it was shown in Chapter Three that almost all of the ligands form neutral iron complexes at physiological pH, the permeability of non-electrolyte (neutral) molecules across the RBC membrane is determined by many variables including lipoid solubility, molecular weight and the presence of steric and electronic substituents, the dominant variable however being lipoid solubility.[68]

In this work lipophilicity has been studied within the context of a partition coefficient. The partition coefficient (P) of a compound AH is defined as the ratio of the concentrations of the unionised species of AH in lipid and water as shown in Scheme 4.1.[69]

Scheme 4.1



Membranes in general hinder the passage of ions but permit that of neutral molecules with a rate depending on their lipophilicity. Thus the higher the lipid/water partition

coefficients the faster they diffuse. In a preliminary study the partition coefficients of the iron complexes were determined by measuring their distribution in water and n-octanol.

The procedure which was used to study the RBC permeability of the ligand iron complexes is similar to that used by Young, S. et al[70] where the ability of ionophores to carry iron(II) across the same system was demonstrated. In this project a ligand complex co-ordinated to ^{59}Iron(III) was prepared, incubated with RBCs and the amount of radio-activity incorporated was monitored at different time intervals. The ligand to iron(III) concentration ratio was varied during this work.

This is the first time that iron(III) chelators were used to monitor the permeability of RBCs to iron(III) complexes. DFB and 2,3-dihydroxy benzoic acid (DHB) were also included in these experiments as in contrast to the ligands developed in this work, they formed charged complexes with iron.

4.2 Partition Coefficient Studies

The partition coefficients of the ligand iron(III) complexes were determined at pH 7.4 using water and n-octanol. The ligands (1×10^{-4}M) were mixed with an equal volume of aqueous $FeCl_3$ (0.33×10^{-4}M) and adjusted to pH 7.4 with Tris HCl (50mM). n-Octanol (10ml) was mixed

with an equal volume of the $Fe(L)_3$ complex and the mixture was shaken for 15 minutes. The cloudy preparations formed were centrifuged for 10 mins at 4000 rpm, samples from both the n-octanol and the water phases were taken using a pasteur pipette. These were transferred to cuvettes and their visible spectra taken. The change in absorbance (ΔA) of the $Fe(L)_3$ complex (X) at certain wavelengths, of the water phase was compared to that of a $Fe(L)_3$ complex solution unmixed with n-octanol. Assuming that a decrease in the absorbance in the water phase is due to the incorporation of an $Fe(L)_3$ complex in n-octanol and vice-versa and since the extinction coefficient of the $Fe(L)_3$ in water and n-octanol determined from the absorption spectra are approximately the same, the n-octanol/water partition coefficient (P) was measured using the following equations.

$$P = \frac{[X]^f_{\text{n-oct.}}}{[X]^f_{\text{water}}} = \frac{[X]^i_{\text{water}} - [X]^f_{\text{water}}}{[X]^f_{\text{water}}} = \frac{[X]^f_{\text{n-oct}}}{[X]^i_{\text{water}} - [X]^f_{\text{n-oct}}}$$

(A) (B) (C)

[X] is the concentration of $Fe(L)_3$

f = final concentration; i = initial concentration

A,B,C = Methods used for estimating P.

The partition coefficients of the ligands, the method and the wavelengths at which these were calculated are shown in Table 4.1.

It was observed that while the characteristic ligand iron(III) coloured complexes of L_1, L_2, L_3 and L_5 were distributed mainly in the water phase and very little in n-octanol, the coloured complex of L_6 was mainly in n-octanol. There were complications in the determination of the partition coefficient of the iron complex of L_4 due to precipitation. Almost all the precipitate was present in the n-octanol/water interphase, while the n-octanol phase was intensely yellow and the water phase slightly yellowish.

The partition coefficient results indicated wide variation in the lipid/water properties of the ligand iron(III) complexes studied. This variation is a reflection of the lipid/water properties of the heteroaromatic rings and the ring substituents. Thus in comparing the 2-hydroxypyridine-1-oxide (L_4) derivatives, the introduction of the -OH substituent (L_3) increased substantially the hydrophilicity of the complex, in contrast to the $-OCH_3$ substituent (L_6) which increased the lipophilicity. The presence of the $-CH_3$ substituent in the pyrone and pyridone ligands did not seem to increase the lipophilicity of their iron complexes. Furthermore, rather unexpectedly L_1 formed a hydrophilic iron complex in spite of the presence of two $-CH_3$ substituents in this ligand. A more detailed study

TABLE 4.1

The Partition Coefficients of the Ligand Iron Complexes

$Fe(L)_3$	P	METHOD	$\frac{\Delta A}{A_{\lambda 1}-A_{\lambda 2}}$	COMMENTS
L_1	0.05	A	$A_{475}-A_{580}$	clear solution
L_2	0.14	A,B,C	$A_{500}-A_{600}$	clear solution
L_3	0.04	A	$A_{405}-A_{580}$	clear solution
L_4	0.95	A,B,C	$A_{470}-A_{600}$	precipi-tation
L_5	0.32	A,B,C	$A_{470}-A_{600}$	clear solution
L_6	4.85	A	$A_{405}-A_{505}$	clear solution

is needed in order to correlate the effects of these and other substituents on the lipid/water properties of the ligands and their iron(III) complexes.

4.3.1 Methodology for Red Blood Cell Permeability Studies

Red blood cells (RBC) were isolated from fresh blood in all the experiments using the following method. Fresh blood in potassium EDTA (1mg/ml) was centrifuged at 3000 rpm for 5 min. using an MSE Coolspin Centrifuge. The plasma and white cells were removed by aspiration leaving behind the RBC, , which were subsequently washed (3x) using equal volumes of phosphate buffered saline (PBS) at the same speed and time period.

To plastic tubes containing $^{59}FeCl_3$ (4-5µl), ligands (0.5ml, concentration as given in Table 4.2, in PBS pH 7.3 or Tris HCl, 0.1M, 0.5ml, pH 7.4) were added and the mixture was left to incubate at room temperature for 30 mins. Packed RBCs (0.5ml) were introduced into the plastic tubes containing the ligand ^{59}iron(III) complex and mixed gently. A sample (0.1ml) from each tube was taken into plastic microcentrifuged cells, silicon oil (0.15ml) was added and spun for 1 minute in a microfuge. After taking this sample, all the tubes were incubated at 37° in a shaking water bath. Subsequently further samples (0.1ml) were then taken at different time intervals and spun through the silicon oil. All the samples in the centrifuged plastic microcentrifuged cells consisted of three layers; the top supernatant layer which was

TABLE 4.2

Experimental Conditions used to Determine the RBC permeability of the ligand iron(III) complexes

Expt	Iron	Ligand	$\frac{[L]}{[Fe]}$	Time interval min^{-1}
Expt. 1 (PBS)	136µg/ml	L_2(4mM)	165	1, 5, 10
	0.07 mCi/ml	L_5(4mM)	165	22, 40, 60
	5µl/tube	L (4mM)	165	
		DHB(4nM)	165	
		DFB (1.3mM)	495	
Expt. 2 (PBS)	10µg/ml	L_1(0.52mM)	75	1, 30, 60
	0.25mCi/ml	L_2(0.52mM)	75	120, 180
	4µl/tube	L_3(0.52mM)	75	
		L_5(0.52mM)	75	
Expt. 3 (PBS)	10µg/ml	L_1(0.5mM)	175	1, 25, 65
	0.20mCi/ml	L_3(0.5mM)	350	120, 180
	4µl/tube	DFB (0.5mM)	350	
Expt. 4 (TRIS)	10µg/ml	L_1(4mM)	2800	1, 5, 10, 16
	0.39mCi/ml	L_3(4mM)	2800	30, 45, 60
	4µl/tube	L_5(4mM)	2800	

colourless, sometimes slightly reddish due to haemolysis, the middle which was that of the oil and the bottom layer which was deep red containing the RBC. The supernatant was transferred into another empty microcentrifuge cell. PBS (0.1 ml) was then used to wash the area above the oil layer and subsequently was transferred to the microcentrifuge tube containing the supernatant. PBS (0.1ml) was again added on the top of the oil layer and both layers were removed by aspiration leaving behind the RBC stroma.

The ^{59}iron content of both the supernatant and that of the RBC was measured using an LKB-WALLAC 1280 ultragamma counting system. For each sample the amount of ^{59}iron incorporated into the RBCs was determined by comparing the radioactivity of the RBC to that of the sum (100% ^{59}iron) of the RBC and the supernatant . An estimation of the rate of permeation of the ligand iron(III) complex was determined by plotting the percentage ^{59}iron incorporated into the RBC against time.

4.3.2 The RBC Permeability Studies of the Ligand iron(III) Complexes

The ability of the various iron complexes to permeate the red blood cell (RBC) membrane is depicted in the Figures 4.1 - 4.5. It is clear from Fig. 4.1 that L_1 and L_3 iron complexes do not permeate even after 3hrs of incubation. However, there was some permeability detected in Experiment 4, in which about 15% of the iron being accumulated with both ligands (Fig. 4.1). This difference

Fig. 4.1

Red Blood Cell (RBC) Permeability studies of the L_1-iron (III) complex (A) and L_3-iron (III) complex (B).

Vertical Axis: Percentage ^{59}iron in the RBC

Horizontal Axis: Time (minutes).

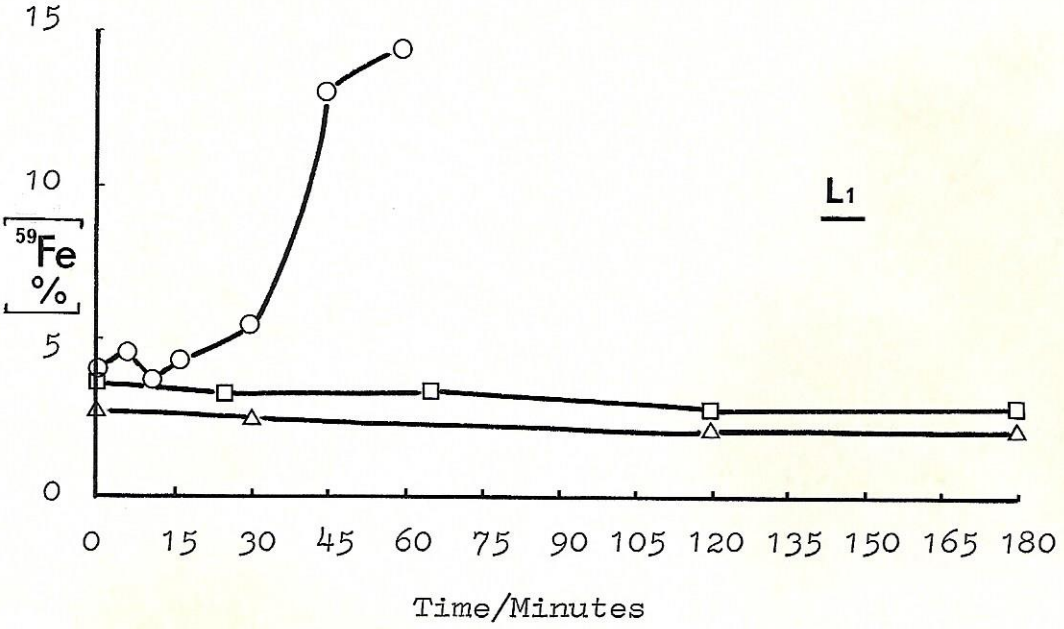
Percentage ^{59}iron is the amount of ^{59}iron incorporated into the RBC at time X compared to the sum of ^{59}iron in the RBC and the supernatant (100%) at this time.

The experimental conditions are described in Table 4.2

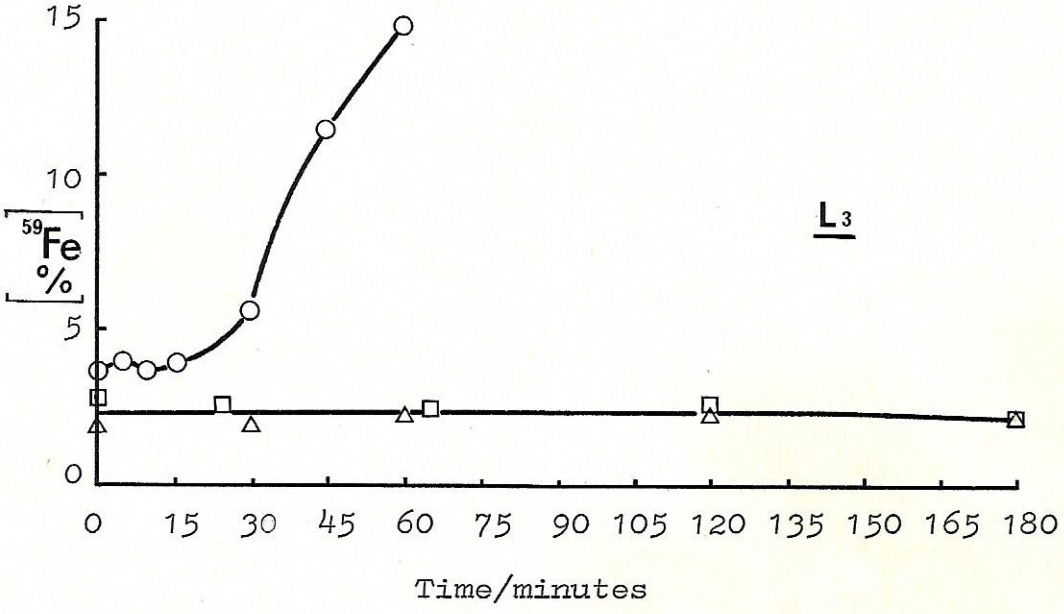
L_1: Expt. 2 (△), Expt. 3 (□), Expt 4. (○).

L_3: Expt. 2 (△), Expt. 3 (□), Expt. 4 (○).

A


15
10
59Fe %
5
0
L1
0 15 30 45 60 75 90 105 120 135 150 165 180
Time/Minutes

B


15
10
59Fe %
5
0
L3
0 15 30 45 60 75 90 105 120 135 150 165 180
Time/minutes

is probably a result of the use of the Tris-HCl as opposed to PBS buffer. The permeability of the L_2 iron complex (Fig. 4.2)proceeds slowly, thus its incorporation into the RBC after 3 hrs of incubation was estimated to be approximately 25%. The rate profiles in experiments 1 and 2 are similar. The L_5-iron complex experiments (Fig. 4. 3) show that this complex readily permeates the RBC membrane, reaching an equilibrium within an hour. The L_6-iron complex diffused through the RBC membrane and reached equilibrium very rapidly as it can be seen from Fig. 4.4. This process took approximately 10min to reach completion and thereafter the level of the ^{59}iron complex was maintained, approximately 50% for another 50 mins.

Two other compounds which were tested under the same conditions were DFB and DHB. The iron complexes of both of these two compounds failed to permeate the RBC membrane. In experiment 1 both these complexes appeared to associate with the RBC at the onset of the experiment. This binding gradually decreased and is completely lost after 20 mins. The DFB iron complex was not incorporated in the RBC even after 3 hrs of incubation (Fig.4.5, experiment 3).

4.4 Discussion

4.4.1 Factors Affecting the Membrane Permeability of Iron

Unlike most other metals and nutrients, the regulation of iron in the body depends mainly on changes in iron absorption, because excretion is limited. The

Fig. 4.2

Red Blood Cell (RBC) permability studies at the L_2-iron (III) complex.

Vertical Axis: Percentage ^{59}iron in the RBC

Horizontal Axis: Time (minutes)

Percentage ^{59}iron is the amount of ^{59}iron incorporated into the RBC at time X compared to the sum of ^{59}iron in the RBC and the supernatant (100%) at this time.

The experimental conditions are described in Table 4.2.

Expt. 2 (□), Expt. 1 (○).

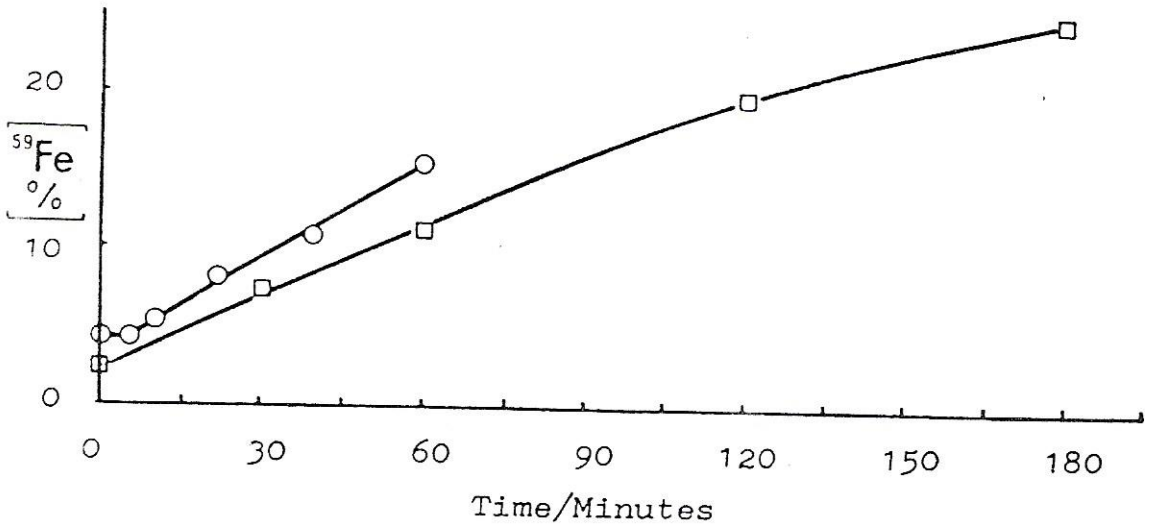


Fig. 4.3

Red Blood Cell (RBC) Permeability Studies of the L_5-iron complex.

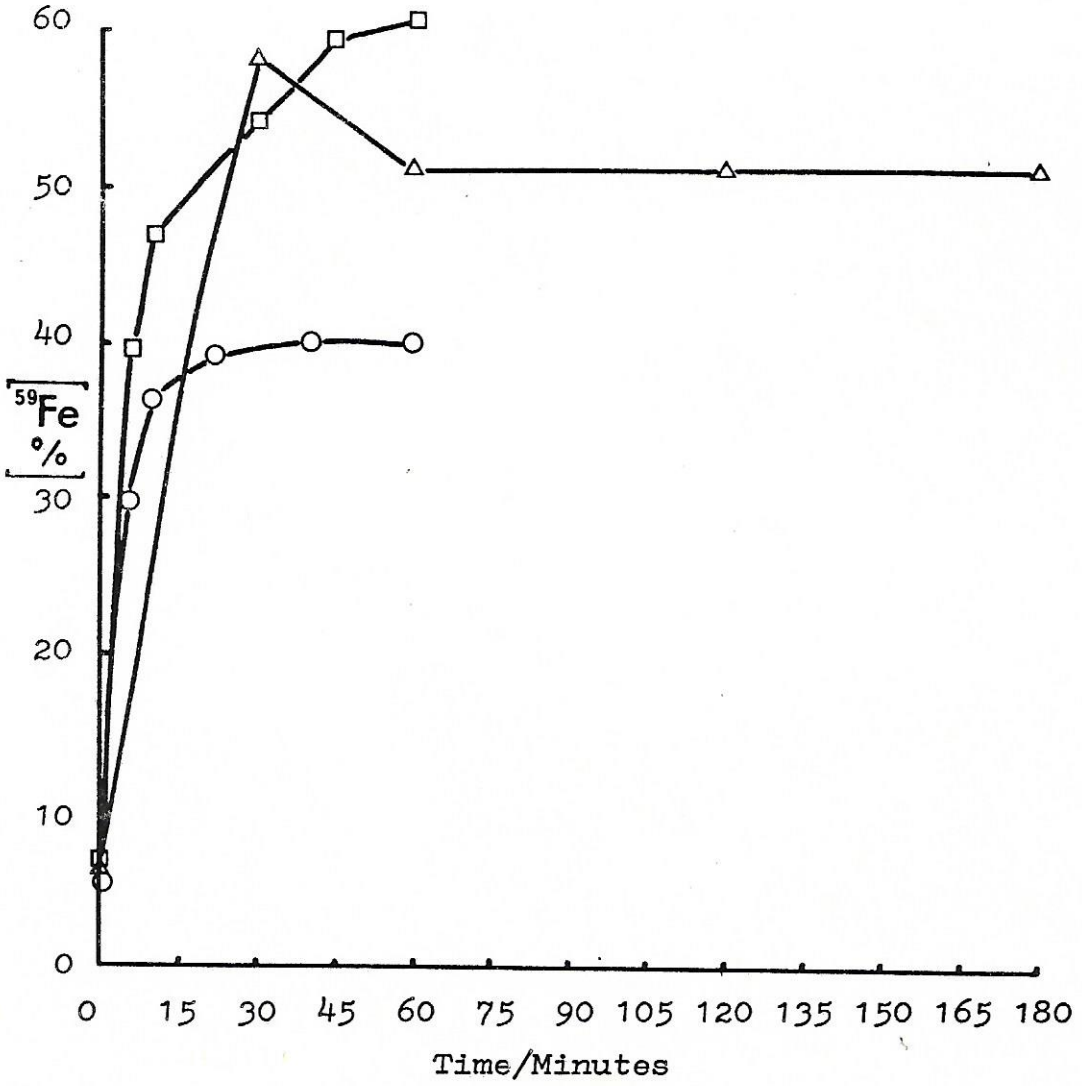
Vertical Axis: Percentage ^{59}iron in the RBC

Horizontal Axis: Time (minutes).

Percentage ^{59}iron is the amount of ^{59}iron incorporated into the RBC at time X compared to the sum of ^{59}iron in the RBC and the supernatant (100%) at this time.

The experimental conditions are described in Table 4.2.

Expt. 1 (○), Expt. 2 (△), Expt. 4 (□).



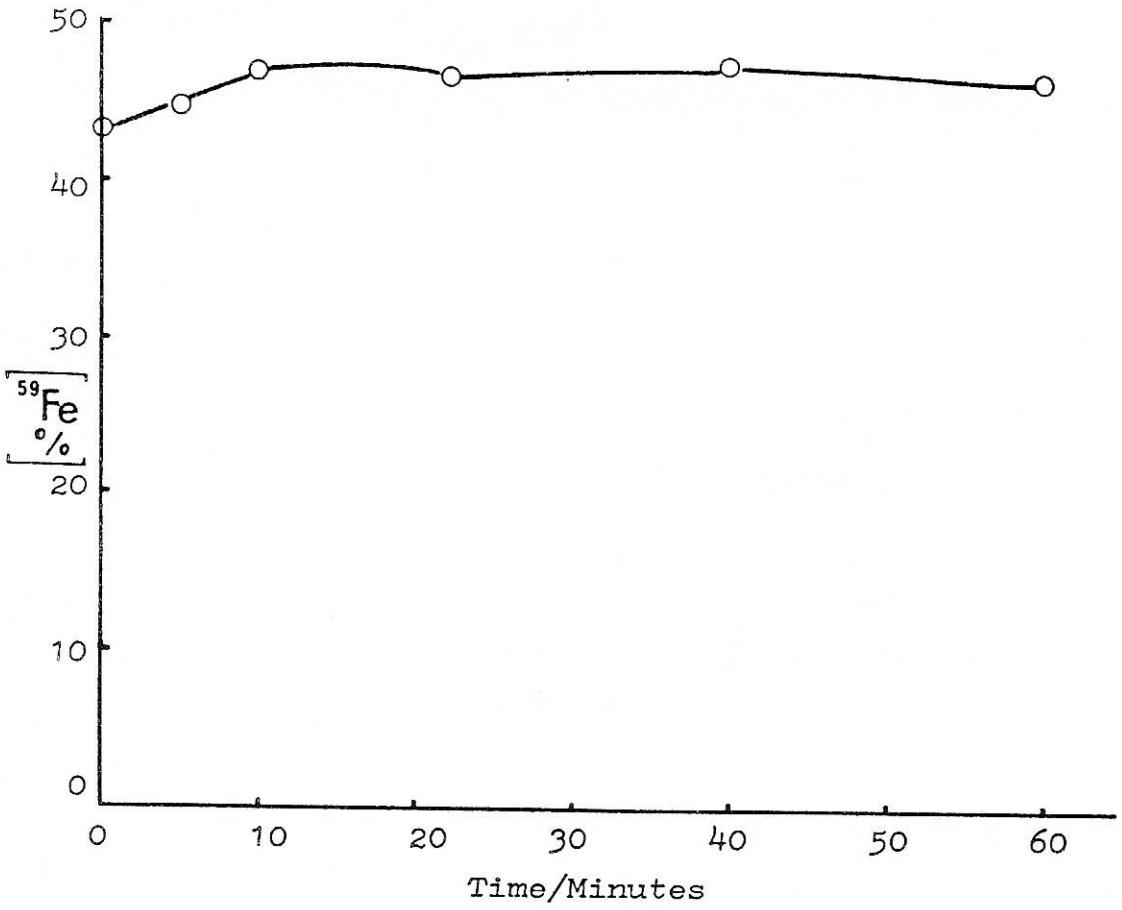


Fig. 4.4

Red Blood Cell Permeability Studies of the L_6-iron (III) complex.

Vertical Axis: Percentage ^{59}iron in the RBC

Horizontal Axis: Time (minutes).

Percentage ^{59}iron is the amount of ^{59}iron incorporated into the RBC at time X compared to the sum of ^{59}iron in the RBC and the supernatant (100%) at this time.

The experimental conditions are described in Expt1 Table 4.2

Fig. 4.5

Red Blood Cell Permeability Studies of DFB-iron (III) complex (A) and the DHB-iron (III) complex (B)

Vertical Axis: Percentage ^{59}iron in the RBC

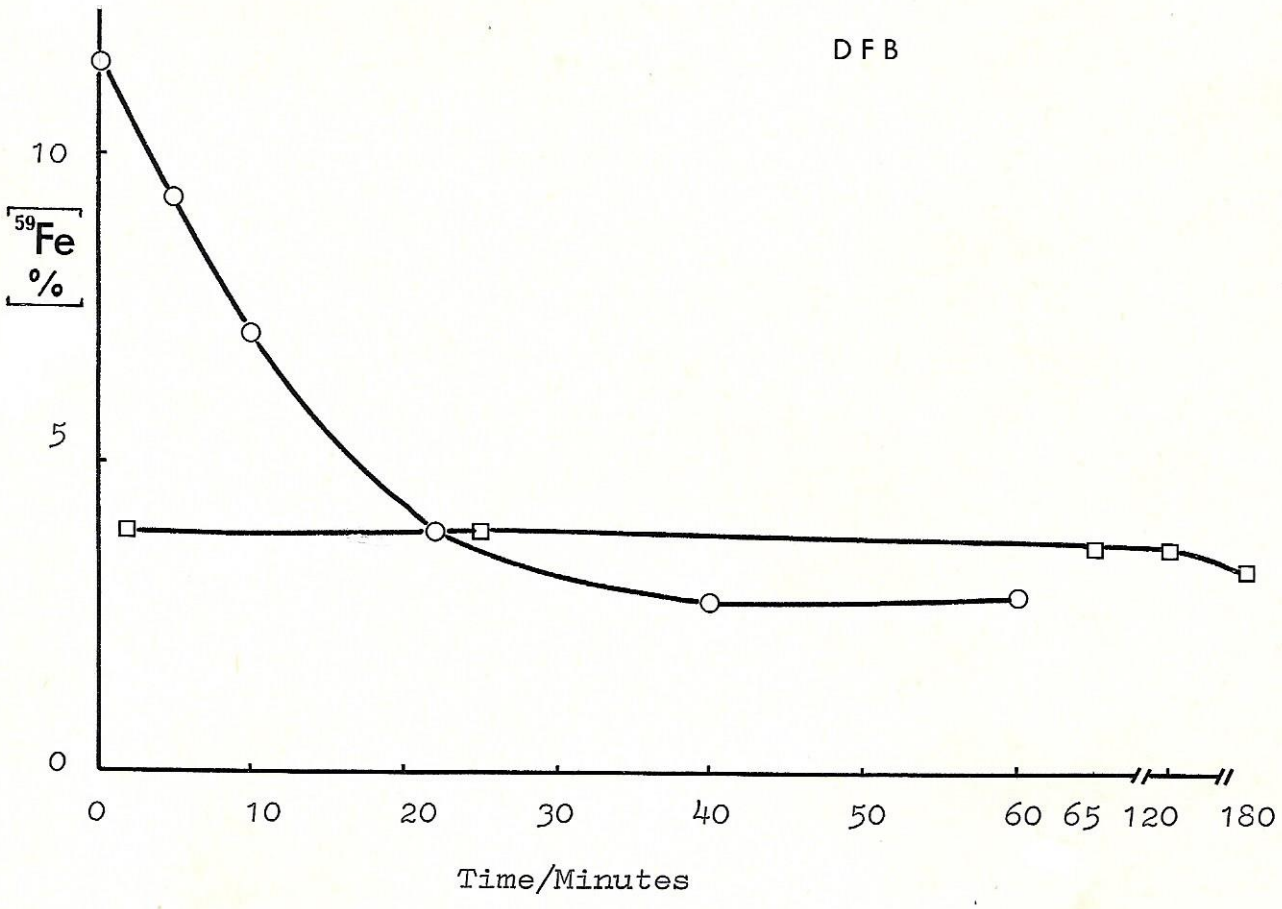
Horizontal Axis: Time (minutes).

Percentage ^{59}iron is the amount of ^{59}iron incorporated into the RBC at time X compared to the sum of ^{59}iron in the RBC and the supernatant (100%) at this time.

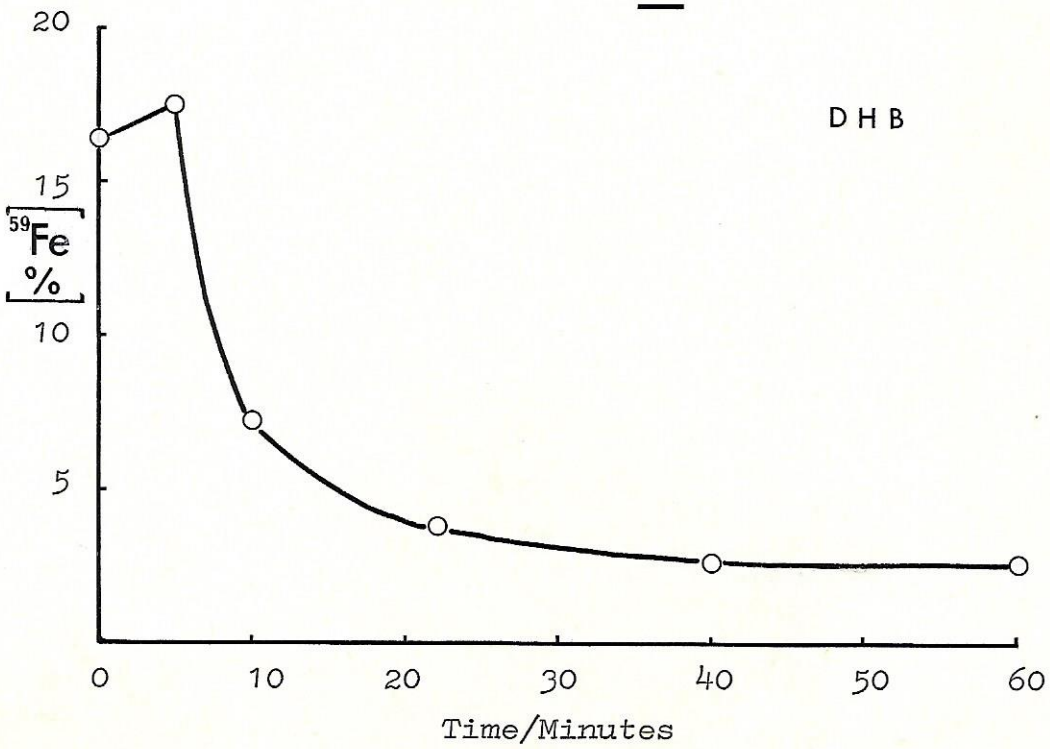
The experimental conditions are described in Table 4.2

Expt. 1 (○), Expt. 3 (□).

A


DFB
10
59Fe %
5
0
0
10
20
30
40
50
60
65
120
180
Time/Minutes

B


DHB
20
15
59Fe %
10
5
0
10
20
30
40
50
60
Time/Minutes

inability of the body to absorb adequate amounts of iron causes iron deficiency anaemia, whereas its inability to excrete iron causes iron overload, mainly in cases of primary and secondary haemochromatosis. The problem associated with iron absorption and excretion is the highly insoluble nature of iron(III) at physiological pH [$Fe(OH)_3$ $K_{sp} = 10^{-39}$]. Solubilisation of iron can be achieved by reduction [$Fe(OH)_2$ $K_{sp} = 10^{-15}$] in which case the absorption and excretion processes could be facilitated. Ascorbic acid, for example, which is both a reducing agent and a chelator[71] increases iron absorption when it is added to food and iron excretion when it is coadministered with DFB, which is the present therapy regime used for the treatment of iron overload in β thalassaemia patients. In addition, the current iron supplement tablets used are mainly ferrous salts of compounds such as fumarate, gluconate, succinate and sulphate.[72]

Another way of solubilising iron, is by using ferric chelators capable of forming water soluble monomeric complexes at physiological pH. It was found, however, that when two such compounds,[6] namely EDTA and DFB, were supplied to different forms of dietary iron, the absorption of this metal was unexpectedly reduced. In contrast, the lipid soluble chelate ferrocene was absorbed even faster than iron(II). Thus water/lipid solubility seems to be a determining factor in the permeability of cell membranes by iron complexes.

The estimated partition coefficients of the iron(III) complexes of the ligands, L_3, L_1, L_2, L_5, L_4 and L_6 depicted in Table 4.1 indicate a range of values of increasing lipophilicity. The L_6 and L_4 iron complexes seem to be the most lipophilic and they could probably partition into membranes at physiological conditions with undesirable toxic effects, because of their rather high partition coefficient. L_5 and L_2 iron(III) complexes are less lipophilic compared to that of L_4 with partition coefficients of 0.32 and 0.14 respectively. L_5 iron(III) complex will therefore be expected to permeate membranes faster than those of L_2.

4.4.2 Red Blood Cell and Jejunum Permeability by the Ligand Iron(III) Complexes

There is a great variation in the RBC permeability of the various ligand complexes as depicted in Figs.4.1 - 4.5. L_1, L_3, DFB and DHB iron(III) complexes did not permeate the RBC membrane even after three hours of incubation whereas those of L_2, L_5 and L_6 did so with different rates. These results are not a reflection of changes in experimental conditions because in these experiments (see Table 4.2) some ligands did permeate the RBC membrane repeatedly whereas others, under the same experimental conditions, did not. This variability could not also be due to size or charge differences because all the new ligands have approximately the same molecular weight and form neutral complexes at pH 7.4. L_2 and DFB could form partially and

DHB completely charged iron(III) complexes at physiological pH. Thus poor permeability of RBC membrane by these complexes may be the result of charge.

L_6, L_5 and L_2 iron(III) complexes were incorporated into the RBCs at decreasing rates respectively. This seems to reflect the order of the lipophilicity of their iron(III) complexes. Indeed, the 4-OCH_3 substituent group in L_6 was found to be highly lipophilic, thus causing fast incorporation of its iron(III) complex into the RBCs. L_5 and L_2 iron(III) complexes were incorporated into the RBCs with rates reflecting differences in their partition coefficients. In contrast, L_1, L_3, DHB and DFB iron(III) complexes failed to permeate, probably due to their low lipophilicity. This observation is further substantiated by a recent experiment in which the uptake by and transfer across rat jejunum of the iron complexes of L_2, L_5 and L_6 was measured (Table 4.3) by monitoring the ^{59}iron incorporation at 30° for 90 min of the ligand iron complexes (1×10^{-4} M), in which the ligands were present in a six molar excess over iron. From these results the L_6-iron(III) complex seems to be partitioning in the intestinal tissue to a much greater extent than the L_5 and L_2 iron(III) complexes, a reflection again of their partition coefficient values. Furthermore, the increased iron transferred across jejunum by these ligands compared to that associated with citrate and catechol renders the possibility of some of these ligands being used in the treatment of iron deficiency anaemia. The most promising possibly being maltol (L_5) which is cheap, commercially available and proven non-toxic.

TABLE 4.3

Uptake and Transfer of Iron Complexes Across Rat Jejunum[c]

COMPOUND	DISTRIBUTION RATIO[a]	
	INTESTINAL TISSUE	SAC[c] CONTENTS
Citrate	0.22	0.05
L_2	0.26	0.27
L_5	0.32	0.21
L_6	0.66	0.27
Catechol	0.44	0.11
Control[b]	0.12	0.04

a = distribution ratio is defined as the concentration of iron chelate in tissue intracellular water (or sac contents) divided by the concentration in the incubation medium. Extracellular space of the jejunum was taken as 10% and total water as 80% of tissue wet weight.

b = no compound added, only buffer (Krebs-Ringer bicarbonate).
c = inverted sac.

A project is now under way, which involves a more detailed study of the ability of the new and other related ligands to donate iron to cells. This work may be extended to cover studies on anaemic rats. [73]

4.4.3 The Significance of the Membrane Permeability by the Ligand Iron Complexes

Although more data are needed regarding the membrane permeability studies of iron(III) chelators, some general deductions and assumptions can be made at this stage;

(a) it is expected that neutral, water soluble and slightly lipophilic (P=0.3) iron(III) complexes could easily permeate membranes. It should be emphasised, however, that the permeability of the iron complexes have only been demonstrated for RBC and jejunum type membranes and the data need not necessarily be true for other cell membrane types;

(b) This study was based on the movement of iron complexes from the exterior to the interior of the cell. It is possible, however, that although certain complexes did not permeate the RBC membrane, their ligands could diffuse through the RBC or other membranes, chelate an iron molecule and diffuse out.

(c) Even if the ligands do permeate the cell membrane, other factors could influence their efficacy in vivo, for example, once they are inside the cell they

may be easily transformed, or interfere with processes associated with intracellular iron metabolism. Furthermore, their iron complex may not diffuse out of the cell.

Some other important information regarding the ligands were derived from these studies. For example, there was no haemolysis observed during the one-hour incubation RBC experiments although there was little haemolysis in all the samples in the 3h experiments. If these ligands are to be used as drugs it is essential for them to lack haemolytic properties.[74]

The results of the RBC permeability studies are of great significance to iron metabolism. It is established that some ligands which can form water soluble iron(III) complexes at physiological pH have the ability to permeate membranes and this can have a possible application in the treatment of iron deficiency anaemia.[75] The direct effect of the ligands ability to permeate membranes and to chelate iron needs to be further studied and to be expanded to include (a) other tissue cultures more suitable for iron removal studies such as Chang cells[76], hepatocytes [77] and reticulocytes.[78]

(b) iron delivery from transferrin to the haemopoietic tissues[79]

(c) microbial iron transport[80, 81, 82]

and (d) accumulation of iron to specific tissues such as heart muscle.

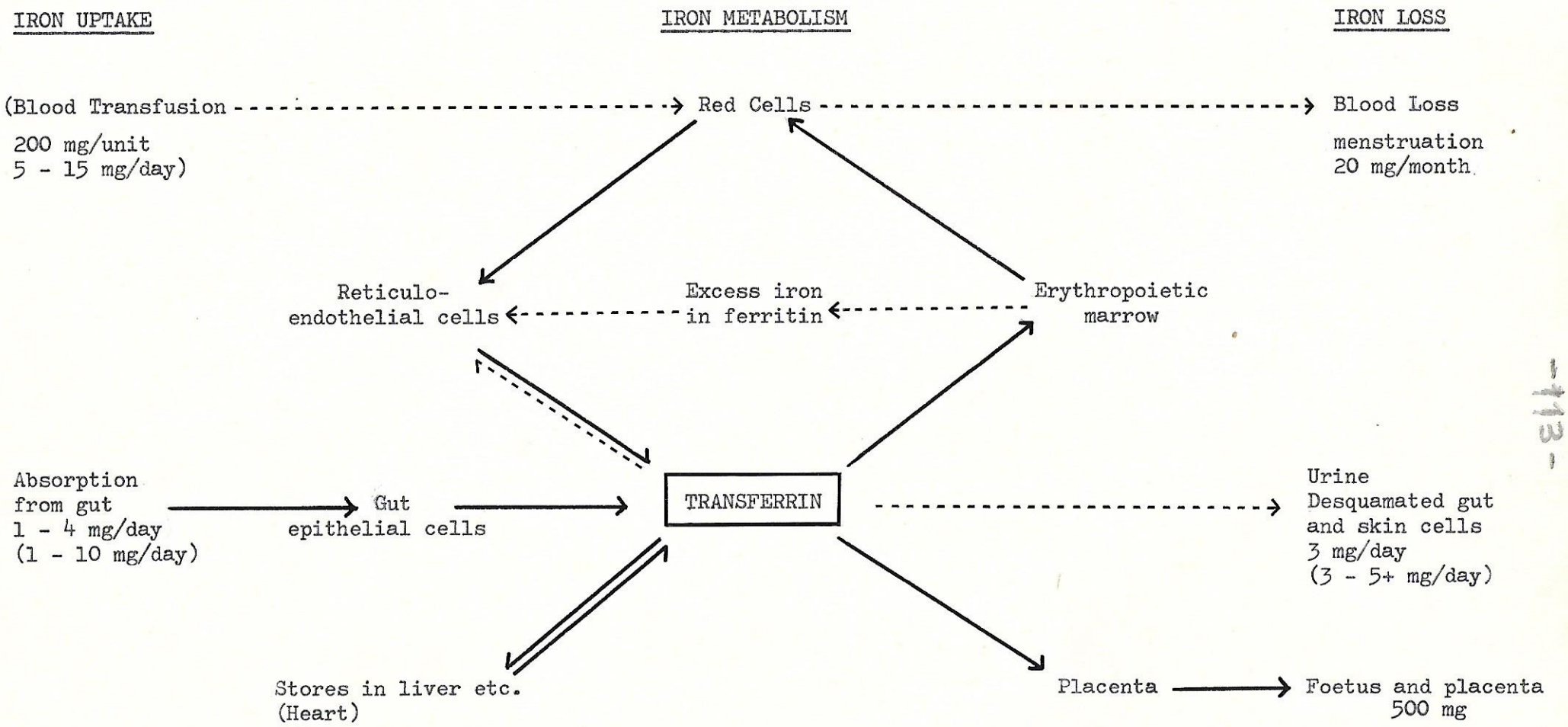


Fig. 5.1. The central role played by transferrin in iron metabolism

Brackets refer to Thalassaemia.(83)

CHAPTER FIVE

IRON MOBILISATION FROM TRANSFERRIN

5.1 Introduction

5.1.1 The Role of Iron Mobilisation from Transferrin in β thalassaemia

The purpose of this work in relation to transferrin was to establish whether the new iron chelators discussed in this thesis can sequester iron from this important protein at physiological pH. Since the present drug for the treatment of iron overload, DFB, is unable to do so, there will be additional advantages in the use of a new chelator capable of mobilising iron from transferrin. It has been suggested[16] that when transferrin is fully saturated, for example, in iron overload, excess iron leaves the bloodstream and is deposited in the tissues with harmful effects. If iron could be mobilised from transferrin there will be:

(a) much less serious iron overload and less adverse effects because it will minimise accumulation in the tissues;

(b) an additional source from which iron can be mobilised;

(c) a more efficient process of iron mobilisation because it will be faster compared to the mobilisation of polynuclear iron complexes, such as ferritin iron;

(d) apotransferrin will in effect be participating in iron mobilisation since under normal conditions it is recharged with iron 6-10 times daily, thus facilitating exchange with the tissues.

A brief account of some of the properties of transferrin and associated controversies with emphasis on iron release will be presented here. The general properties of transferrin and the discussion surrounding this subject have been well reviewed.[84, 85, 86, 87, 88, 89]

5.1.2 Transferrin Structure and Function

Transferrin is the protein involved in the transport of iron in the sites of absorption, utilisation and excretion. In doing so, it plays a vital and central role in iron metabolism. Transferrin is found in the blood of all the vertebrates examined, including mammals, birds, reptiles,amphibians and cyclostomes. It is a single polypeptide chain of molecular weight of about 80.000 and it contains 6% carbohydrate. Apotransferrin binds two molecules of iron(III) in the presence of HCO_3^- with the release of 6 protons. It is suggested that the iron binding site of this protein is provided by 2-3 tyrosyl residues, 1-2 nitrogen ligands (histidyl residues) and one bicarbonate molecule. When the iron binds to the transferrin molecule the latter becomes more compact and more spherical in shape, with the consequent properties of being more resistant to heat, high urea concentrations and proteolytic enzymes. The amino acid sequence of human

serum transferrin is not complete but a comparison of the sequences within the N-terminal and C-terminal portions of the molecule reveals about 40% homology, suggesting that transferrin probably has evolved from a gene duplication of an ancestral single metal binding site.

Transferrin can bind many metals e.g. Cu, Zn, Gr, Co, Mn, Cd, Ni, Ga and Sc, but if iron is added it will displace these metals. Thus, transferrin is more specific for iron than these metals. Specific binding of iron can only occur in the presence of a suitable anion. Under physiological conditions carbonate or bicarbonate is thought to fulfil this requirement but other anions can substitute for it. Such anions include oxalate, malonate, EDTA, Nitalotriacetate (NTA) and indeed Schlabash and Bates[90] found about twenty such anions. Dicarboxylic acids were the main anions able to form stable iron-transferrin-anion complexes which were only slowly displaced by carbonate. Because the strong binding to transferrin could only be achieved in the presence of both the metal ion and the anion the term synergistic has been used to describe these anions.

It is apparent that transferrin can fulfil other physiological roles apart from the transport of iron in the serum (Fig.5.1). It seems to be involved in:

(a) the transport of other metals such as Zn, Mn, and Cr;

(b) detoxification, by sequestering other toxic metal ions;

(c) the defence against infection

by restricting the availability of iron to invading micro-organisms. Indeed, transferrin is an essential ingredient for cell cultures. The central role of transferrin in iron metabolism is mainly the transport of iron to the haemopoietic tissues. Although iron(III) bound to transferrin in the extracellular pool in man is 4mg, this iron pool has to be turned over several times a day in order to provide the 20-25 mg of iron essential for the production of haemoglobin. Transferrin also exchanges iron(III) reversibly with the stored (ferritin and haemosiderin) and transient iron pools. Adult plasma transferrin binding sites are normally 25-35% saturated with iron, but are generally much higher in cases of iron overload. Like the other plasma proteins transferrin is distributed throughout most of the extra-cellular fluid of the body with a continuous circulation from plasma to the interstitial fluid and then back through the lymph vessels.

5.1.3 Iron Release from Transferrin in vivo

The best studied model of transferrin/cell interaction is that of the transferrin/reticulocyte, where transferrin makes iron available for haemoglobin synthesis. The first step of this interaction is an adsorption of transferrin on receptors present on the reticulocyte surface. This process is non-specific. The second step is a specific binding of transferrin to these receptors, which is affected by temperature, pH and is an energy

dependent process. The binding strength of the diferric, monoferric and apotransferrin with the reticulocyte is in the order Fe_2T > FeT > ApoT, thus Fe_2T is preferentially bound to reticulocytes. The last step involves the release of iron into the cell and the release of the iron-depleted transferrin to the plasma. The mechanism of iron release is unknown and there is a controversy as to whether transferrin enters the cell by endocytosis and subsequently releases its iron to the interior of the cell, returning to the plasma by exocytosis or iron release is mediated at the cell surface by other iron chelators. Other tissues which seem to extract iron from transferrin via receptors are liver, placenta and lymphocyte cells.

Another question which led to an abundant but contradictory literature is whether the two sites of transferrin function differently. This argument originated with the hypothesis put forward by Fletcher and Huehns in 1968.[91] (Fig. 5.2). They proposed that one of the transferrin sites arbitrarily called A delivers iron preferentially to red cell precursors, or to receptors on the placenta of pregnant animals. The other site, B, delivers its iron preferentially to storage sites in hepatocytes and to intestinal mucosa. By expanding their hypothesis and relating the heterogeneous sites of transferrin to iron absorption and mobilisation, they postulated that the A site, under conditions of increased marrow activity, repeatedly absorbs iron from the intestinal mucosa subsequently delivering it to the bone marrow. In contrast under conditions of low marrow activity the iron loaded transferrin would not remove

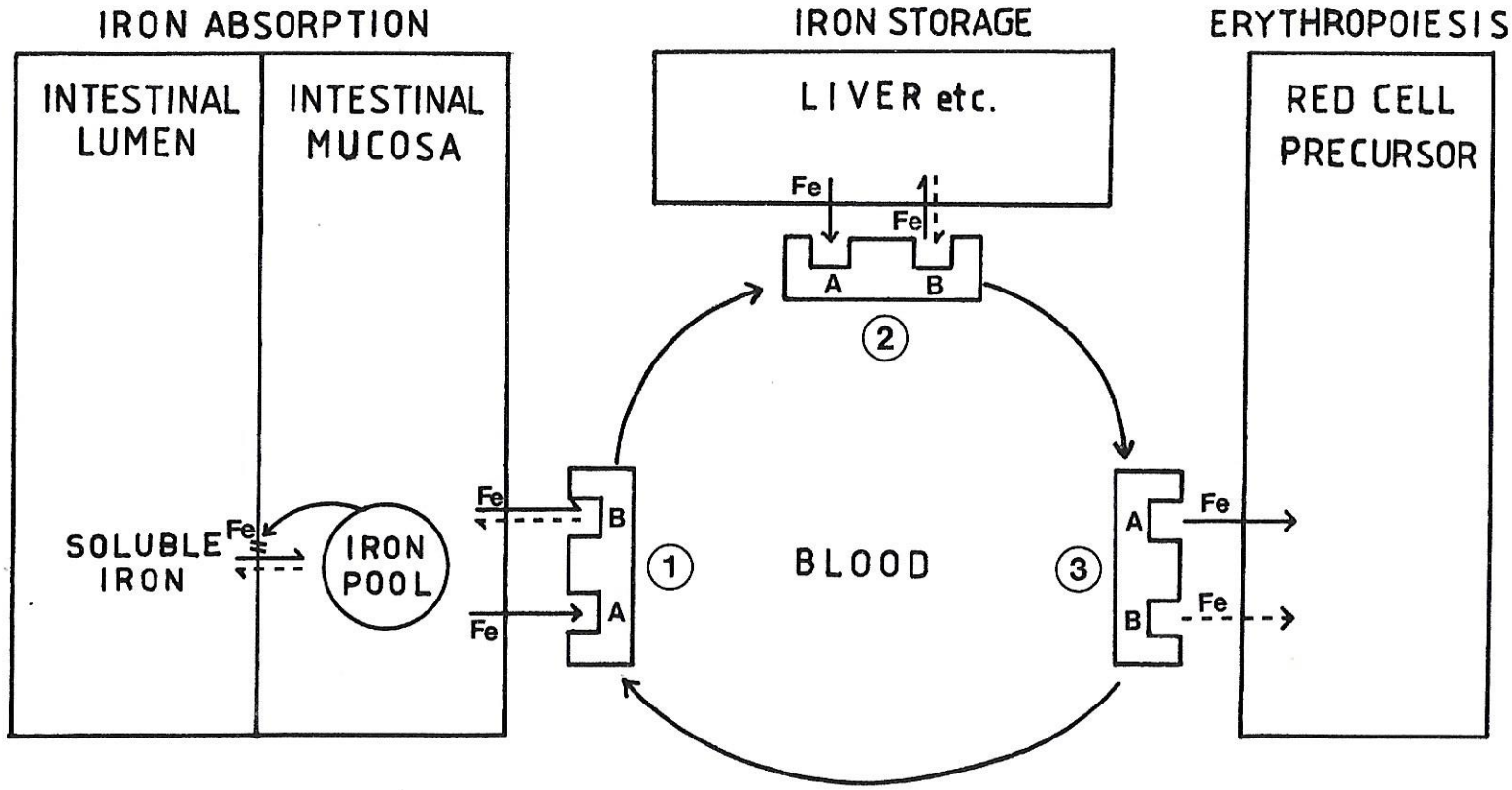


FIG. 5.2 Functional Heterogeneity of the two Transferrin Sites (A and B)

Schematic Representation of the Fletcher-Huehns Hypothesis.[91]

Addition: Mechanism 3 is thought to be involved in the delivery of iron to placenta.

iron from the intestinal mucosa. When sufficient iron is present in the B site the circulating transferrin will shut down iron absorption and will only deliver iron to hepatocytes when the other site is also filled. In a series of experiments with conflicting results this hypothesis has neither been proven nor disproven. Some of the more important experiments are described below.

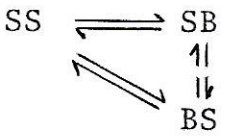
Some of the major variations are associated with the transferrin animal species used. For instance, the sites are able to donate iron in an equivalent manner when studies with human transferrin and human reticulocytes, or rabbit transferrin and rabbit reticulocytes are made.[92,93] In a heterologous system, however, using human transferrin and rabbit reticulocytes the ease with which the one site gives its iron, is five times greater than the other.[84,91,94] It would appear that the experimental procedures are among the main reasons for these disagreements. Other sources of variation are: the method of iron loading, the method of selective iron labelling of the iron sites, the method of formation of monoferric species[122] and the existence of possible transferrin isomers. Some of these differences will now be discussed in the context of similar or different sites in relation to iron loading and removal under different conditions.

5.1.4 Iron Loading of Transferrin

Although iron loading was one of the reasons leading to problems in interpreting results, there is a clearer understanding of this process and there are currently two methods favoured. It was suggested that the best method[95] of loading transferrin with iron is by adding NTA-Iron(III) complex to apotransferrin followed by bicarbonate, thus displacing NTA forming a carbonate-iron-transferrin complex. Another method[96] is the addition of $Fe^{II}(NH_4)_2(SO_4)_2$ to apotransferrin in the presence of carbonate. Care must be taken in this case to keep the solutions under anaerobic conditions and at low pH before its addition to the protein. Although some specific binding of iron(III) to apotransferrin can be achieved even in the absence of bicarbonate, there is also some non-specifically bound iron(III).[97] A similar situation occurs with $Fe^{II}(NH_4)_2(SO_4)_2$ and $Fe^{III}Cl_3$. This is, however, contradicted in a recent report[98] where complete and specific binding of $^{59}FeSO_4$ by transferrin was shown after the addition of $Fe^{II}(NH_4)_2(SO_4)_2$ in saline at pH 2 both *in vitro* and *in vivo*. Furthermore, this polynuclear iron could easily be formed during the loading procedures[99] of transferrin and it could be removed satisfactorily by gel filtration. Iron(III) bicarbonate (100mM) can also bind in the absence of a competing chelator to both metal binding sites.[100]

5.1.5 The Nature of the Specific Iron Binding Sites of Transferrin

In recent years mounting evidence in support of the suggestion that the two sites behave differently under certain conditions has been reported. Price and Gibson[101] using EPR suggested that $NaClO_4$ causes conformational changes to iron transferrin and proposed an equilibrium in which in the presence of $NaClO_4$, only one site in diferric transferrin can exist in this conformation (B). They suggested that the following equilibrium system existed.



EPR was also used to show differences between the sites when they were occupied by $Gr^{(II)}$, $VO^{(II)}$ and $Cu^{(II)}$.[102] Princiotto[103] and Zapolski showed that the dissociation of iron under acidic conditions is biphasic, and at pH 5.8 only one iron(III) is co-ordinated, to the protein. A similar observation[104] was found in a more detailed study where it was suggested that in both metal sites there is a strong positive co-operativity in the release of protons from functional groups with apparent pKa values near physiological pH (7.4). Aisen and co-workers[64] using equilibrium dialysis, determined the apparent stability constants at pH 7.4 and atmospheric pCO_2 as $K_1 = 4.7 \times 10^{20} M^{-1}$ and $K_2 = 2.4 \times 10^{19} M^{-1}$. They also suggested that although the a site, namely the C-terminal site

(TfFeC) binds iron stronger[105] than the b site, namely the N-terminal site (FeNTF), the accessibility to all iron complexes is different. Thus ferric nitrilotriacetate is directed towards the a site[116] whereas ferric citrate, oxalate, chloride and ferrous ammonium sulphate towards the b site. Differences between the sites are also displayed in the exchange of transferrin bound bicarbonate[106] with ambient bicarbonate in solution. Thus in monoferric transferrin the rate of exchange is monophasic but for diferric it is biphasic, one rate greater and the other smaller than in the situation with monoferric transferrin. Site differences were also observed when ferric transferrinwas reduced in excess dithionite[107] and the resulting iron(II) was subsequently sequestered by bathophenanthroline sulphonate.

5.1.6 Iron Removal from Transferrin using Chelators

Aspects which could be involved in influencing iron release from ferric transferrin are protonation, reduction, destabilisation of anion binding and conformational changes.[117] It is difficult to conceive of a situation where iron release from transferrin *in vivo* occurs without the involvement of a chelator. Iron release from transferrin using chelators, is also the problem facing scientists involved in the design of drugs for the treatment of iron overload.

DFB is unable to remove iron from transferrin unaided but it can do so under in vitro conditions in the presence of nitriloacetate.[108] Studies of iron release from human transferrin triggered by EDTA[109] have shown that the two sites are kinetically similar but not identical and that in the presence of salts and detergents the release was partly heterogeneous and partly homogeneous, depending on the salt or detergent added. This was interpreted as release from different conformation states.

Various anions[111] such as sodium citrate, ATP and pyrophosphate have also been used to facilitate iron transfer from transferrin to DFB. These anions do not form stable ternary complexes with iron transferrin but HCO_3^- substitution and subsequent chelation is suspected. It seems that polyphosphate compounds[111] bearing a pyrophosphate[115] moiety can remove iron from transferrin in a bimodal manner.[112,118] This can be achieved in the presence and absence of iron chelators. Enterobactin and synthetic tricatechols[113] have recently been employed to remove iron from transferrin. It was suggested that a ternary compex was formed between these and transferrin before iron removal occurred. Ligand size apparently did not affect the reaction. This is in agreement with the formation of another ternary complex which has been observed between transferrin and VO^{II}-xylenol orange[114] and VO^{II}-semixylenol orange, two other large molecules. It seems that the binding sites of transferrin are located at the surface or in a large crevice on the molecule.

In the light of the contradictory literature on reaction conditions regarding transferrin, various methods were adopted in order to study the effect of the ligands on this protein. Thus different transferrin samples (purchased from different suppliers), iron loading procedures, buffers, temperatures and ligand concentrations were employed in a series of experiments basically aiming to establish whether or not there was a reaction between transferrin and the ligands. ^{59}Fe-transferrin was also prepared to show the extent of iron removal by the ligands, while under the same conditions DFB, EDTA and catechol were used as markers. Furthermore, spectrophotometric studies were undertaken in order to examine the rates and the mechanism of these reactions.

5.2.1 Methods of iron loading of transferrin

The preparation of iron transferrin was carried out by two different procedures. The first one is based on the iron loading of apotransferrin using iron nitrilotriacetic acid, previously described by Bates et al.[95,119] In a typical experiment $FeCl_3$ was mixed with nitrilotriacetic acid (NTA) at a ratio of 1:2, made 0.1M in Tris HCl and mixed for 15 min. Apotransferrin was dissolved in Tris HCl, $NaClO_4$ (0.1M, pH 7.4) and $NaHCO_3$ was added to give a final concentration of 10mM. Iron-NTA (0.1 ml) was added to apotransferrin (3ml). This corresponded to 115% saturation. The mixture was allowed to stand at room temperature for not less than 30 min and it was

sequentially passed through two Sephadex G.25 columns equilibrated with $NaClO_4$ (0.1M) and Tris HCl (0.1M, pH 7.4) respectively at 4°. This chromatographically homogeneous transferrin solution was mainly used for kinetic experiments.

In the second method of iron loading of apo transferrin with ferrous ammonium sulphate, apotransferrin was dissolved in Tris-HCl (0.1M, pH 7.4) and made 10mM in $NaHCO_3$. Ferrous ammonium sulphate was dissolved in water and brought to pH 4 using HCl. The solution was then degassed and N_2 added. For approximately 100% saturation of apotransferrin with iron the two solutions were mixed in the proportion of 1.4µg of iron per 1mg of apotransferrin. The mixture was then incubated at 37° for 10 min and left to stand at room temperature for 30 min in a shaking water bath.

^{59}Fe-transferrin was prepared as follows:

$FeCl_3$(5µCi/13µl, 10µg/ml) was mixed with freshly prepared $FeCl_3$ aqueous solution. NTA was dissolved in Tris-HCl (0.1M, pH 7.4) and added to the iron mixture at a molar ratio of 2:1. The iron-NTA complex was stirred for at least 15 min before it was added to apotransferrin, which was made 10mM with $NaHCO_3$, in the same buffer. After the addition of ^{59}Fe-NTA to apotransferrin the mixture was placed in a shaking water bath and mixed for at least 1h at room temperature. Various percentages of iron saturation were prepared as shown in the Table 5.2.

5.2.2 Methods for Studying the Reaction of the Ligands with Transferrin

The transferrin solution was examined spectrophotometrically to confirm its iron loading as well as to estimate the iron(III) concentration in transferrin [88] (Fe_2T E_{470}= 2500 $M^{-1}cm^{-1}$). The ligand solutions in the same buffer, unless otherwise stated, were then mixed with transferrin and the absorption spectra taken at different time intervals. The change in absorbance (ΔA) at a selected wavelength was plotted against time and the pseudo first-order rate constants of the reaction were estimated from the plot of $\log(\Delta A_\infty - \Delta A_t)$ versus time. The conditions used in the study of the reaction of transferrin with the ligands are illustrated in Table 5.1.

The reaction of ligands with ^{59}Fe-transferrin was studied by mixing equal volumes of their solutions in Tris HCl (0.1M, pH 7.4) and by shaking them continuously in a constant temperature water bath for different time intervals. The reactive mixtures (2ml) were then placed in dialysis tubes which were previously washed with EDTA (0.1mM) and dionised water, and dialysed against buffer for several hours at 4°. The contents of the dialysis tubes were then transferred to a plastic tube and the radioactivity monitored (as in Chapter 4), sometimes the dialysed reaction mixture was monitored alone and on other occasions it was monitored together with the empty dialysis tube which was pushed down to the bottom of the plastic tube. With each experiment, control samples of the transferrin without

TABLE 5.1

Spectrophotometric Studies of the Reaction of Transferrin with Various Ligands

EXPT	LIGAND	$[L]x10^{-3}M$	$[Fe_2T]x10^{-5}M$	$\frac{[L]}{[Fe_2T]}$	PROCEDURE	TEMP.	LIGAND BUFFER[c]
1	L_1	10.3	3.60	286	2	RT	TRIS
2	L_1	5.15	3.60	143	2[a]	RT	TRIS
3	L_1	0.50	2.72	18.4	1	RT	TRIS/PER
4	L_1	3.00	3.00	100	2	RT	PBS
5	L_2	1.70	3.40	50	1	37°	TRIS/PER
6	L_2	3.00	3.00	100	2	RT	PBS
7	L_3	0.50	2.72	18.4	1	RT	TRIS/PER
8	L_3	3.00	3.00	100	2	RT	PBS
9	L_4	1.70	3.40	50	1	RT	TRIS/PER
10	L_4[b]	1.70	3.20	53	1[a]	37°	TRIS/PER
11	L_5	3.00	3.00	100	2	RT	PBS
12	L_5	1.05	3.60	292	2	RT	TRIS
13	L_6	3.00	3.00	100	2	RT	PBS

a = 1 week old transferrin (Fe_2T) stored at 4°.

b = suspensions occurred during the reaction.

c = TRIS - Tris HCl (0.1M, pH 7.4); TRIS/PER - Tris HCl, $NaClO_4$ (0.1M, pH 7.4); PBS - Phosphate Buffered Saline.

1 = Method of preparation using iron-NTA and apotransferrin from Sigma Co.

2 = Method of preparation using $Fe^{II}(NH_4)_2(SO_4)_2$ and apotransferrin from Behring Diagnostics.

ligand were monitored in an identical fashion and their average ^{59}iron content was taken as 100% for comparison with the other samples.

The experimental conditions of the reaction of ^{59}Fe-transferrin with the ligands is shown in Table 5.2. In certain experiments, oversaturation of transferrin was employed to test the removal of non-specifically bound iron by the ligands and the markers under the set experimental conditions.

5.3.1 The Reaction of Transferrin with the Ligands

The main objective in this study was to establish whether or not these ligands could remove iron from this protein and if so, to what extent. The experiments, using ^{59}Fe were designed to quantitatively demonstrate iron removal from transferrin,and the spectrophotometric studies to give an indication of the rates and the mechanism of this reaction.

With the spectrophotometric studies, the progress curves in the visible region (350-600nm), of the reaction of transferrin with the different ligands, show a gradual change from the iron-transferrin spectrum to that of the iron ligand. Thus after the addition of ligands to iron-transferrin (Fig.5.3-5.9) the characteristic spectrum of iron transferrin, together with that of the ligand, changes particularly in the vicinity of the λ_{max} of the iron ligand complex. This was anticipated as the extinction

TABLE 5.2

The Conditions of the Reaction of ^{59}Fe-Transferrin with the Ligands

	EXPT.I	EXPT.II	EXPT.III[b]	EXPT.IV	EXPT.V
LIGAND	L_1, L_2, L_3, L_4, L_5, L_6, DFB	L_1, L_2, L_3, L_4, L_5, L_6, L_7, DFB EDTA	L_1, L_2, L_3, L_4, L_5, L_6, DFB EDTA, CATECHOL	L_1, L_2, L_5, DFB	L_1, L_2, L_3, L_4, L_5, L_6
[L] x 10^{-3}M	4.0	4.0	4.0[a]	4.0	4.0
[Transferrin] x 10^{-5}M	1.2	1.1	0.55	0.27	1.21
% iron saturation	115	110	110-90	100	100
Reaction time/h	3-4	4-5	6-7	18	16
Temperature	RT	RT	RT	RT	37°
Dialysis Buffer volume Time	PBS 2L 16h	TRIS HCl 2L 16h	PBS 2L 24h	PBS 2L 24h	PBS 2L 20h

a = [catechol] 2 x 10^{-3}M

b = The samples of ^{59}Fe-transferrin in Expt.II which did not lose substantial ^{59}Fe, i.e. with no ligand(A);with DFB (B); with L_5(C); with L_2(D); and with EDTA (E) were used for Expt.III. Thus part of sample A was incubated with catechol and with no ligand; of B with DFB; of C with L_5 and of D with L_2 and L_3; and of E with EDTA, L_4 and L_6. The % ^{59}Fe removal from ^{59}Fe-transferrin in each was estimated from the ^{59}Fe present in their original samples (100%).

coefficients of the iron-ligand complexes are higher than that of iron-transferrin in this wavelength region.

By plotting the change in absorbance (ΔA) of the progress curves versus time an exponential curve was obtained in all the reactions of transferrin with the different ligands (Fig. 5.10-5.11). The plot of log ($\Delta A_\infty - \Delta A_t$) versus time showed that almost all of the reactions are biphasic (Fig. 5.10-5.11) The two phases are of equal amplitude and each is characterised by a slow and a fast phase. The pseudo first-order rate constant of the slow phase k_1 was calculated using

$$k_1 = \frac{0.693}{T_{\frac{1}{2}}}$$, where $T_{\frac{1}{2}}$ is the half-life of the slow phase.

In the case of the pseudo first-order rate constant for the fast phase (k_2), a plot of $\log(\Delta A_{total} - \Delta A_{slow\ phase})$ versus time was used to estimate $T_{\frac{1}{2}}$ of the fast phase and subsequently k_2 as above (Table 5.3).

There were a few single phase reactions observed (Table 5.3), with associated pseudo first-order rate constants falling between those of the slow and the fast rates of the biphasic reactions. In a low ligand to transferrin concentration ratio, there is a decrease in the rate constants compared to the other reactions.

Two reaction mixtures, those corresponding to L_1-transferrin and L_5-transferrin (Expts. 1 and 12) were dialysed after the completion of the reaction and their absorption spectra recorded. These were compared to the

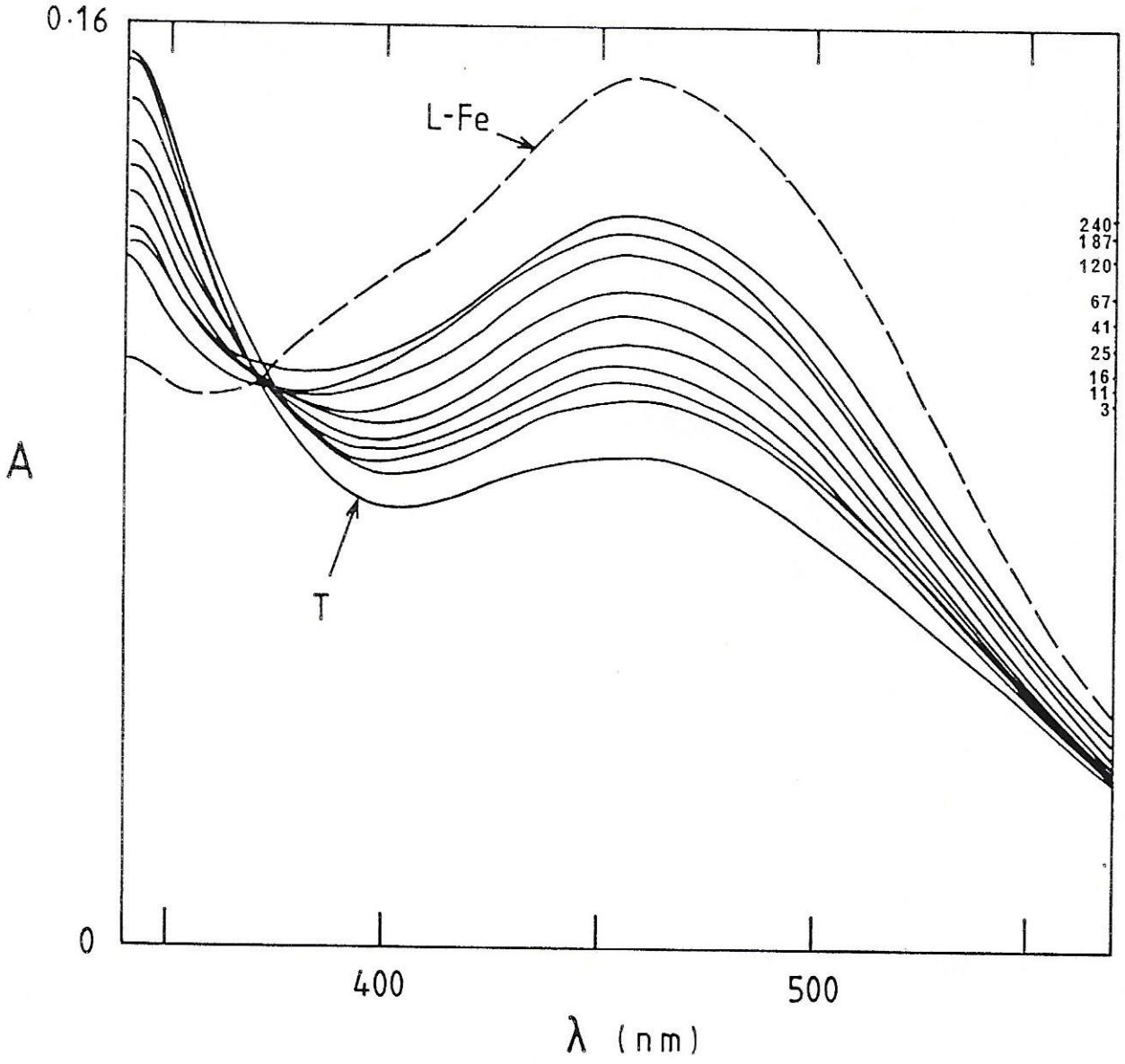


Fig. 5.3

Spectrophotometric study of the reaction of L_1 with transferrin (T).

Vertical Axis: Absorbance

Horizontal Axis: Wavelength

The time(min) corresponding to each of the progress curves is indicated at the right hand vertical axis.

L-Fe: Is the ligand-iron (III) complex absorbance spectrum. The iron concentration of the complex was chosen to be approximately equal to that present on transferrin.

The experimental conditions of this reaction are described in Expt. 3, Table 5.1.

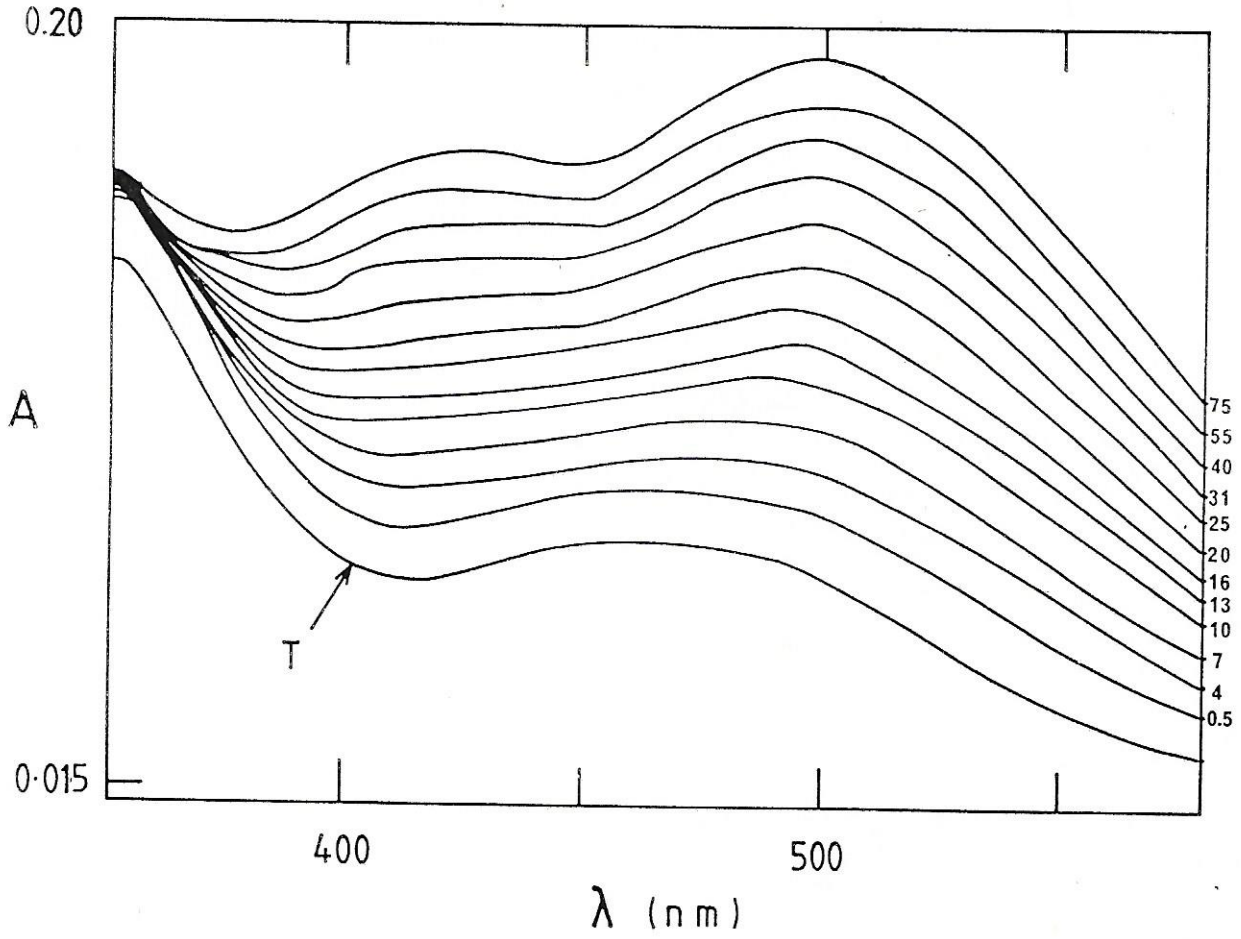


Fig. 5.4

Spectrophotometric study of the reaction of L_2 with transferrin (T).

Vertical Axis: Absorbance
Horizontal Axis: Wavelength

The time/min corresponding to each of the progress curves is indicated at the right hand vertical axis.

The experimental conditions of this reaction are described in Expt. 6, Table 5.1.

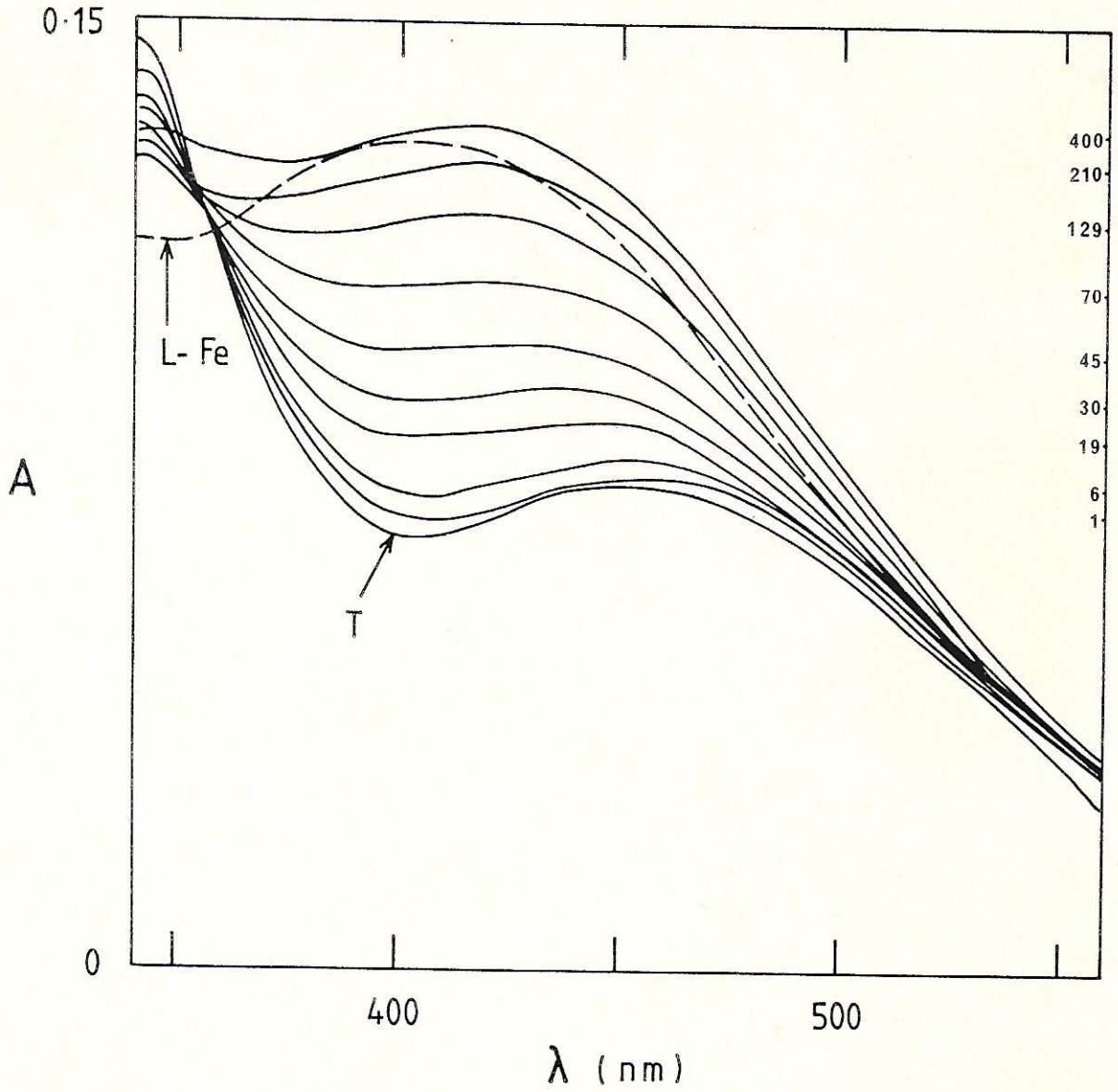


Fig. 5.5

Spectrophotometric Study of the reaction of L_3 with transferrin (T).

Vertical Axis: Absorbance
Horizontal Axis: Wavelength

The time/min corresponding to each of the progress curves is indicated at the right hand vertical axis.
L-Fe: Is the ligand-iron(III) complex absorbance spectrum. The iron concentration of the complex was chosen to be approximately equal to that present in transferrin.
The experimental conditions of this reaction are described in Expt. 7, Table 5.1

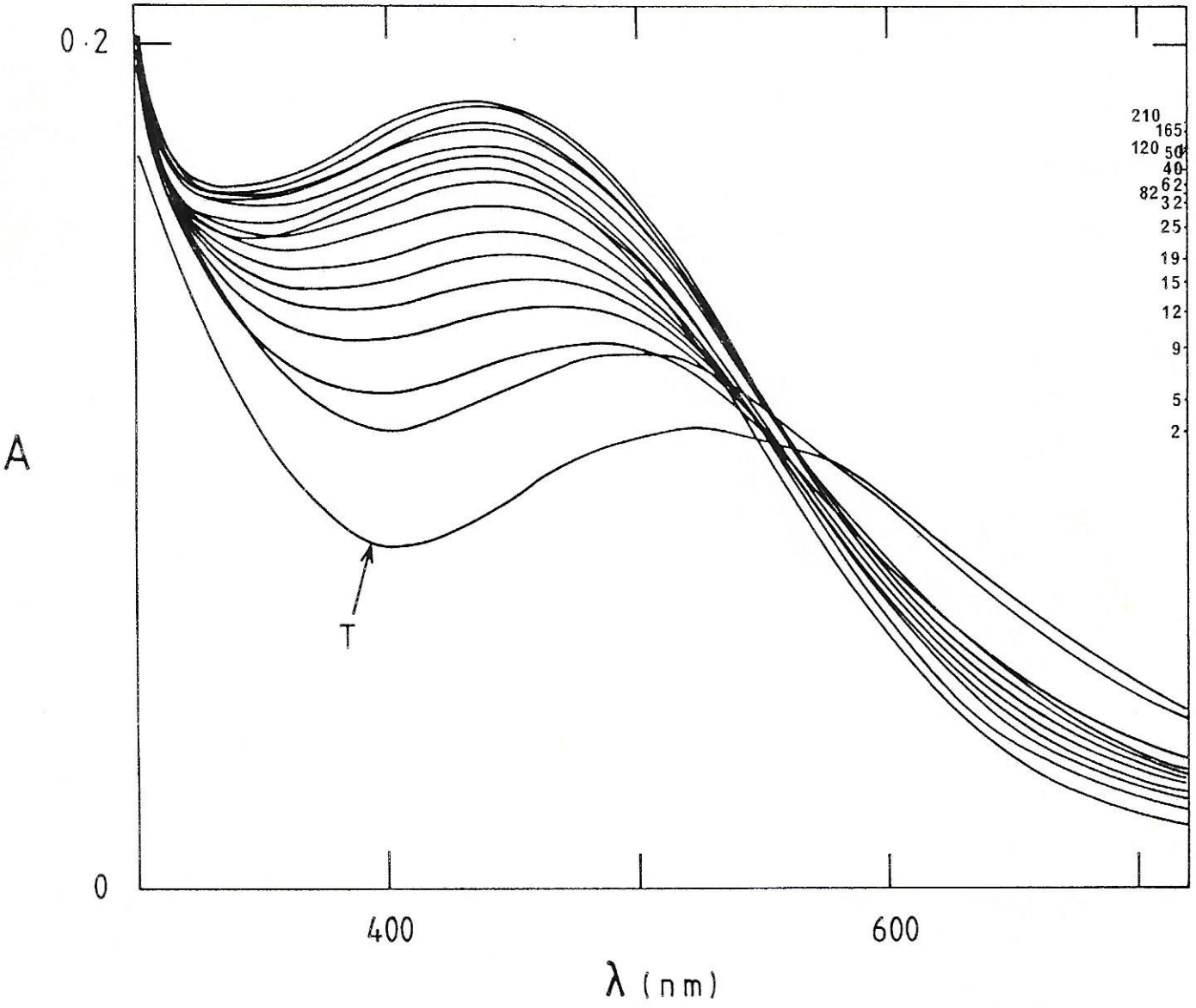


Fig. 5.6

Spectrophotometric study of the reaction of L_4 with transferrin (T)

Vertical Axis: Absorbance

Horizontal Axis: Wavelength

The time/min corresponding to each of the progress curves is indicated at the right hand vertical axis.

The experimental conditions of this reaction are described in Expt. 10, Table 5.1

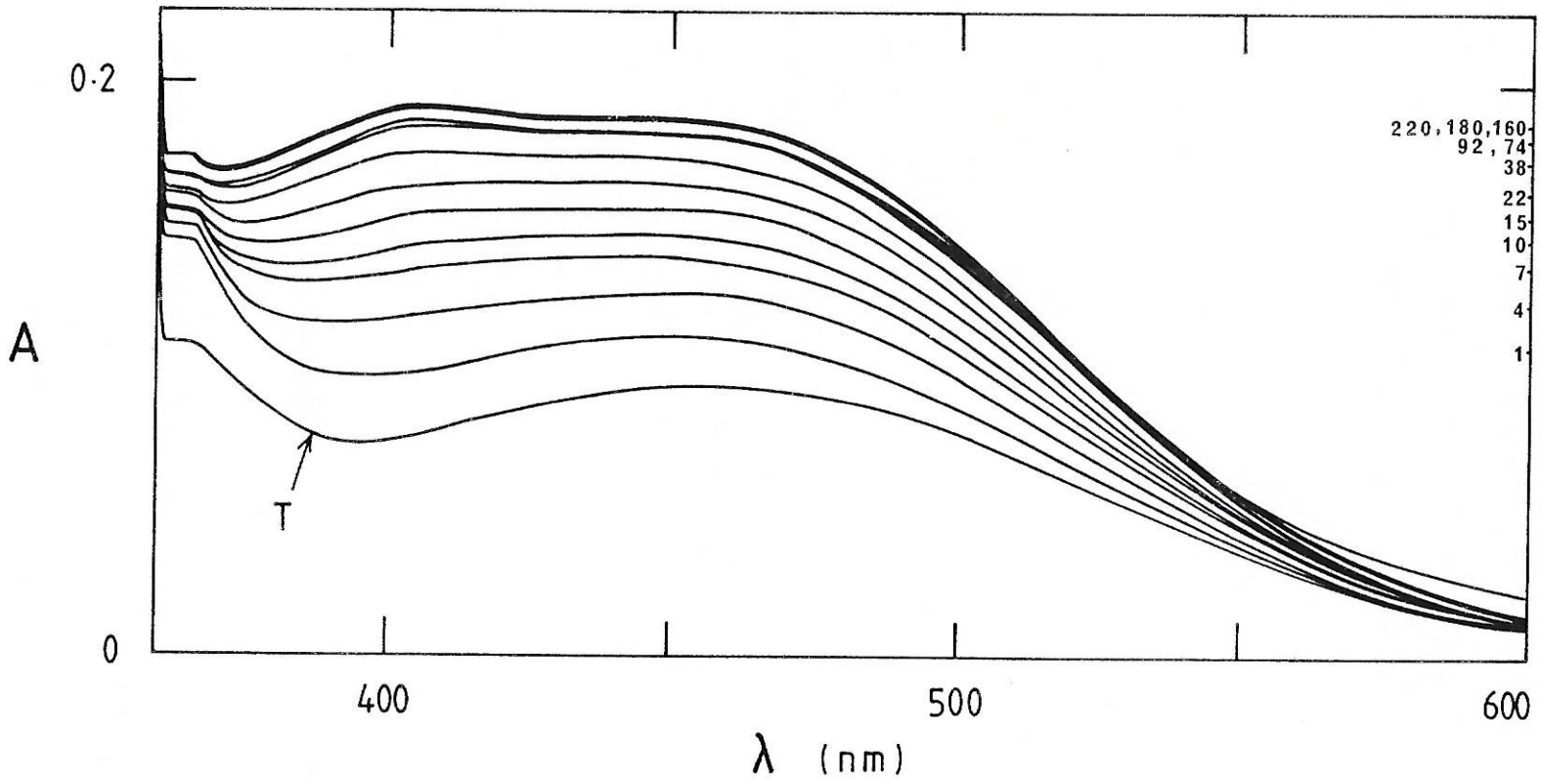


Fig. 5.7

Spectrophotometric Study of the Reaction of L_5 with Transferrin (T).

Vertical Axis: Absorbance

Horizontal Axis: Wavelength

The time/min corresponding to each of the progress curves is indicated at the right hand vertical axis.

The experimental conditions of this reaction are described in Expt. 12, Table 5.1.

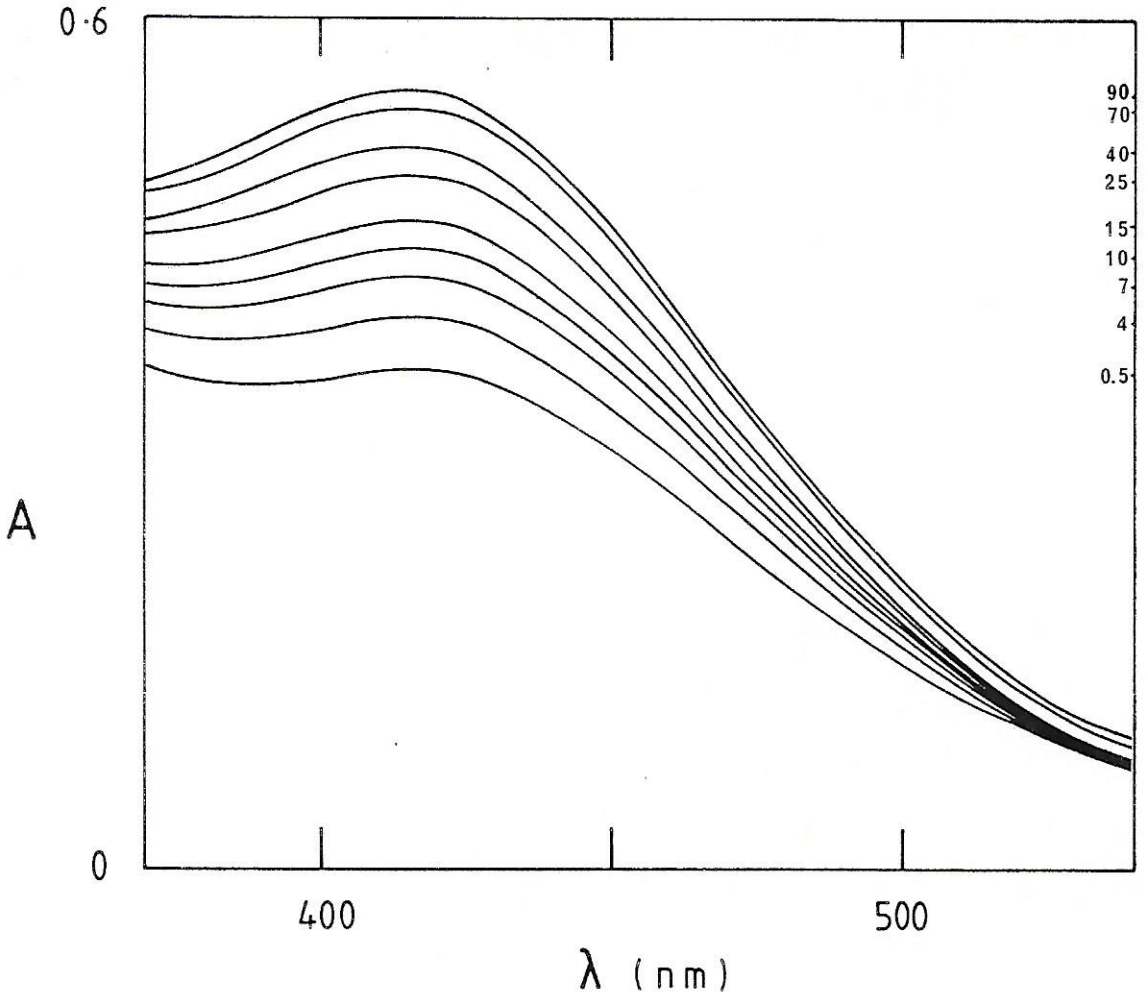


Fig. 5.8

Spectrophotometric study of the reaction of L_6 with transferrin(T)

Vertical Axis: Absorbance

Horizontal Axis: Wavelength

The time/min corresponding to each of the progress curves is indicated at the right hand vertical axis.

The experimental conditions of this reaction are described in Expt. 13, Table 5.1.

Fig. 5.9

The kinetic profile of the spectrophotometric study of the reaction of L_1 with transferrin.

A:

A plot of the change in absorbance[a] against time from where the infinite change in absorbance of the reaction was estimated.

B:

The biphasic mode of the reaction as shown from the log plot of the change in absorbance[a] at infinity minus the change in absorbance at time T of the reaction.
The experimental conditions of the reaction are described in Expt. 4, Table 5.1

a = Arbitrary units

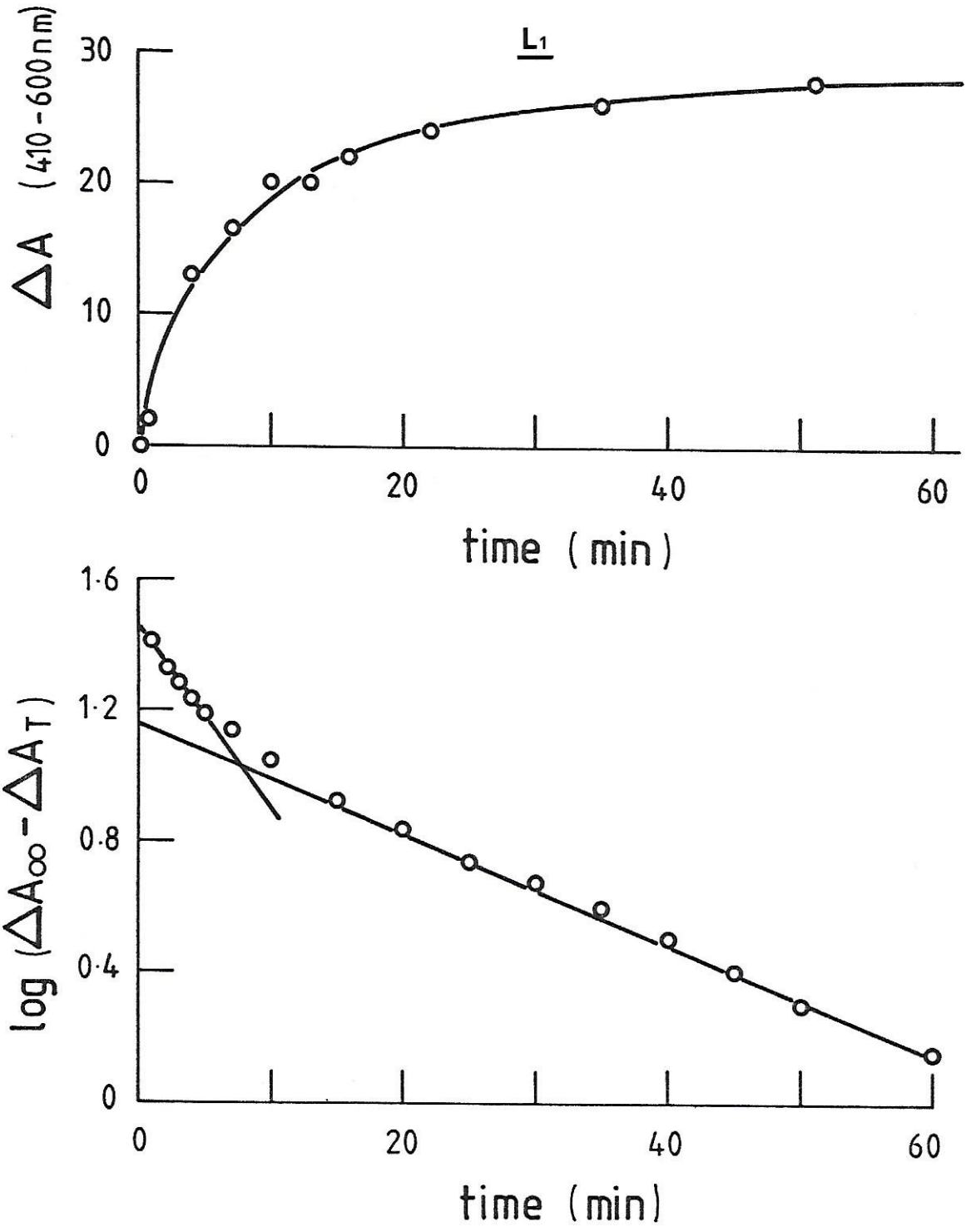

L1
ΔA (410-600nm)
30
20
10
0
0
20
40
60
time (min)
log (ΔA∞-ΔAT)
1·6
1·2
0·8
0·4
0
0
20
40
60
time (min)

Fig. 5.10

The kinetic profile of the spectrophotometric study of the reaction of L_3 with transferrin.

A:

A plot of the change in absorbance^{a} against time from where the infinite change in absorbance of the reaction was estimated.

B:

The biphasic mode of this reaction as shown from the log plot of the change in absorbance^{a} at infinity minus the change in absorbance at time T of the reaction.

The experimental conditions of the reaction are described in Expt. 8, Table 5.1

a = Arbitrary units.

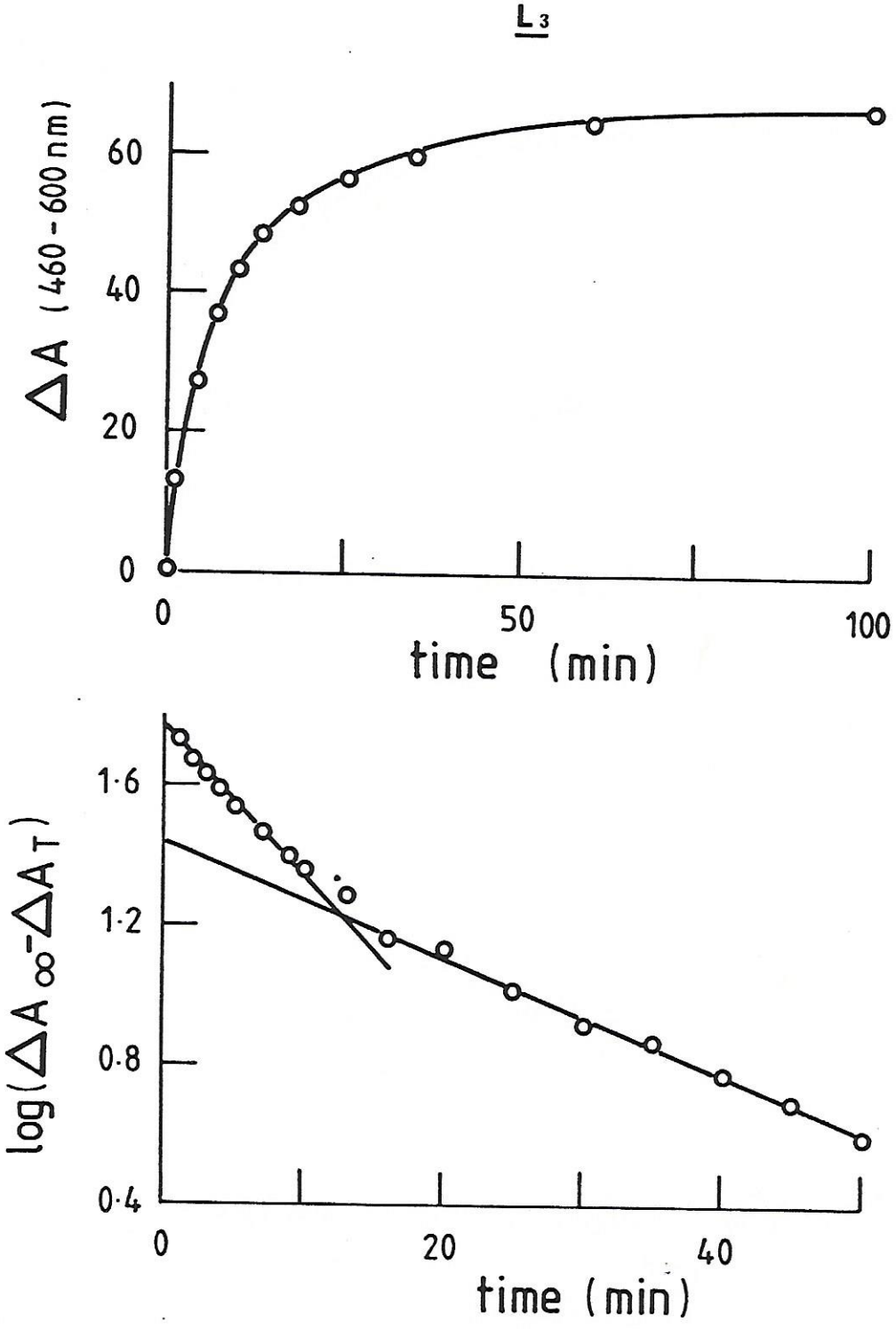

L3
ΔA (460-600 nm)
60
40
20
0
0
50
100
time (min)
log(ΔA∞-ΔAT)
1·6
1·2
0·8
0·4
0
20
40
time (min)

TABLE 5.3

Results of the Spectrophotometric Studies of the Reaction of Transferrin with the Ligands

EXPT.	LIGAND	$\frac{[L]}{[Fe_2T]}$ [a]	MODE [b]	k_1/s^{-1} x 10^{-4}	k_2/s^{-1} x 10^{-4}	k_i/s^{-1} x 10^{-4}
1	L_1	286	Biphasic	8.05	27.2	
2	L_1	143	Biphasic	9.24	56.3	
3	L_1	13.4	Biphasic	1.04	7.65	
4	L_1	100	Biphasic	6.6	48.1	
5	L_2	50	Biphasic	1.82	13.6	
6	L_2	100	Monophasic			8.55
7	L_3	18.4	Biphasic	1.62	4.62	
8	L_3	100	Biphasic	6.42	30.3	
9	L_4	50	Biphasic	6.24	19.2	
10	L_4	53	Monophasic			14.8
11	L_5	100	Monophasic			7.8
12	L_5	292	Biphasic	6.4	24	
13	L_6	100	Biphasic		8.8	

a = Molar ratio of [L] : [Transferrin]

b = The two phases of the biphasic reactions are of equal amplitude except from experiments 3 and 13.

spectra of apotransferrin and transferrin of equal concentrations, which underwent the same dialysis procedure. In the case of the L_1-transferrin mixture the absorption spectrum after dialysis was similar to the apotransferrin solution whereas that of L_5-transferrin mixture still gave evidence for the presence of some iron(III).

The reaction of L_1 with transferrin was studied on four different occasions, showing biphasic reactions with equal amplitude and similar pseudo first-rate constants in three cases, whereas at low ligand concentrations the amplitudes were not equal. L_3 is another ligand showing biphasic reactions with transferrin at both high and low ligand concentrations, again both phases were of equal amplitude. In contrast, although three other ligands - L_2, L_4 and L_5 showed biphasic reactions under certain conditions, sometimes monophasic reactions were observed. In the biphasic reactions the amplitude of both phases were equal. L_6 and L_7 also reacted efficiently with transferrin as shown from Fig.5.8. In the case of L_6 there was a fast phase, but it did not correspond to half of the amplitude, and in L_7 some problems in determining the rates took place, probably due to oxidation of this ligand.

The quantitative estimation of iron removal from transferrin was carried out in five different experiments using ^{59}Fe radioactively labelled transferrin, and ligands at various concentrations and conditions. The results of

these experiments are depicted in Table 5.4. The reported percentage values are those of the radioactivity remaining after dialysis, compared to the samples where ligands were not added (100%).

In these experiments L_1 removed the highest amount of ^{59}iron from ^{59}Fe-transferrin, between 80-90% over 3h and under all the set experimental conditions. Its efficacy of iron removal from transferrin decreased slightly at 37°(Expt. V). L_3 was also effective in mobilising iron from transferrin, in all the experiments. Its efficacy increased with both reaction time and temperature, thus releasing 70-90% ^{59}Fe from transferrin. Two other ligands L_4 and L_6 reproducibly removed equivalent amounts of iron from transferrin(60-70%). Both increased their efficacy slightly at longer reaction times. L_7 mobilised similar amounts of iron to that of L_6 (approximately 70%). L_2 and L_5 did not remove substantial amounts of iron in short incubation periods, however, their efficacy approaches that of other ligands after longer reaction times (18h), this is particularly true for L_2. Furthermore, the efficiency of L_2 is further enhanced with increasing the temperature in contrast to L_5. In experiment III where duplicate samples of these ligands were allowed to react for shorter periods than their counterparts, the iron removed was substantially less.

The other three ligands used for comparison, DFB, EDTA and catechol, failed to remove substantial quantities of iron from transferrin, under the same experimental

TABLE 5.4

Iron Mobilisation from Transferrin

LIGAND	Percentage ^{59}Fe left in the ligand ^{59}Fe-Transferrin reaction mixture after dialysis[a]					
	EXPT.I	EXPT.II	EXPT.III	EXPT.IV	EXPT.V	
					A	B
None	100	100	100	100	100	100
L_1	16.7 -	10.1 10.7	12 -	9.6 -	23.2 -	23.5 -
L_2	81.2 80.4	89.4 88.9	56 80[b]	38	33.2	39.5
L_3	23.8 24.2	18.5 20.6	17		11.3	10.1
L_4	37.9 37.3	31.9 34.5	18		28.0	29.4
L_5	83.8 78.5	95.4 95.7	79 100[b]	42.6	79.1	77.5
L_6	32.7 32.1	30.6 30.8	27		37	34.4
L_7		30.5				
DFB	77.1	82.5 84.9	100 100	78		
EDTA		74.9 72.1	100 100			
CATECHOL			89			

a = For reaction conditions, see Table 5.2.
b = Reaction time 2h instead of 6-7h.

A = Counting of samples with the dialysis tube.

B = Counting of samples without the dialysis tube.

conditions. In experiment III, however, where the transferrin saturation was about 100%, DFB and EDTA did not seem to remove any iron. The iron removed by catechol was probably non-specifically bound as its "mother" sample (from experiment II) was ^{59}Fe-Transferrin control, i.e. incubated in the absence of any ligand.

5.4 The Significance of Iron Removal from Transferrin

These preliminary experiments show that all the new chelators react with transferrin and indeed some of them remove substantial amounts of iron at physiological pH values. The quantitative studies using ^{59}Fe-transferrin suggest that there is variability amongst the ligands in their efficacy to remove iron from transferrin.

Most of the competition reactions appear to be biphasic when studied spectrophotometrically and each phase corresponds to approximately half of the total change in absorbance (ΔA). This type of reaction can be interpreted as starting with the formation of a ligand-iron-transferrin ternary complex, followed by iron removal. The biphasic nature of the process is indicative of a differential iron reaction rate from each site of transferrin.

L_1 showed reactivity and substantial iron removal consistently in all the experiments with iron transferrin. Thus it would seem to be a promising compound, not only because it can be used for studying iron removal from iron-transferrin which is a property not shared by other iron chelators, but also because this property is directly

related to an effective treatment of iron overload diseases. L_3 is another ligand with consistency in its reaction with transferrin. It is the second most effective chelator in this aspect, showing substantial iron removal from transferrin with biphasic rates.

L_4, L_6, and L_7 are slightly less effective in iron mobilisation from transferrin compared to L_1 and L_3 and further work will be needed to clarify their kinetic properties. Problems associated with the interpretation of the kinetic results of the L_2 and L_5 reactions with transferrin need also to be further examined. These two latter ligands remove little iron when exposed to transferrin for short periods (6-7h). This was rather unexpected because when their reaction was followed spectrophotometrically, changes were shown within minutes of mixing. However, at longer incubation times, iron removal increased substantially especially in the case of L_2.

The other known iron chelators, namely DFB, EDTA and catechol, seemed to be rather ineffective in mobilising iron from transferrin, thus confirming previous reports.[120]

Some other general trends can be observed in this study. It seems that all the ligands at high concentrations have similar slow and fast rates when reacting with transferrin (Table 5.3). Furthermore, the monophasic reaction rates are always of intermediate values compared to the slow and the fast rates of reactions, when measured under similar conditions. The difference between the two phases in each biphasic reaction is usually 3 to 8

fold thus allowing for distinction between the two sites.

A possible general mechanism which can account for most of the experimental results in this section is depicted in Scheme 5.1. Thus a ternary complex is formed when transferrin, bearing iron accessible to the ligand, reacts with the latter. This complex seems to be stable in the case of L_2 and L_5 since spectral changes do not fit chronologically with iron removal. To account for the biphasic reaction, the mechanism depicted in Scheme 1 is thought to take place with two different rates in the second step, each corresponding to one iron site of transferrin. Furthermore, if steps 3 and 4 are very fast compared to 2, the biphasic reaction could be identified with biphasic iron release, which is likely to occur in the case of L_1, L_3, L_4, L_6 and L_7. For the monophasic reaction, a conformational change due to different solution conditions could be assumed, which causes iron release of rates almost identical from both sites.

Clearly the reaction with transferrin of the ligands synthesised in this work can be used to shed light on the mechanism of iron removal both <u>in vivo</u> and <u>in vitro</u>. Thus the biphasic reactions are in agreement with the observation by other workers that the two sites of transferrin are functionally different.[91]

SCHEME 5.1

Mechanism of the Reaction of Transferrin with Ligands

$$FeT^* \rightleftharpoons FeT \quad (1)$$

$$FeT + L \rightleftharpoons LFeT \quad (2)$$

$$LFeT \rightleftharpoons T + LFe \quad (3)$$

$$LFe + 2L \rightleftharpoons L_3Fe \quad (4)$$

FeT represents both sites or any site of transferrin bearing iron in a state accessible to the ligand and FeT* in a state inaccessible to the ligand. The second step involves the ligand binding to the iron site to form a ternary complex. The third step involves iron removal which can be fast or slow depending on the ligand. The fourth step is the eventual formation of the 3:1 ligand to iron complex usually found at physiological pH.

Many mechanisms have been implicated so far in the release of iron from transferrin. These mechanisms mainly involve the protonation of a functional group of amino acid (e.g. tyrosine) binding to iron in transferrin; the breaking of the carbonate-iron bond; the reduction of iron; conformational changes; and the formation of a ternary complex between a ligand and iron-transferrin.[113] Which mechanism or which combination is involved *in vivo* is still unknown. However, in designing compounds for the mobilisation of iron from transferrin, one should note that these compounds not only should have the thermodynamic ability to do so but in addition, they should be kinetically efficient, i.e. possess an appreciable rate under physiological conditions.

Thermodynamic and kinetic parameters may also govern the functional ability of transferrin receptors, present on the cell surface, to mobilise iron from transferrin. Thus the number, the affinity and the kinetic efficiency of transferrin receptors, the saturation of transferrin, and the presence of molecules mediating intracellular and extracellular iron release may be the determining factors in iron mobilisation from this protein *in vivo*.

The technique developed to study iron mobilisation from ^{59}Fe-transferrin has proved to be extremely useful for screening new iron chelators. Furthermore, the ligands prepared in this work were found to be the most efficient bidentate iron chelating agents ever reported in relation to iron mobilisation from transferrin.

CHAPTER SIX

IRON MOBILISATION FROM FERRITIN

6.1.1. Introduction to Ferritin

Cooley's anaemia and certain other disorders are characterised by secondary iron overload due to prolonged blood transfusions which result in the accumulation of excessive iron in the liver, spleen, heart and other tissues. In such cases iron is stored mainly in the form of ferritin and haemosiderin, the latter predominating after prolonged treatment.[14] The mobilisation of iron from ferritin, is one of the main tasks facing workers involved in the design and therapeutic use of iron chelators. An ideal drug should be able to directly or indirectly mobilise iron from the iron overloaded organs and eventually facilitate its excretion, without causing any toxic effects.

The storage of iron accomplished by ferritin is in a water soluble form and that of haemosiderin in a form of an insoluble variable mixture of iron protein and other organic material.[149] Indeed, haemosiderin is thought to be the product of lysosomal proteolytic digestion of ferritin.[146] Both proteins are used as tools for other scientific purposes,[123] for instance:

(a) in morphological studies of tissues and cell components, because of their electron dense appearance under the microscope;[123]

(b) in the determination of the state of the reticulo-endothelial iron stores, by estimating the ferritin level in the serum;[147]

(c) for monitoring the presence and progress of malignant diseases by examining the phenotypes of ferritin in the serum;[126]

and (d) in aspects of protein structure and synthesis, in the wider context of iron absorption and regulation in iron metabolism.[146]

In this study the prime interest is iron release from ferritin in vitro. Before embarking on this subject some general aspects of the ferritin biochemistry will be introduced, which have recently been reviewed.[86,87,123,124,125]

6.1.2 Ferritin: Occurrence, Structure and Function

Ferritin is widespread in nature. It has been identified in fungi, plant cells, annelid worms, shellfish, insects, fish, amphibians and mammals. In mammals it is mainly in the liver, spleen and bone marrow, but also at low concentration in serum and other tissues, including tumour cells.

Isoelectric focussing shows a great amount of heterogeneity among ferritins. Each tissue has a characteristic isoferritin profile, but many tissues have several isoferritins in common. A simple two subunit model was proposed to account for this heterogeneity.[126] In this model it is suggested that all tissue ferritins consist of only two polypeptide chains, with the molecular weights 21,000 and 19,000 designated H and L respectively. The H type predominates in the more acidic human isoferritins, e.g. the heart, and L in the more basic, e.g. liver and spleen. The relative proportions of these in any tissue will account for the heterogeneity which is correlated

with the isoelectric point distribution. (Fig. 6.1). The characteristic isoferritin profile changes to that characteristic of basic tissues in situations of iron overload,and to more acidic in cancer. This model, however, is now questioned.

Recently amino acid analysis and tryptic peptide maps indicated that the H and L subunits had extensive sequence homologies[127]and that after treatment of isoferritins with neuraminidase the acidic ferritins disappeared, probably due to the removal of sialic acid residues.[128] Further to this, a third glycosylated subunit "G" of molecular weight 23000 was identified in human serum ferritin from patients suffering with idiopathic haemochromatosis, suggesting that microheterogeneity of serum ferritin is largely due to glycosylation[129] rather than to variation in the proportion of H and L subunits.[148]

Although there is no mechanism in the human body for the physiological excretion of iron, this essential transition metal which is harmful if not effectively chelated in vivo, is temporarily stored in ferritin in a microcrystalline form consisting of ferric oxyhydroxide-phosphate[130] and micelles used as a reserve for later use. The iron content can vary from zero (apoferritin) to 4500 iron atoms per ferritin. Native ferritin is usually in a non-saturated state.

Fig. 6.1

Isoferritins

The two subunit (H and L) model proposed by Drysdale et al [126] to account for the heterogeneity of ferritins observed in isoelectric focussing.

Fig. 6.2

The role of the channels in ferritin.

In this model of ferritin the size of the ferritin channels are compared to the size of known molecules. Direct access to the iron core in ferritin is thought to be possible only if the chelators could pass throught the channels.

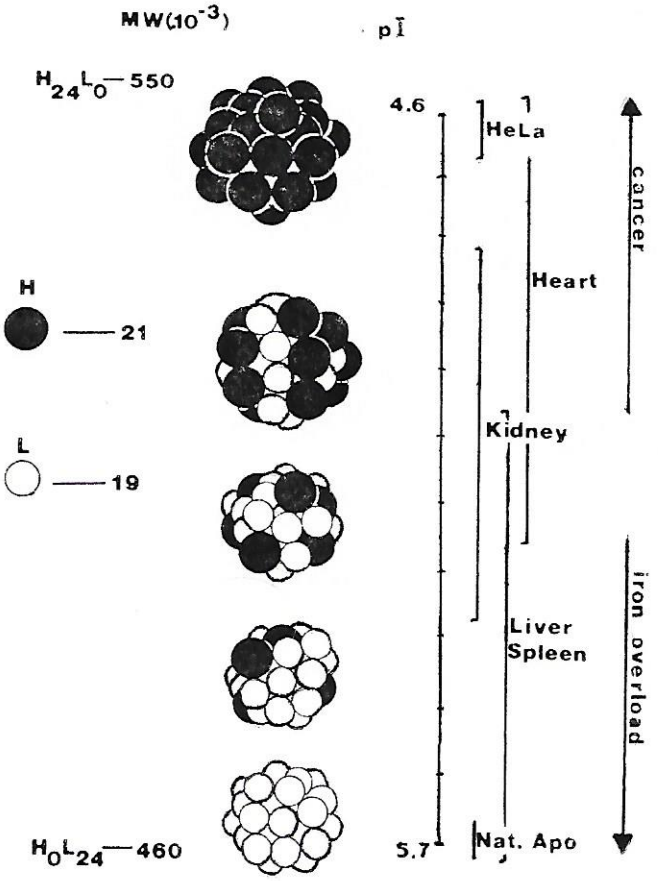

MW(10^{-3})
pI
$H_{24}L_0$—550
H
—21
L
—19
H_0L_{24}—460
4.6
5.7
HeLa
Heart
Kidney
Liver
Spleen
Nat. Apo
cancer
iron overload

FIG.6.1

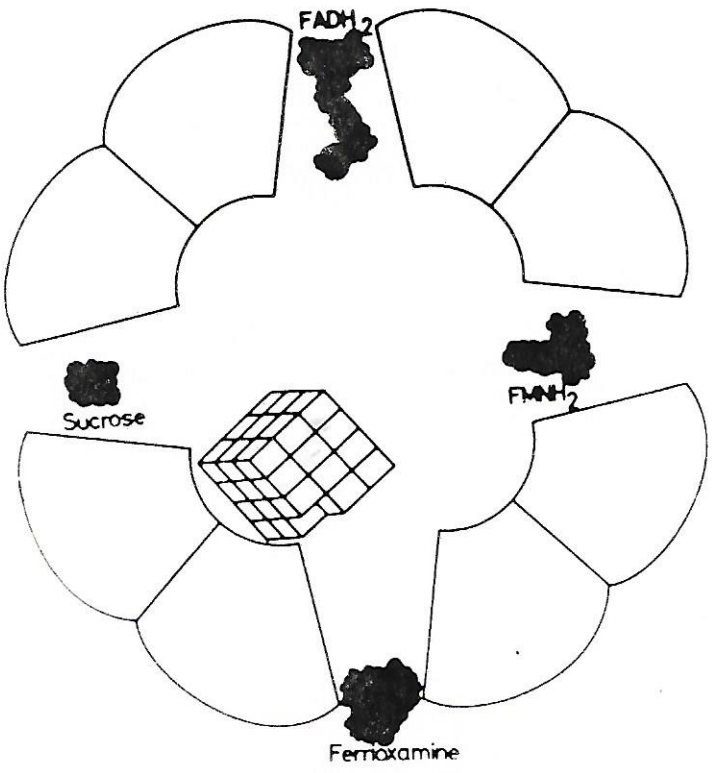

$FADH_2$
$FMNH_2$
Sucrose
Ferrioxamine

FIG.6.2

Apoferritin has a molecular weight of 450,000 with a sedimentation coefficient of 17-18 S. It has a compact spherical shape and is stable to heat and 10M urea at physiological pH. It consists of 24 subunits, and contains six channels which are thought to be the route of iron deposition and removal (Fig. 6.2). The protein shell has an internal and external diameter of 70-90 Å and 120-130 Å respectively. The channels have 9-13 Å and 17 Å external and internal diameters respectively, thus restricting entry of molecules larger than the channels.

6.1.3 Iron Deposition in Ferritin

The mechanisms involved in iron deposition in ferritin *in vivo* and *in vitro* are so far unknown. Ferritin formation and iron deposition were originally studied in relation to an *in vivo* response to iron administration.[131] There are, however, different opinions regarding the timing of the two events. In one of the mechanisms, formation of an iron aggregate is first thought to take place, followed by a subunit assembly, which forms a shell to enclose the iron core.[132] However, the generally accepted mechanism is the "protein first" hypothesis, that is, the formation of apoferritin occurs first, which is followed by a gradual uptake of iron with subsequent polymerisation in the central protein cavity.[125] Although keeping iron in a reduced state under physiological conditions is difficult, most theories of iron deposition involve the hypothesis that the entry of iron via the channels into

the protein cavity proceeds while iron is in the ferrous state. Oxidation is then thought to occur on catalytic sites on the walls of the channel[133] with the aid of an oxidising agent, or in the inner surface of the protein subunits.[134]

Once nucleation sites in the protein are formed, ferrous ion can be deposited and oxidised on the surface of the lattice, layer by layer with a rate dependent on the surface area. Iron removal will then be expected to proceed according to the "last-in, first-out" principle. That is, the iron molecules on the surface of the iron micelle will be the first to be removed.

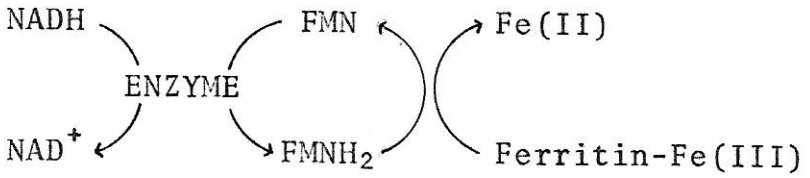
A new approach to the mechanism of iron deposition came about by the observation that iron(III) can be directly deposited in ferritin.[135] This was shown in experiments with ^{59}iron(III) where about 200 iron atoms per molecule remained bound to ferritin after extensive dialysis. These molecules were thought to be deposited on the micellar surface and not on to the protein. It seems, therefore, that the in vivo mechanism of iron storage in ferritin is still unsettled.

Another unexplained phenomenon associated with ferritin biochemistry is the presence of phosphate which is thought to be distributed on the micellar surface and throughout the iron core,[136] but there is no clear explanation of its role, if any, in iron storage.

6.1.4 Iron Mobilisation from Ferritin

The physiological processes involved in iron removal from ferritin *in vivo* are stillunknown. Iron, however, can be mobilised *in vitro* by a variety of mechanisms, mainly involving iron reduction, chelation and/or combination of both. The initial rate of iron release using 1,10-phenanthroline from ferritins of different iron content varies, a maximum being reached when ferritin is about one-third saturated (approximately 1300 iron atoms per molecule).[134] Thus ferritins of various iron content could, in principle, be involved in the regulation of iron release *in vivo*.

Reducing agents such as dithionite and thioglycollate are known to release iron from ferritin at pH 5 or below and naturally occurring intracellular reducing agents such as cysteine,glutathione and ascorbic acid could also facilitate iron release. However, in practice, such compounds are only capable of yielding less than 20% in 24h of the total ferritin iron.[137] Sirivech *et.al*[138] showed by measuring the formation of an iron(II) bipyridyl complex that reduced flavins can mobilise iron rapidly at physiological pH. An initial fast rate was attributed to the preferential reduction of the less polymerised iron present in the ferritin population. The following reductive mechanism was proposed:



The rate for iron(II) release by dihydroflavins, using ferritin loaded with 1200 iron atoms was about 42 atoms per minute per ferritin molecule. Thus it was estimated that total iron mobilisation by this process could be achieved within 1h.[123] Electron transfer and not the rate of diffusion through the channels was thought to be the rate determining step in the reaction because dihydroflavin derivatives covalently bound to sephatose failed to mobilise ferritin iron.[139]

In the past, xanthine oxidase and ferriductase were both thought to be involved with the iron release from ferritin in vivo, but at present, reduced flavins coupled to an NADH-FMN oxidoreductase[140] or ubiquinol FMN oxidoreductase[141] coupled with an iron(II) complexing agent are considered more likely to assume this role. Apart from reductants, iron(III) chelators are capable of mobilising iron from ferritin but at much slower rates. For this reason, these chelators have not been systematically studied, except for those involved or tested for the treatment of iron overload diseases.

In early studies Pape and co-workers[142] showed that iron could be mobilised from ferritin using high concentration of NTA, EDTA and sodium citrate, and more recently DFB and other iron(III) chelators were the subject of a study of iron mobilisation from ferritin.[143] DFB and rhodotorulic acid were found to be the most effective, whereas DHB, bipyridyl and pyridine-2-aldehyde-2-pyridyl hydrazone (PAPHY) removed substantially less iron. After the addition of FMN there was a substantial inhibition of iron mobilisation in the case of DFB and rhodotorulic acid but enhancement in the case of PAPHY and bipyridyl. DHB was a poor chelator of ferritin iron and it was also inhibited by FMN. Ascorbate but not oxalate and citrate had a positive effect on DFB and rhodotorulic acid iron mobilisation. It seems, therefore, possible that reducing agents, or other mediators, may have positive or negative effect in the iron release from ferritin.

A new group of synthetic chelators resembling the microbial siderophore enterobactin has recently been tested for iron release from ferritin but were found to be ineffective when used alone.[144] In the presence of ascorbic acid the rate of iron release was dependent on the ascorbic acid concentration, which was therefore assumed to act not only as a reductant, but also as a chelator, transporting iron from the ferritin core through the channels to the synthetic chelators on the outside.

TABLE 6.1

Iron Mobilisation from Ferritin by Known Chelators

LIGAND	[LIGAND] mM	ASCORBIC ACID m M	% IRON REMOVED			REF
			5h	6h	24h	
		6.0		0.0		144
		5.0	3.3		11.9	137
		100	14		45.4	137
DFB	1.0	-			10	143
	1.0	1.0		1.2		144
	1.0	6.0		2.6		144
DHB	1.0				2	143
Rhodotorulic Acid	1.0				11.5	143
PAPHY	1.0				4	143
3,4 LICAMS	6.0			0.0		144
3,4 LICAMS	6.0	6.0		5.0		144
$[Me]_3$MECAMS	0.3	6.0		5.2		144
EDTA	10			0.6	1.0	142
NTA	10			4.5	9.5	142
Sodium Citrate	10			0.3	0.4	142
Glutathione	5.0		2.1		7.0	137
Cysteine	5.0		4.7		15.3	137

Iron mobilisation from ferritin can therefore be achieved by using a variety of compounds or combinations of these, such as reducing agents, iron(II) and iron(III) chelators. Furthermore, the efficiency of these compounds will depend on their size and their ability to solubilise polynuclear iron. This process can be physiologically significant only if the rate of iron mobilisation is rapid and also if the iron complex formed after the mobilisation is stable.

6.2 Methods used to Study the Iron Mobilisation from Ferritin

Horse spleen ferritin (SIGMA TYPE I) was used without further purification. The iron content was estimated spectrophotometrically at λ = 420nm ($A^{1\%}_{1cm}$ = 100)[145] Ligand solutions were freshly prepared for each experiment. The iron mobilised was estimated using exctinction coefficients of the ligand iron(III) complexes (Chapter 3). The concentration of the ligand iron complex formed at different time intervals was estimated according to Beer Lambert's Law. Baseline corrections were carried out for each absorbance measurement and the appropriate buffer was used as a blank. Two general methods were adopted:

(A) Monitoring of the change in absorbance of the ligand-ferritin mixture, by taking the absorption spectrum of aliquots at different time intervals and then replacing them in the mixture;

TABLE 6.2

Conditions Employed in the Ferritin Experiments

EXPT.	LIGAND	METHOD	CONDITIONS	TEMP. °C
1	L_1	A	TRIS-HCl, $NaClO_4$ (0.1M, pH 7.4)	29.5
2	L_1	B	PBS pH 7.3	37
3	L_2	A	PBS, pH 7.3	26
4	L_2	B	PBS, pH 7.3	37
5	L_3	A	TRIS, HCl, $NaClO_4$ (0.1M, pH 7.4)	24.5
6	L_3	B	PBS, pH 7.3	37
7	L_5	A	PBS, pH 7.3	26
8	L_5	B	PBS, pH 7.3	37
9	L_6	A	TRIS HCl, $NaClO_4$ (0.1M, pH 7.4)	24.5
10	L_6	B	PBS, pH 7.3	37
11	L_7	A	TRIS-HCl, $NaClO_4$ (0.1M, pH 7.4)	24.5
12	L_4	A	PBS, pH 7.3	26

(B) Monitoring of the change in absorbance of the ligand iron complex alone and not of the ferritin ligand mixture as in (A).

Thus the ferritin solution (1.4ml) was enclosed in a dialysis tube and dialysed against a constant volume of ligand solution. The system was incubated at 37° and the absorption spectrum of dialysate aliquots were monitored at different times and then replaced in the total dialysate.

6.3 The Mobilisation of Iron from Ferritin by the Ligands

The progression curves of the reacting mixtures show a gradual increase of absorbance in the spectral region characteristic of the ligand iron(III) complexes. In the case of the L_4-ferritin mixture, the absorbance decreased due to precipitation of the L_4-iron complex. In figures 6.3 - 6.5 the plot of the change in absorbance (ΔA) versus time shows a gradual formation of the iron complex. The curves obtained in these plots show a similar kinetic mobilisation pattern for the majority of the newly synthesised ligands, which was characterised by an initial rapid removal of iron (7-10h) followed by a slower removal rate. The results of the experimental method B differ from A in that a lag phase in B was observed during the first 3h. The percentage iron removal data at 6 and 24h are shown in Table 6.3. More iron removal occurs under the conditions of method B, where the concentration of the ligands and the temperature of the reaction were higher.

Fig. 6.3

Iron mobilisation from ferritin using L_1, L_2, L_3, L_5 and L_6

Vertical Axis: Change in Absorbance i.e.

A_X (ligand-iron III) $-$ A_X (ligand)

where A is the absorbance at wavelength X

(X used for L_1 is 460, L_2 is 510, L_3 is 400, L_5 is 410, L_6 is 420).

Horizontal Axis: Time/h.

The experimental conditions are described in Table 6.2 and 6.3. Method B.

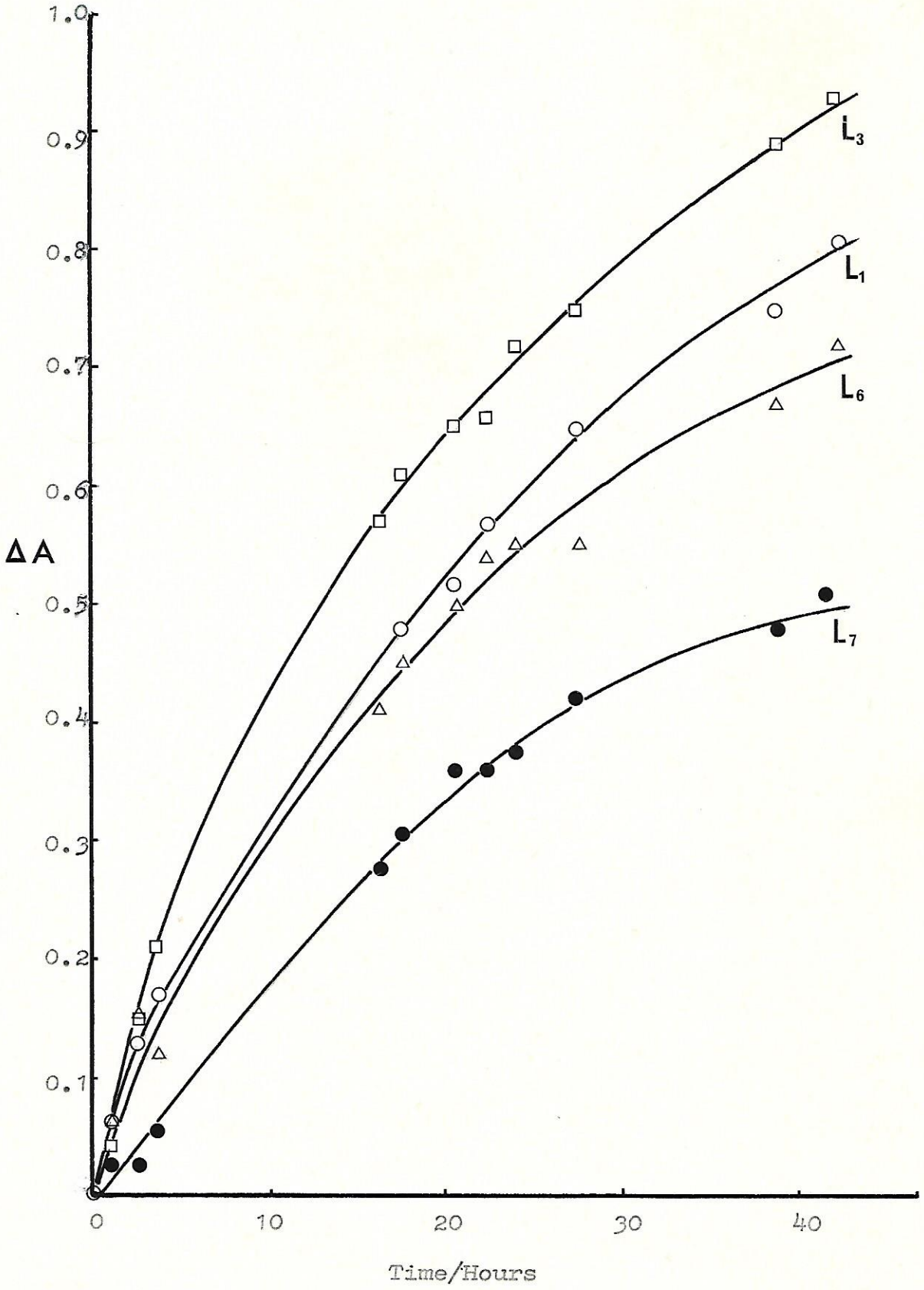

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ΔA
0
10
20
30
40
Time/Hours
L3
L1
L6
L7

Fig. 6.4

Iron mobilisation from ferritin using L_1, L_3, L_6 and L_7.

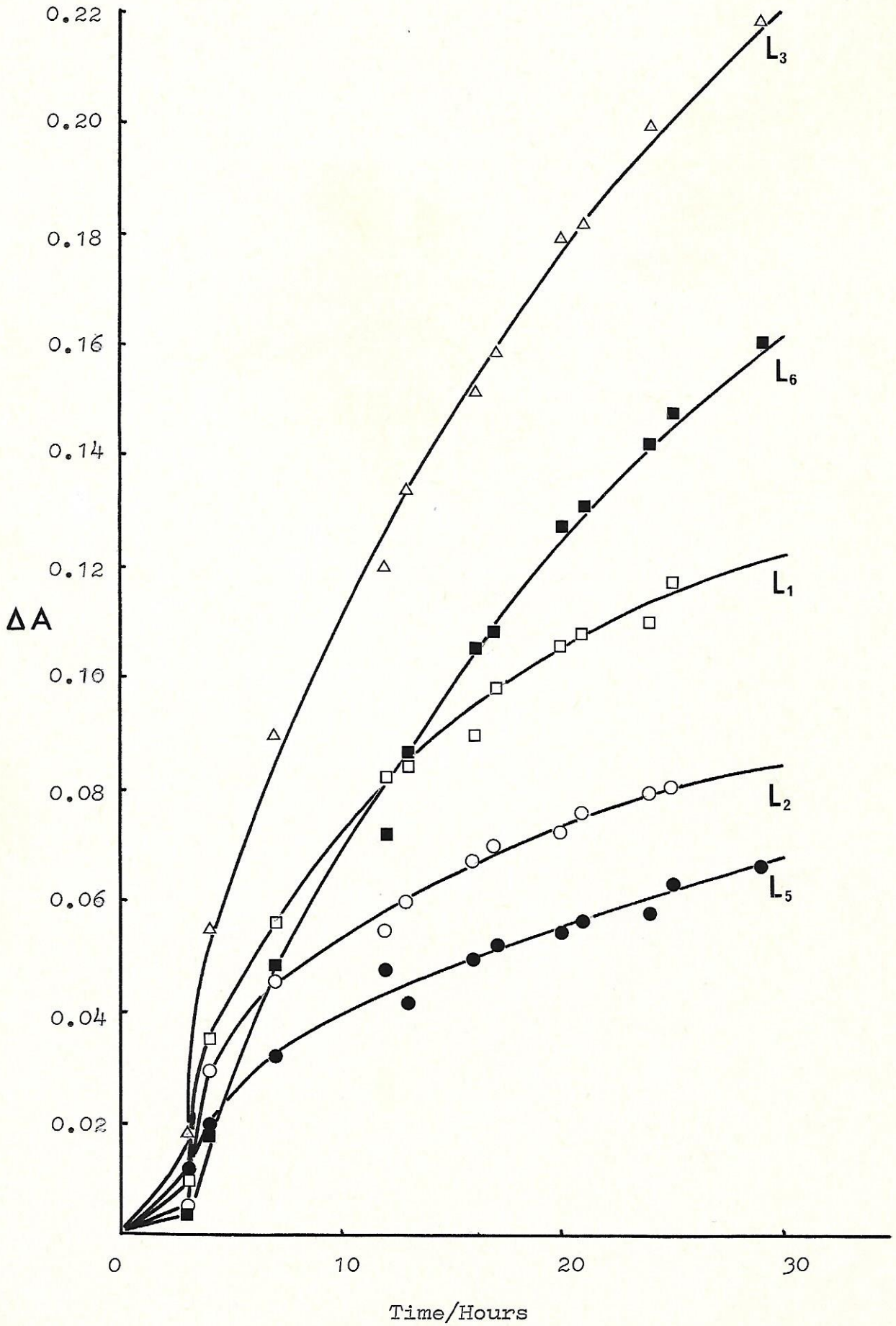
Vertical Axis: Change in absorbance i.e.

$Ax_{(at\ t\ =\ zh)} - Ax_{(at\ t\ =\ oh)}$

Where A is the absorbance of the ligand ferritin mixture at wavelength X, measured at different time intervals (z) after mixing (time zero) and where X for L_1 is 460, for L_3 400, for L_6 450 and for L_7 420nm.

Horizontal Axis: Time/h

The experimental conditions are described in Table 6.2 and 6.3 method A.


0.22
0.20
0.18
0.16
0.14
0.12
ΔA
0.10
0.08
0.06
0.04
0.02
L3
L6
L1
L2
L5
0
10
20
30
Time/Hours

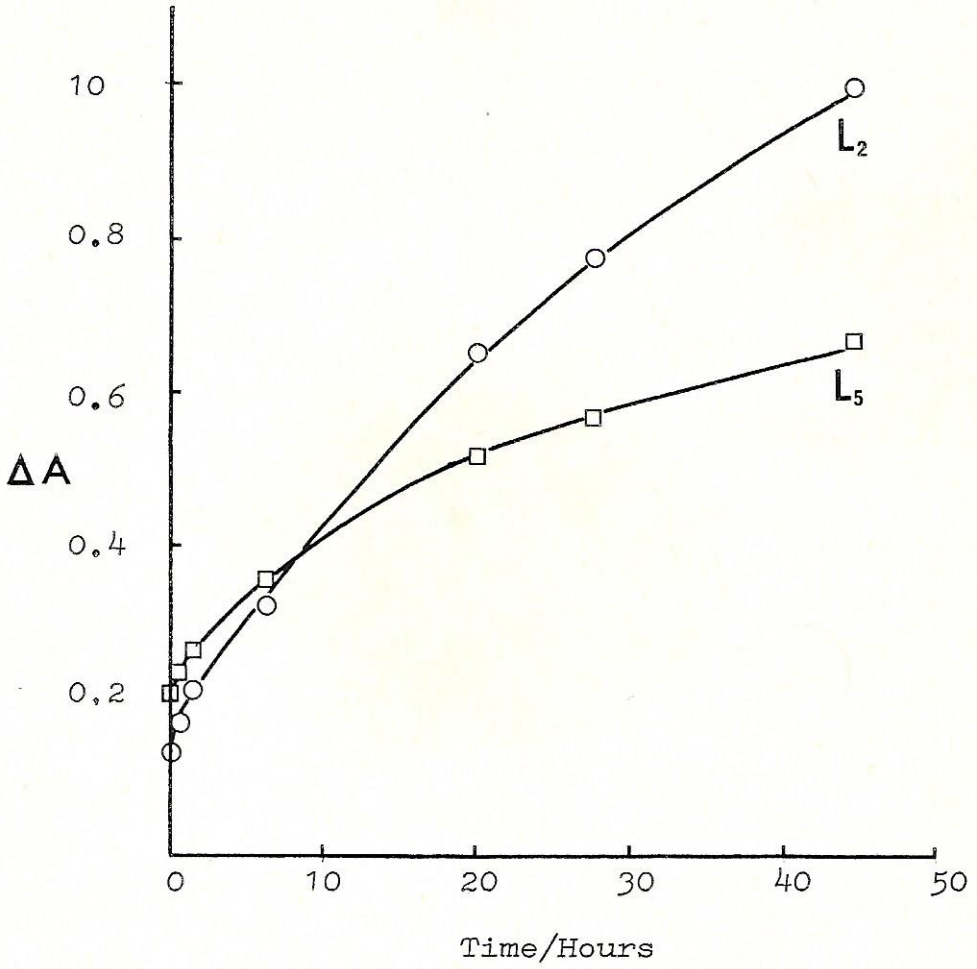


Fig. 6.5

Iron mobilisation from Ferritin using L_2 and L_5

Vertical Axis: Change in absorbance i.e.
Ax (ligand-Ferritin)-Ax (Ferritin)
where Ax is the absorbance at 510 nm for L_2 and 420 nm for L_5.

Horizontal Axis: Time/h.

The experimental conditions are described in Table 6.2 and 6.3 Method A.

TABLE 6.3

Percentage of Iron Removal from Ferritin by the Ligands

LIGAND	METHOD	[LIGAND]	[FERRITIN]	[Fe] IN FERRITIN	% IRON REMOVED	
		$X10^{-3}$ M	$X10^{-6}$ M	$X10^{-3}$ M	6h	24h
1	A	1.0	0.80	1.42	4.4	10.8
	B	3.8	0.98	1.74	13.9	31.4
2	A	4.0	1.22	1.78	2.6	8.2
	B	3.8	0.98	1.74	11.1	22.2
3	A	1.0	0.80	1.42	5.8	12.2
	B	3.8	0.98	1.74	21.9	53.5
4	A	4.0	1.22	1.78		>34[a]
5	A	4.0	1.22	1.78	1.2	3.7
	B	3.8	0.98	1.74	7.3	15.2
6	A	1.0	0.80	1.42	4.2	11.3
	B	3.8	0.98	1.74	12.9	46.3
7	A	1.0	0.80	1.42	2.0	7.5

a = the iron(III) complex was precipitating. Thus the percentage iron removed was estimated from the iron left in ferritin in the supernatant.

Although there is a great variation amongst the ligands in the amount of iron they remove from ferritin, they were all found to mobilise significant amounts in both experiments. Furthermore, temperature and ligand concentration are other factors contributing to this variation.

L_3 seemed to be the most efficient chelator of ferritin iron mobilisation in both types of experiments (A and B). Its efficiency increased with increasing concentration and temperature, causing the release of over half the ferritin iron in 24 h with B. L_6 and L_1 were the second best chelators in both experiments, showing similar pattern of iron release to that of L_3 regarding concentration, but not temperature in the case of L_1 where there was a drop in its efficacy at 37° for 24h probably due to ligand oxidation. L_2, L_4, L_5 and L_7 although they mobilise appreciable amounts of iron from ferritin, they were not as effective as the above ligands. Furthermore, L_4 iron complex was precipitating.

6.4 The Significance of Iron Mobilisation from Ferritin

From the results of the two preliminary experiments it is clearly established that all the new iron chelators are capable of mobilising iron from ferritin. This is an important additional property for iron chelators intended for clinical use in the treatment of iron overload.

Although more ligands and more experiments will be needed in order to establish a structure/activity correlation between the four classes of chelators and iron mobilisation from ferritin, it seems that the 2-hydroxy-pyridine-1-oxide derivatives (L_3, L_4, L_6 and L_7) are more efficient in this process than the 3-hydroxy-pyrid-4-one derivative (L_1). This is significant because the iron mobilisation efficiency from transferrin was the other way around. The order of efficiency of iron mobilisation from ferritin of the other two classes of ligands was similar to that of transferrin thus the 3-hydroxy-pyrid-2-one derivative (L_2) was the third most efficient in this respect and the 3-hydroxy-pyr-4-one- derivative (L_5) the last.

These two kinetic profiles, as characterised in experiments A and B, were also observed in other iron removal studies involving ferritin.[138,143] Furthermore, the amounts of iron mobilised from ferritin by the new synthetic ligands are comparatively higher than those reported for other iron chelators, even when the latter were facilitated by the synergistic effect of mediators (Table 6.1). Although hexadentate ligands like 3,4 LICAMS and DFB should be more effective chelators than the newly synthesised bidentate ligands of this work, because of the higher stability constants of the former, additional factors seem to be involved.

Kinetic accessibility is thought to be one major factor which influences the efficiency of chelators in mobilising iron from transferrin and ferritin. Thus the new synthetic ligands and the synthetic tricatechols[113] are capable of mobilising iron from transferrin whereas DFB is not without the presence of a mediator (NTA),[108] probably due to kinetic reasons. Iron mobilisation from ferritin was shown to be carried out by the new synthetic ligands and by DFB to a lesser extent,but not by the synthetic tricatechols, without the involvement of a mediator (ascorbic acid).[144] This ineffectiveness of the latter ligands seems to be due to their large size, thus it should always be borne in mind that molecular size is important in the design of new iron chelators, if ferritin iron mobilisation is desirable. It should also be emphasised that in most of the experiments where reducing agents like flavins were employed, the use of anaerobic conditions as well as that of an iron(II) chelator were essential in the iron mobilisation. However, such anaerobic conditions and effective iron(II) chelators are not likely to prevail in vivo.

The fact that the new chelators are clearly mobilising appreciable amounts of iron(III) from ferritin without the mediation of a reductant is of considerable relevance to the mode of iron mobilisation and deposition in ferritin. Further detailed studies involving this particular problem require investigation.

In the light of these results some other important observations and useful conclusions could be made regarding ferritin biochemistry in general and the design of new iron chelators in particular. During this study it was shown that in some cases the extent of iron removal from ferritin depended on the ligand concentration. Although a more detailed experiment is needed to substantiate this statement, it will be of great interest in vivo to find whether increased non-toxic concentrations of ligand could result in higher iron excretions.

The rate of iron mobilisation from ferritin is very slow compared to that of transferrin. This could, in a way, be explained in the kind of iron these proteins carry, namely, polynuclear in the former in which case chelation proceeds slowly, and mononuclear in the latter, in which chelation is faster.[57] Since these proteins are thought to be in equilibrium with the transient iron pool, an iron(III) chelator would be more efficient if it could mobilise iron from transferrin and the "transient" iron pool in a short time than from ferritin alone. However, ideally, in critical situations of iron overload, it may be best to have direct access to ferritin bound iron.

The dialysis technique developed and used in the experimental method B, for the determination of mobilised iron is simple and an affective procedure which could be used for future screening programs of new iron chelators.

The ability of some of the new compounds to remove iron from ferritin *in vitro*, much more efficiently than any other known iron(III) chelators previously reported, further increases the prospects of some of them being useful in the clinical treatment of iron overload diseases.

CHAPTER SEVEN

ANIMAL STUDIES

7.1 *In vivo* Systems for Screening Iron Chelators

The encouraging results from the *in vitro* studies with ferritin and transferrin, where iron was removed by a range of different ligands, prompted the examination of the ligands' ability to remove iron from iron-overloaded animals. Several methods have been developed for screening iron chelators *in vivo*.

Cumming *et al*[150] induced iron overload in rats by daily injections of 5mg of iron in the form of iron sorbitol citrate for a period of two weeks. The rats were left to stabilise for one week and then DFB was administered daily (10mg/day) for a two week period. At the end of a further week the rats were killed and their total ferritin and iron ferritin was estimated from homogenates of liver, spleen, kidneys and intestinal mucosa. The levels were compared to other groups of rats which were iron overloaded but did not receive any DFB and to normal rats, some of which were treated and some not treated, with DFB. From this study it was calculated that the iron removed by DFB from the liver amounted to 70 per cent of the total iron removed.

Rats were also the animals selected for screening iron chelators by the clinical biochemists of the Rockefeller University, New York, namely Graziano,[151] Grady,[27] Cerami[152] and their co-workers. Donor rats were exsanquinated

by cardiac puncture and their blood stored in acid citrate dextrose at 4°C. Prior to transfusion by intraperitoneal injection the blood was heated to 50°C for 5 min and the recipient rats received the equivalent of one blood volume per week for three weeks and half blood volume per week for at least a further six weeks. One week prior to drug testing the rats were placed on "low iron diet" and housed individually in metabolic cages. The urine and faeces were collected over 24h periods and the iron, together with other metals, was quantified by atomic absorption.

Gralla[153] has suggested a bioassay system for screening iron chelators based on organ examination in addition to iron excretion. In a preliminary study to establish an animal model, tissues, species, sex and age variants were used to examine iron accumulation. Initially it was thought that female hemisplenectomised mice were the suitable model. It was shown that when compounds were administered for seven consecutive days to such mice, which were concurrently transfused with two volumes of heated mouse blood, iron accumulation in the spleen for an active chelator was <0.025µg/mg of wet splenic tissue, compared to at least 100µg/mg for an inactive one.

The screening system was then extended to incorporate other procedures useful in the determination of iron excretion. Thus forty mice were divided into eight groups of five. A non-transfused, non-treated group and a transfused non-treated group were used as controls. Another

transfused group was treated with a drug standard, e.g. DFB 200mg/kg/day and the remaining groups received an experimental drug at the LD10 dose daily for seven days. The mice were housed in groups in metabolic cages and daily collections of urine were analysed throughout the treatment period. A day after the last drug injection the mice were killed by exsanquination and their liver and spleen iron determined by atomic absorption. The experiment was repeated so that ten mice were tested in all with each drug and compared to the same number of control ones. The main task of the above experiment termed "the primary screening system", was to show inhibition in the uptake of transfused iron by drug administration during transfusion. It was suggested that a secondary screening system should include the treatment of animals after transfusion, and a third, the depletion of naturally accumulating tissue iron especially haemosiderin . In all the screening systems the iron content of the excreta should provide additional evidence for iron depletion activity.

A modified method of the above[153] was recently introduced by Pitt et al.[31] in which, DFB was included as a reference standard in each assay and furthermore, the time of the administration of the test compound was changed to two days after the last transfusion rather than the same time as the transfusion.

Hoy and co-workers[32] used a different animal model to test the oral ability of pyridoxal isonicotinoyl hydrazone (PIH) to mobilise iron in vivo. Rats were injected subcutaneously first with iron-sorbitol-citric acid complex (jactofer, 10mg/day) for two successive days and then, immediately after the second injection, ^{59}iron(III) citrate (5μCi). The rats were left for a week and then placed in metabolic cages 4 days per week. Different test substances were administered at different doses by a stomach tube or subcutaneously at the beginning of the fourth day after overnight starvation. The radioactivity of the urinary and faecal excretions of each animal was monitored separately and each day's counts were expressed as a percentage of the first day. Finally, the animals were killed and their livers and spleens homogenised. The non-haem iron content was determined. From this study it was concluded that PIH increases iron faecal excretion in rats by mobilising iron from the liver.

In an attempt to localise the site of iron mobilisation of DFB in rats, Hershko[22] differentially labelled the liver reticuloendothelial cells and the parenchymal cells using ^{59}iron labelled heat-damaged erythrocytes for the former cells and ^{59}Fe-ferritin or ^{59}Fe-transferrin for the latter ones. In both cases hypertransfusion was carried out by two intravenous injections of 2ml of packed red blood cells per 100g body weight on four days and one day prior to the administration of ^{59}iron in the prescribed form. One day after the ^{59}iron injection almost

all of the radioactivity was recovered in the ferritin fraction of tissue homogenates, except for about 25% which was soluble non-ferritin iron. Although iron from the reticulo-endothelial cells was recycled into the plasma within a few days, the release of iron from the parenchymal cells was very slow. To avoid the possible interference in the interpretation of the results due to this difference in iron release, DFB was continuously infused for 24h immediately after the labelling. In this study it was concluded that there are two independent pathways of iron chelation by DFB in the rat in vivo. The first involves intracellular chelation of iron in the parenchymal cells and eventual biliary excretion and the second, extracellular iron chelation from reticuloendothelial cells when transferrin is saturated, resulting in urinary excretion.

A similar procedure for labelling liver parenchymal cells by injecting intravenously ^{59}Fe-ferritin was recently adopted by Pippard et al.[33] for screening iron chelators using normal and iron loaded rats. Iron loading was carried out by feeding the rats with a diet containing 10g of iron per kg in the form of ferrous sulfate, for five days per week over a 5-6 week period. A regular diet was given on the other two days each week. In all their studies food was removed the night before the chelator test. Administration of the chelator was carried out 2h after the injection of ^{59}Fe-ferritin, because iron was found to be in maximum availability between the second and sixth hour after the injection. They suggested that iron was

chelated while in transit within the hepatocyte and not when it was accumulated in endogenous ferritin where it was relatively unavailable. After the administration of the chelators (DFB, DHB, DTPA, EDHPA, PIH) almost all of the iron was excreted through the bile. The ratio of ^{59}iron to total bile iron was thought to be independent of both the chelator and its doses in normal rats, whereas that ratio was reduced in iron loaded rats. In this study, it was concluded that normal rats could be used for a rapid assay of iron chelating agents and also, that EDHPA and PIH are comparable iron chelators to DFB. Furthermore, they were reported to be orally effective.

In the study reported in this thesis experiments were undertaken in conjunction with University College Medical School, Department of Clinical Haematology, where a method of testing iron removal from mice had been previously developed. The procedure adopted was based on that developed by Young et al.[154] but differed insofar as ^{59}Fe-lactoferrin was used instead of ^{59}Fe-citrate. This change was adopted because it had been previously shown that when human ^{59}Fe-lactoferrin is injected in rats ^{59}iron mainly accumulated in the liver as ferritin rather than in red blood cells.[155] It was therefore considered that by using this procedure the ^{59}iron removed by any ligands would be that associated with iron stores. This animal model is relatively inexpensive, reliable and convenient. Basically male albino mice were overloaded with iron dextran, subsequently labelled with ^{59}Fe-

lactoferrin, housed individually in plastic cages and their total excretion (faeces plus urine) of ^{59}iron was measured before and after the drug administration.

Initially a preliminary toxicological study was undertaken as several compounds used in this work had not been previously tested for toxicity in animals. Thus compounds L_1 - L_6 were administered intraperitoneally at a dose of 12.5 mg per mouse. Those ligands, which were apparently non toxic, were subsequently screened for their ability to remove iron. The drugs were administered via two routes intraperitoneally and intragastrically. The percentage of ^{59}iron excreted was measured before and after the ligand administration and was used to compare the ability of different ligands at iron removal. Mice, to which no ligand was administered, were adopted as controls. Further to this, DFB was included in the study in order to compare its well documented ability to remove iron with the new compounds reported in this work. Three other aspects concerning the removal of iron were studied, namely the influence of starvation, the repetitive application of the ligands, on their efficiency of facilitating the removal of iron and the duration of ligand action.

7.2.1 Toxicity Studies

The melting point, NMR and IR spectra were used as criteria for the purity of the compounds. No further purification was undertaken for commercial samples (L_5 and DFB). The different ligands were dissolved in PBS by

TABLE 7.1

Toxicity of Ligands when Administered Intraperitoneally to Normal Mice

LIGAND	DOSE mg/mouse	NO. OF MICE	SURVIVAL TIME
L_1	12.5	2	> 4 months
L_2	12.5	2	> 4 months
L_3	12.5	2	> 4 months
L_4	12.5	2	20 minutes
	1.33	1	> 4 months
	0.67	1	> 4 months
	0.13	2	> 4 months
	0.01	2	> 4 months
L_5	12.5	2	> 4 months
L_6	12.5	2	10 minutes
	1.33	1	> 4 months
	0.67	1	> 4 months
	0.13	2	> 4 months
	0.01	2	> 4 months
PBS	1 ml	2	> 4 months

mixing at 20°C (L_1, L_2, L_4) or at 60°C (L_3, L_5, L_6). After standing for 1h at 20°C the ligands were administered intraperitoneally to the mice (25g). The amounts of ligand used and the survival times are shown in Table 7.1. It was observed that L_4 and L_6 were toxic at doses of 500 mg kg^{-1}.

7.2.2 Iron Loading and Labelling of Mice

Two groups of mice, A and B, were studied at two chronologically different periods using the procedure outlined in Table 7.2. Iron loading was induced by injecting a total of 8mg of iron dextran over a three week period and ^{59}iron labelling by administering ^{59}Fe-lactoferrin intravenously (2μCi ^{59}iron per mouse).

7.2.3 ^{59}Iron-Lactoferrin Preparation and Profile of Excretion in Mice

Lactoferrin (16mg) isolated from human milk[156] was dissolved in Tris HCl (pH 7.3 0.1M) containing $NaHCO_3$ (10mM). $^{59}Fe\ Cl_3$ (32μCi) was mixed with ferrous ammonium sulphate (total iron 22.4μg) and added to the lactoferrin solution (1.4μg iron/1mg lactoferrin), thus generating lactoferrin virtually saturated with iron(III).[155] PBS was added making a total of 3.2 ml of ^{59}Fe-lactoferrin solution.

Mice were warmed under an infra-red lamp for five min and then injected via the tail vein (0.2ml, 2μCi, 1mg ^{59}Fe lactoferrin per mouse). This procedure was difficult causing some loss of ^{59}Fe-lactoferrin during injection

TABLE 7.2

Typical Procedure of Iron Loading and Ligand Testing

DAY	ADMINISTRATION	CONDITIONS	
		No. of mice per cage	Collection of excreta
1	iron dextran	7	
8	iron dextran	7	
15	iron dextran	7	
22	iron dextran	7	
36	^{59}Fe-lactoferrin	7	
46		1	
47		1	✓
52	Ligand ip or ig	1	✓
58	Ligand ip or ig	1	✓

ip = intraperitoneal administration
ig = intragastric administration.

Dose per injection:

iron dextran: 2 mg (5mg/ml)

^{59}Fe-lactoferrin: 0.2ml, 2μCi ^{59}Fe, 1mg/mouse

Ligand: 10mg/mouse

and occasional bleeding. However, these difficulties do not fully account for the great variation in the level of ^{59}iron excretion observed (Table 7.3). Such fluctuations must be largely attributed to the differential capacity of individual mice to excrete iron.

As the work described in this thesis was the first time that mice had been labelled with ^{59}Fe-lactoferrin, a study was undertaken to examine the excretion profile of ^{59}iron of five mice following the ^{59}Fe-lactoferrin injection for a period of 14 days (Fig. 7.1). There was no ligand administration to these five mice during this period. Thus the excreta of each mouse were collected daily and placed in a large centrifuge tube. The radioactivity per tube per 200 secs was recorded manually using a Harwell 2000 Modular Single Channel Counter. Each tube was placed in the same geometric position for counting and all the readings were corrected for ^{59}iron decay.

The pattern and the level of excretion of ^{59}iron (Fig. 7.1) differs between the five mice studied. In each individual there is relatively high ^{59}iron excretion rate over the first few days which gradually decreases and reaches a relatively constant level after about a week. Thus it can be suggested from these results, that a relatively steady excretion can be achieved prior to ligand administration, using this procedure.

The comparative aspects of the rate of ^{59}iron excretion was also studied in all the mice which had been administered with ^{59}Fe-lactoferrin. Table 7.3 shows the main daily

Fig. 7.1

Administration of ^{59}Fe-lactoferrin.

The ^{59}iron excretion profile of five mice of group B, from the day after ^{59}Fe-lactoferrin administration to the day before the ligand administration.

Vertical Axis: Counts per minute (CPM)

Horizontal Axis: Time/days.

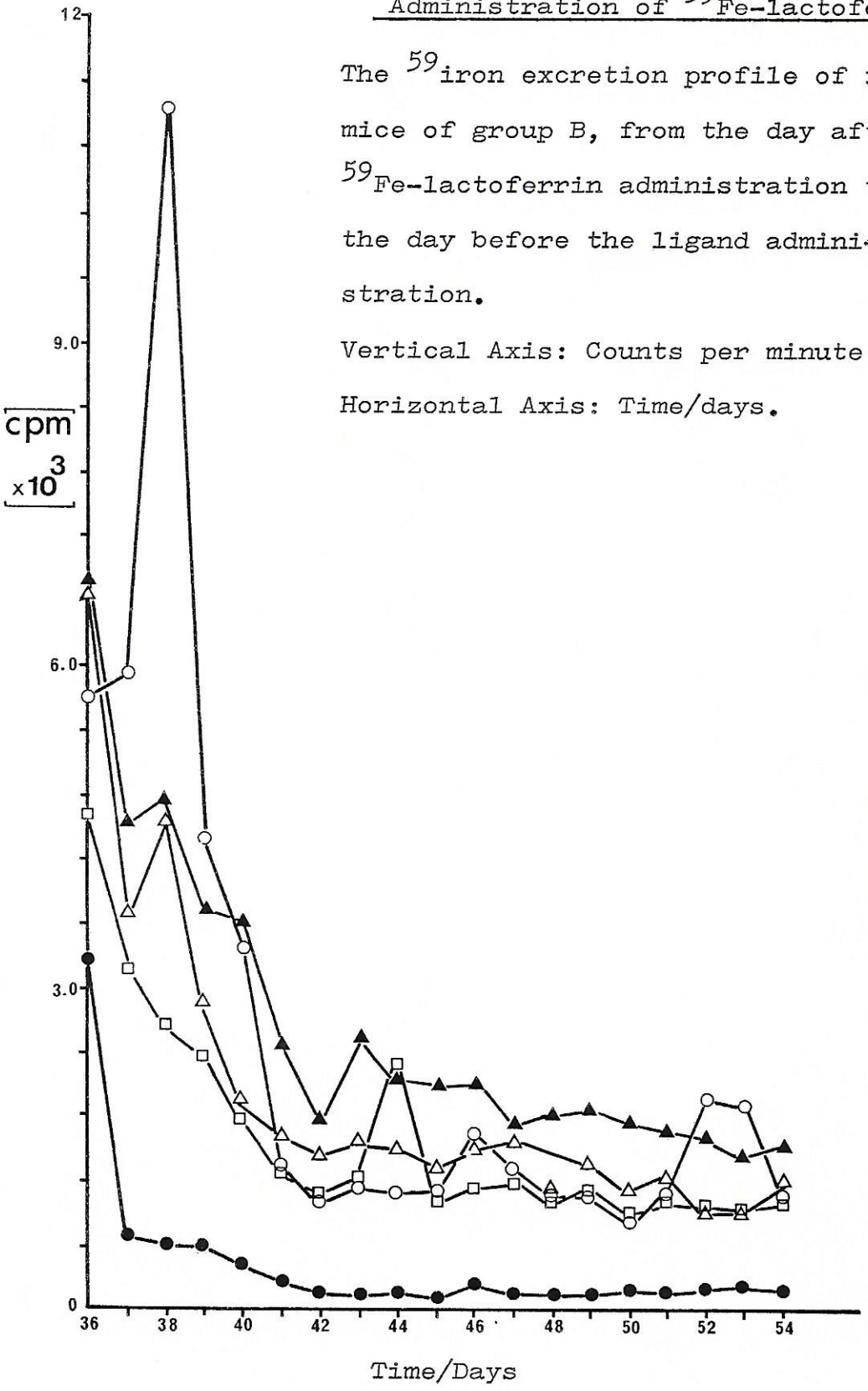


TABLE 7.3

Differential ^{59}iron Excretion of Mice

MICE GROUP A			MICE GROUP B	
MOUSE	Expt.1	Expt.2	MOUSE	Expt.3
	($\bar{X}$ ^{59}Fe/cpm)[a]			($\bar{X}^{59}$Fe cpm)[a]
M_1	1832	1927	M_1	2250
M_2	2764	2806	M_2	1222
M_3	1623	2910	M_3	1083
M_4	2191	2249	M_4	577
M_5	2159	2389	M_5	2085
M_6	1811	2044	M_6	608
M_7	1759	1812	M_7	491
M_8	2828	2258	M_8	2126
M_9	1595	1300	M_{10}	863
M_{10}	2244	3148	M_{13}	1859
M_{11}	1530	1555	M_{14}	1518
M_{12}	2125	1742	M_{15}	186
M_{13}	2173	2266	M_{16}	1214
M_{14}	1521	1369	M_{17}	928
			M_{18}	1522
			M_{19}	1650
			M_{20}	676
			M_{21}	856
			M_{23}	1126

a = $\bar{X}$ ^{59}Fe cpm - is the mean of ^{59}iron excretion 3 days (Group A) or 4 days (Group B) before the ligand administration.

Mice group A and B: Two groups of mice studied in different chronological order.

Mx = Number of an identified mouse after it was caged individually.

Expt X = Number of an experiment involving ligand administration, see later pages.

^{59}iron excretion of either three or four days. There is considerable variation in the excretion levels of the group A mice (mean of 3 days) ranging from 1290 to 3150 cpm and even greater in the group B mice (mean of 4 days) ranging from 180 to 2250 cpm. In the second group B, there were two mice of very low ^{59}iron excretion, M_{15} and M_7 of 186 and 491 cpm respectively. The mice in group B were graded as high (1650-2250 cpm) M_1, M_5, M_8, M_{13}, M_{19}, medium (1083-1522 cpm) M_2, M_3, M_{16}, M_{14}, M_{18}, M_3 and low (577-928cpm) M_4, M_{21}, M_{10}, M_{17}, M_6, M_{20} ^{59}iron excretors.

There was some variation in the iron excretion profile of mice used as standards. However, the degree of variation was relatively small 98% ± 12% SD (Fig. 7.2). These fluctuations are of minor significance compared to the ^{59}iron excretion enhancement observed due to ligand administration.

7.3 Experiments Designed to Test the Ligands' Ability to Remove Iron

Two groups of mice, A and B, were studied at two different chronological periods. First group A and then group B, for ^{59}iron removal using the different ligands. The series of experiments which were carried out are described in Table 7.4. The average weight of the mice, in both groups was 33g.

Fig. 7.2

^{59}Iron excretion profile of mice used as standards*.

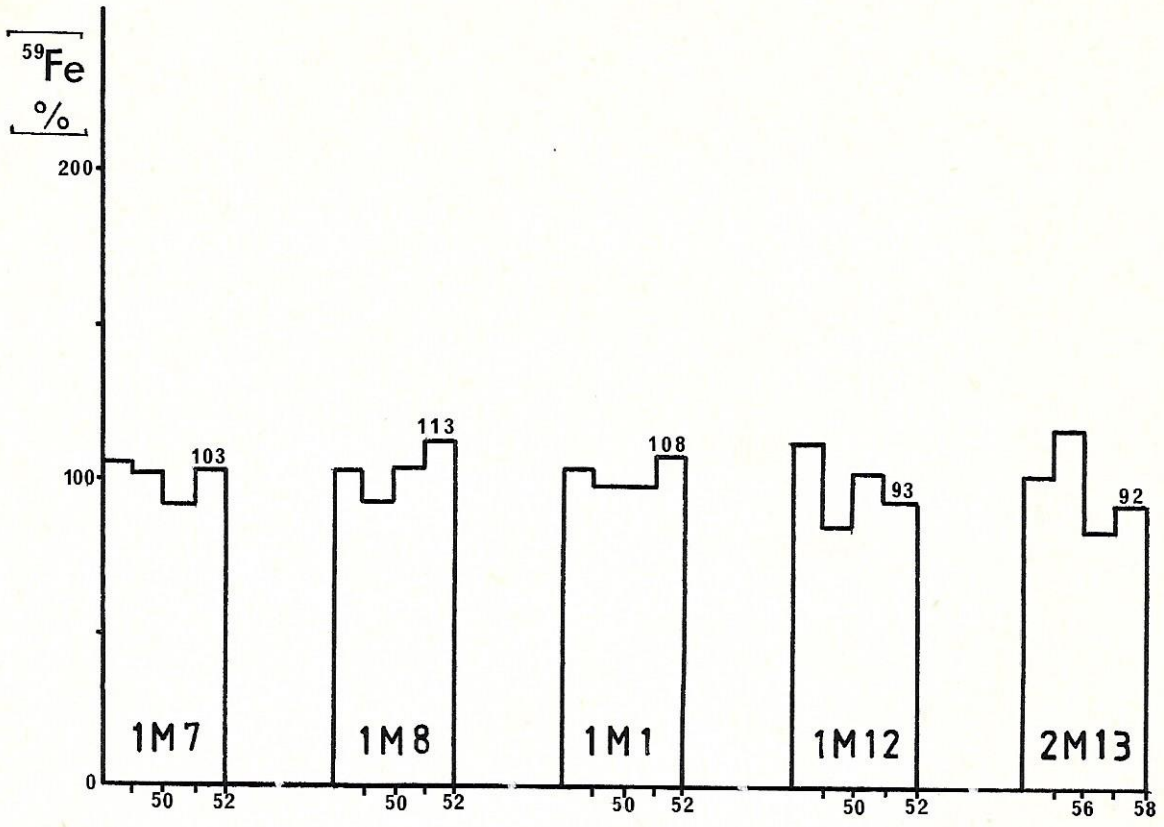
Vertical Axis: Percentage ^{59}Iron excretion.

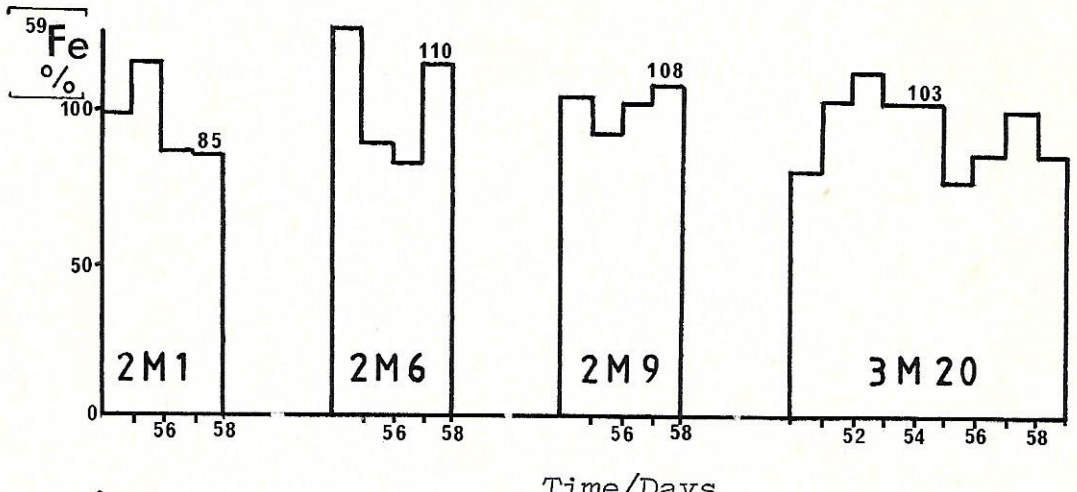
Horizontal Axis: Time/days.

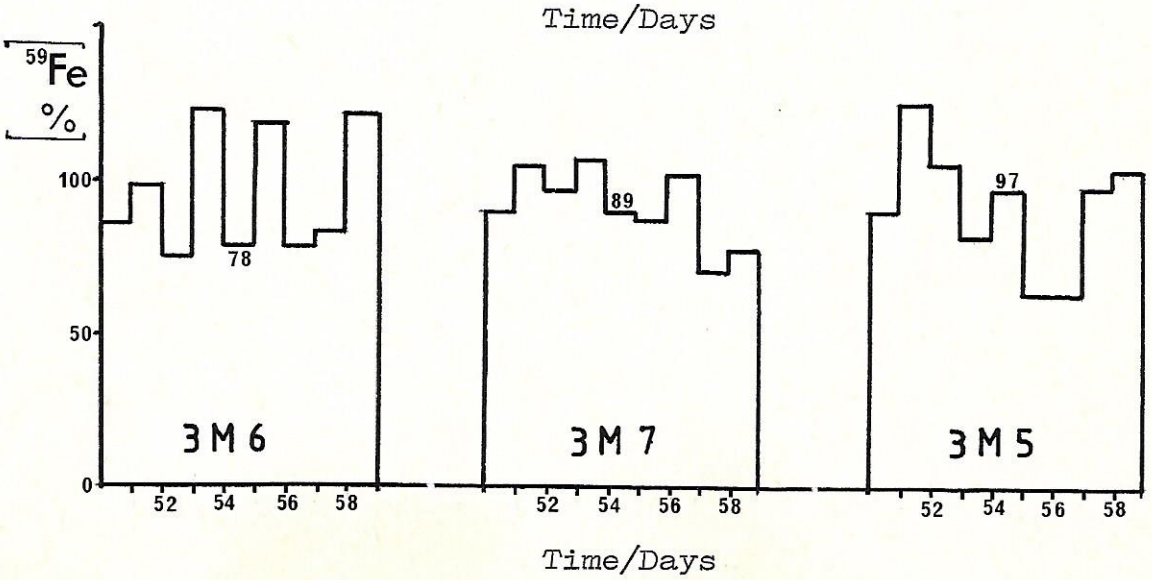
XMn: X is the experiment number, Mn is the mouse number.

Percentage ^{59}Iron excretion: Is determined on each individual day by taking the mean value (100%) of 3 (Expt. 1 and 2) or 4 (Expt. 3) days before the recorded value of the specified day (indicated by the % value at the head of the column).

*No ligand was injected in the periods shown.


59Fe
%
200
100
0
103
113
108
93
92
1M7
1M8
1M1
1M12
2M13
50
52
50
52
50
52
50
52
56
58
Time/Days


59Fe
%
100
50
0
85
110
108
103
2M1
2M6
2M9
3M 20
56
58
56
58
56
58
52
54
56
58
Time/Days


59Fe
%
100
50
0
78
89
97
3M 6
3M 7
3M 5
52
54
56
58
52
54
56
58
52
54
56
58
Time/Days

7.3.1 Experimental Procedure Associated with Group A Mice

Fifteen mice were loaded with iron and labelled using iron dextran and ^{59}Fe-lactoferrin respectively as described in Table 7.2. Ten days after the ^{59}Fe-lactoferrin injection, the mice were housed individually in plastic cages with a nylon film wrap attached to the bottom. These mice then received two sets of intraperitoneal injections of ligand, six days apart, termed experiment 1 and experiment. 2. The excreta were collected every 24h before and after the administration of the ligands. Each nylon film wrap was removed and the excreta then placed in a large plastic centrifuge tube. The radioactivity was counted as described earlier in this chapter. The mean value ($\bar{X}$) of the counts over the three days immediately prior to drug administration was taken as a 100% excretion. Thus all the daily excretions were recorded as percentages of this arithmetic mean.

7.3.2 Experimental Procedure Associated with Group B Mice

The same procedure of iron loading and ^{59}iron labelling as that of group A was carried out for the 24 mice of this group. Before drug administration, mice were selected and placed in five groups so that for each of the five ligands, three mice of relatively high, medium and low ^{59}iron excretion rates were selected (section 7.2.3). The mice M_6, M_7, M_{15}, M_{20} were excluded from the ligand testing and were used as standards.

Each ligand was administered both intraperitoneally and after 72h intragastrically (Table 7.4, expt. 3). Collection of excreta and counting was carried out in an identical manner to that described for the group A mice. With group B the mean value of ^{59}iron excretion of four days before the intraperitoneal administration was taken as a 100% excretion and as before, all the daily excretions after correction for ^{59}Fe decay, were recorded as percentages of this arithmetic mean.

Three other aspects affecting ^{59}iron excretion were briefly studied, namely, the duration of ligand action, the repetitive administration of ligands and the effect of starvation, as shown in expt. 2, expt. 4 and expt. 5 respectively in Table 7.4.

7.4.1 Comparative Efficiency of Ligands

Figures 7.3 - 7.7 depict the efficacy of different ligands to remove iron from iron overloaded mice.

1,2 Dimethyl-3-hydroxy-pyrid-4-one (L_1) was the most successful compound as judged by its ability to remove iron.When it was applied intraperitoneally to seven mice there was an increase in the percentage of ^{59}iron excretion (267 ± 68% SD) (Fig. 7.3). There was a similar response from four mice which had received the same dose of L_1 intragastrically. One mouse ($3M_{23}$) had the highest excretion in this study of about 425% when L_1 was applied intragastrically. The results depicted in Fig. 7.3 are clearly reproducible.

TABLE 7.4

Experiments Involving Ligand Administration to Iron Loaded Mice

EXPERIMENT	GROUP OF MICE	NUMBER OF MICE	LIGANDS	ROUTE OF ADMIN.	DOSE OF LIGANDS mg	COLLECTION INTERVALS h	OTHER COMMENTS
Expt.1	A	M_1, M_2	L_1	ip	10	24	
		M_3, M_4	L_2	ip	10	24	
		M_5, M_6	L_3	ip	10	24	
		M_7, M_8	L_4	ip		24	
		M_9, M_{10}	L_5	ip	10	24	
		M_{11}, M_{12}	L_6	ip		24	
		M_{13}, M_{14}	DFB	ip	10	24	
Expt.2	A	M_1, M_2	L_1	ip	10	12 & 24	
		M_3, M_4	L_2	ip	10	12 & 24	
		M_5, M_6	L_3	ip	10	12 & 24	
		M_9, M_{10}	L_5	ip	10	12 & 24	
		M_{13}, M_{14}	DFB	ip	10	12 & 24	
Expt.3	B	M_1,M_2,M_{23}	L_1	ip & ig	10	24	ig 3 days after ip
		M_3, M_4,M_5	L_2	ip & ig	10	24	ig 3 days after ip
		M_8,M_{21},M_{16}	L_3	ip & ig	10	24	ig 3 days after ip
		M_{10},M_{13},M_{14}	L_5	ip & ig	10	24	ig 3 days after ip
		M_{17},M_{18},M_{19}	DFB	ip & ig	10	24	ig 3 days after ip
Expt.4	A	M_3, M_4	L_1	ig	10	24	Administration of L_1 ig 24h after L_2 ip
Expt.5	A	M_{11}, M_{12}	L_3	ig	10	24	starvation
		M_7, M_8	-	-	-	24	starvation
STANDARDS	A	M_1,M_6,M_7,M_8 M_9,M_{11} M_{12},M_{13}	-	-	-	24	-
	B	M_5,M_6 M_7,M_8	-	-	-	24	-

ip = intraperitoneal administration
ig = intragastric administration

Fig. 7.3

Profile of percentage ^{59}iron daily excretion from mice treated with L_1.

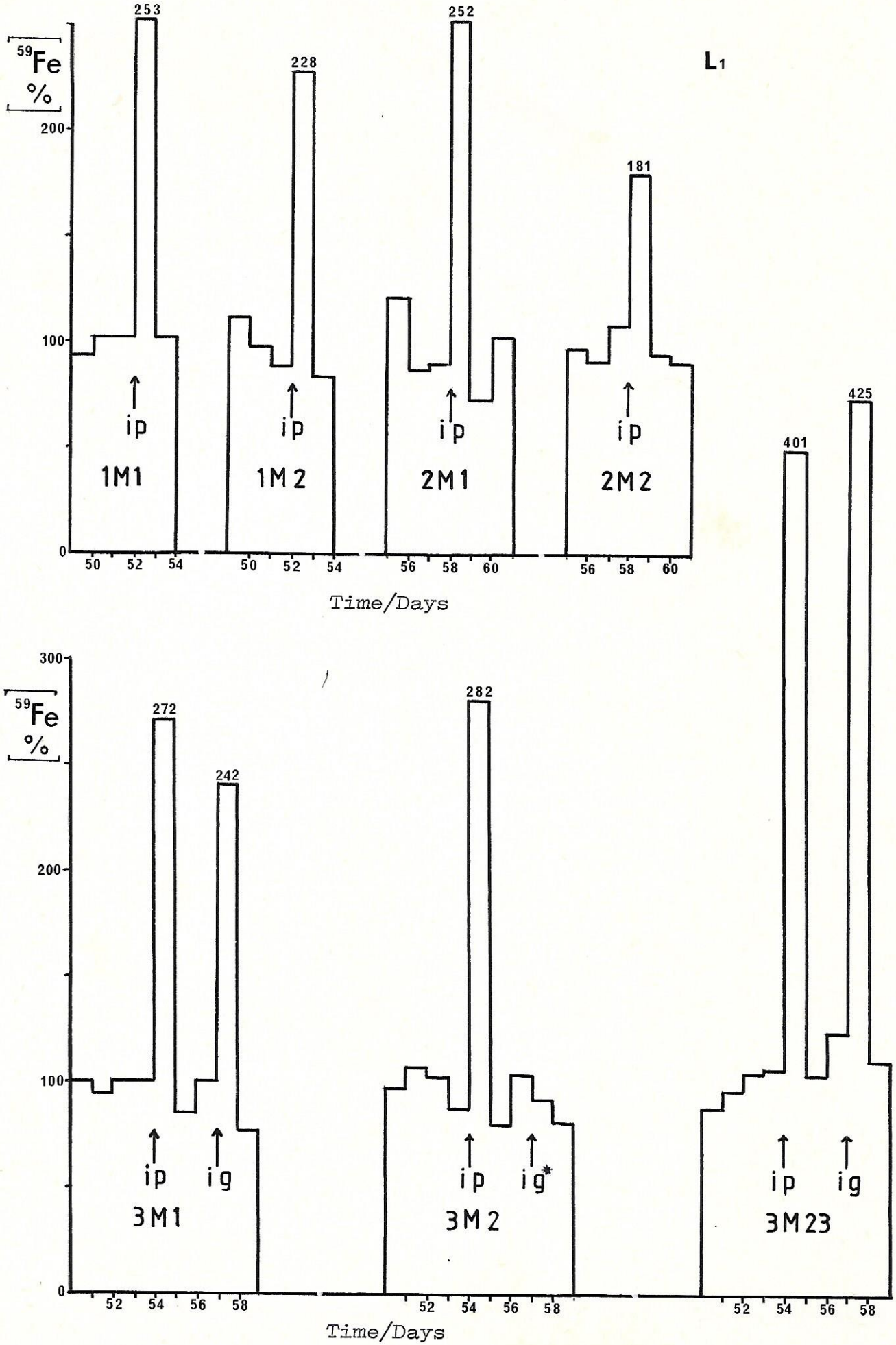
Vertical Axis: Percentage ^{59}iron excretion.

Horizontal Axis: Time/days.

XMn: X is the experiment number, Mn is the mouse number.

Percentage ^{59}iron excretion: Is determined on each individual day by taking the mean value (100%) of 3 (Expt. 1 and 2) or 4 (Expt. 3) days before the recorded value of the specified day (indicated by the % value at the head of the column).

ip and ig are intraperitoneal and intragastric administration respectively of 10 mg/mouse (ig* 1.5 mg/mouse).


59Fe %
L1
253
228
252
181
425
401
272
242
282
ip
1M1
1M2
2M1
2M2
3M1
3M2
3M23
ig
ig*
Time/Days

When 1-methyl-3-hydroxy-pyrid-2-one (L_2) was administered intraperitoneally there was an increase in the percentage of ^{59}iron excretion (146 ± 31% SD, seven mice), but this increase was not uniform in all mice. For example, mice $2M_4$ and $3M_5$ experienced only low excretion increases (107% and 118% respectively) whereas $2M_3$, $3M_3$ experienced much higher rate enhancement (178% and 192% respectively). This non-uniform response to L_2 is also apparent in the intragastric administration of this ligand (182 ± 95% SD). Although mice $3M_3$ and $3M_4$ had high excretion (232% and 242% respectively) mouse $3M_5$ failed to demonstrate a response (Fig. 7.4).

2,4-Dihydroxy-pyridine-1-oxide (L_3) is another chelator which consistently caused a substantial increase in ^{59}iron excretion in all the mice studied (Fig. 7.5). When it was administered intraperitoneally in seven mice and intragastrically to four mice it caused (217 ± 59% SD) and (201 ± 78% SD) respectively.

2-methyl-3-hydroxy-pyr-4-one (L_5) was injected in seven mice intraperitoneally causing only a slight increase in ^{59}iron excretion (Fig. 7.6) (131 ± 28% SD). In one case there was a decrease in ^{59}iron excretion (mouse $3M_{10}$). Intragastric administration of L_5 to three mice showed no detectable enhancement in ^{59}iron excretion (100 ± 21% SD).

Desferrioxamine (DFB) enhanced the excretion of iron when applied intraperitoneally (Fig. 7.7), (341 ± 85% SD). It was effective in all the mice studied, although there

Fig. 7.4

Profile of percentage ^{59}iron daily excretion from mice treated with L_2.

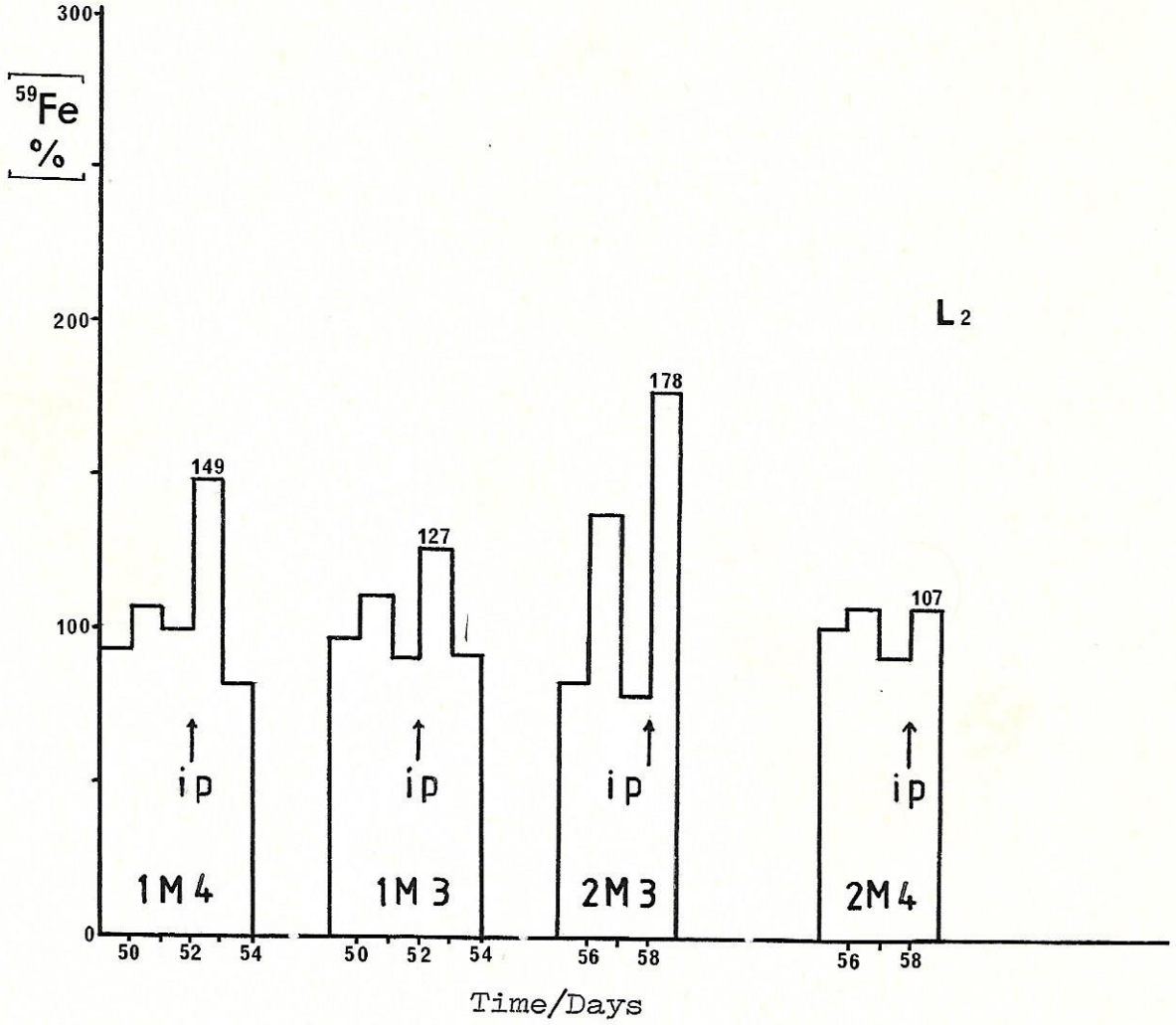
Vertical Axis: Percentage ^{59}iron excretion.

Horizontal Axis: Time/days.

XMn: X is the experiment number, Mn is the mouse number.

Percentage ^{59}iron excretion: is determined on each individual day by taking the mean value (100%) of 3 (Expt. 1 and 2) or 4 (Expt. 3) days before the recorded value of the specified day (indicated by the % value at the head of the column).

ip and ig are intraperitoneal and intragastric administration respectively of 10 mg/mouse.


59Fe %
300
200
100
0
L2
149
127
178
107
ip
ip
ip
ip
1M4
1M3
2M3
2M4
50 52 54
50 52 54
56 58
56 58
Time/Days

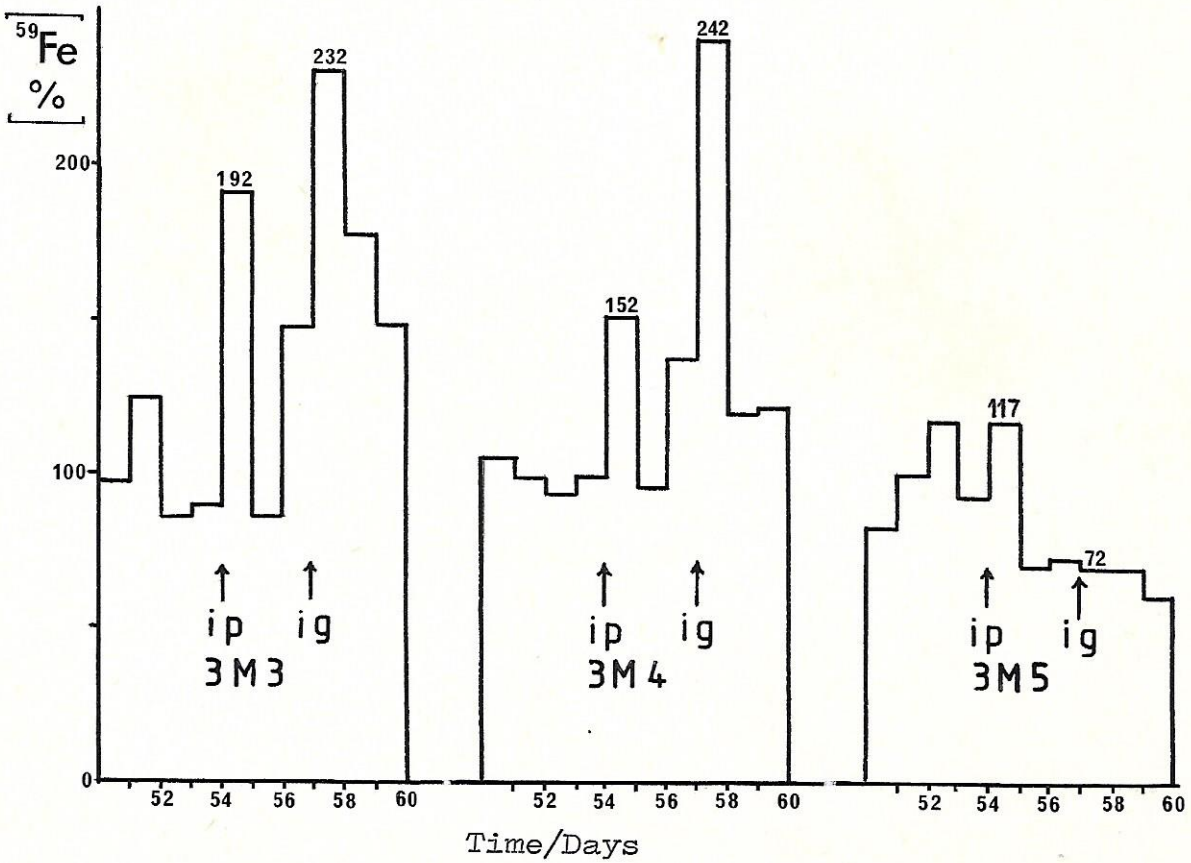

59Fe %
200
100
0
192
232
242
152
117
72
ip ig
ip ig
ip ig
3M3
3M4
3M5
52 54 56 58 60
52 54 56 58 60
52 54 56 58 60
Time/Days

Fig 7.5

Profile of percentage ^{59}iron daily excretion from mice treated with L_3.

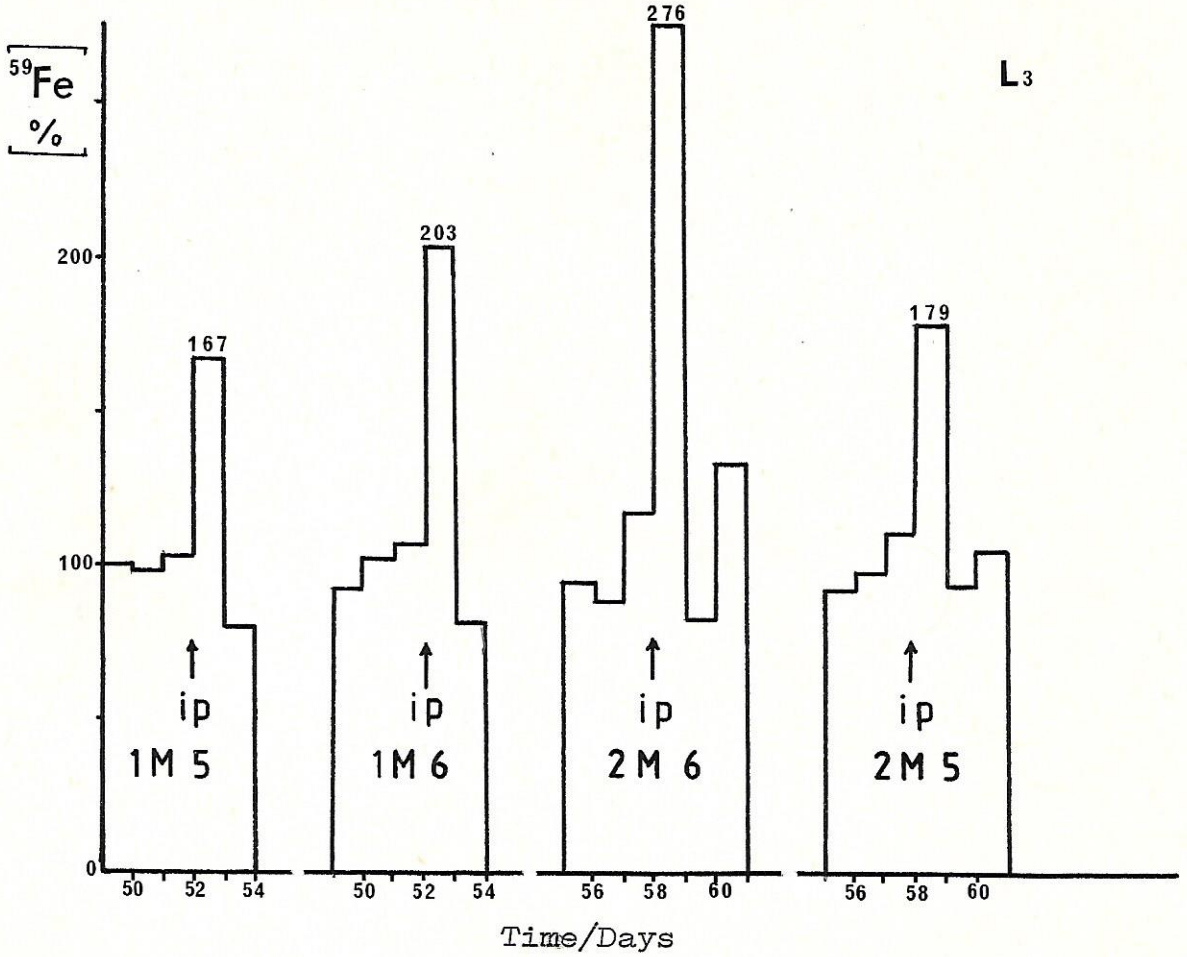
Vertical Axis: Percentage ^{59}iron excretion.

Horizontal Axis: Time/days.

XMn: X is the experiment number, Mn is the mouse number.

Percentage ^{59}iron excretion: is determined on each individual day by taking the mean value (100%) of 3 (Expt. 1 and 2) or 4 (Expt. 3) days before the recorded value of the specified day (indicated by the % value at the head of the column).

ip and ig are intraperitoneal and intragastric administration respectively of 10 mg/mouse.


59Fe
%
L3
276
203
179
167
200
100
0
ip
1M 5
ip
1M 6
ip
2M 6
ip
2M 5
50 52 54
50 52 54
56 58 60
56 58 60
Time/Days

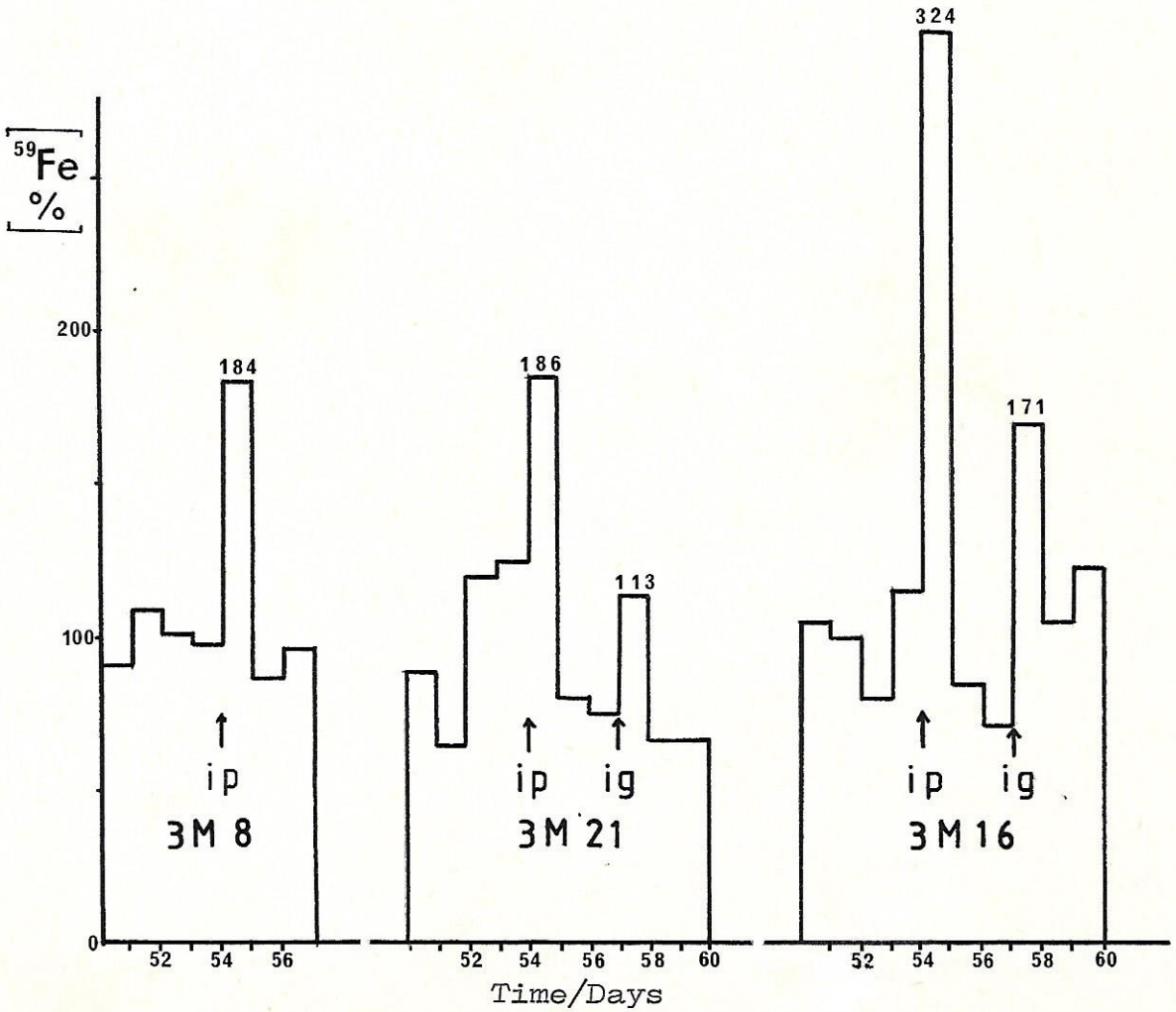

59Fe
%
324
184
186
171
113
200
100
0
ip
3M 8
ip ig
3M 21
ip ig
3M 16
52 54 56
52 54 56 58 60
52 54 56 58 60
Time/Days

Fig. 7.6

Profile of percentage ^{59}iron daily excretion from mice treated with L_5.

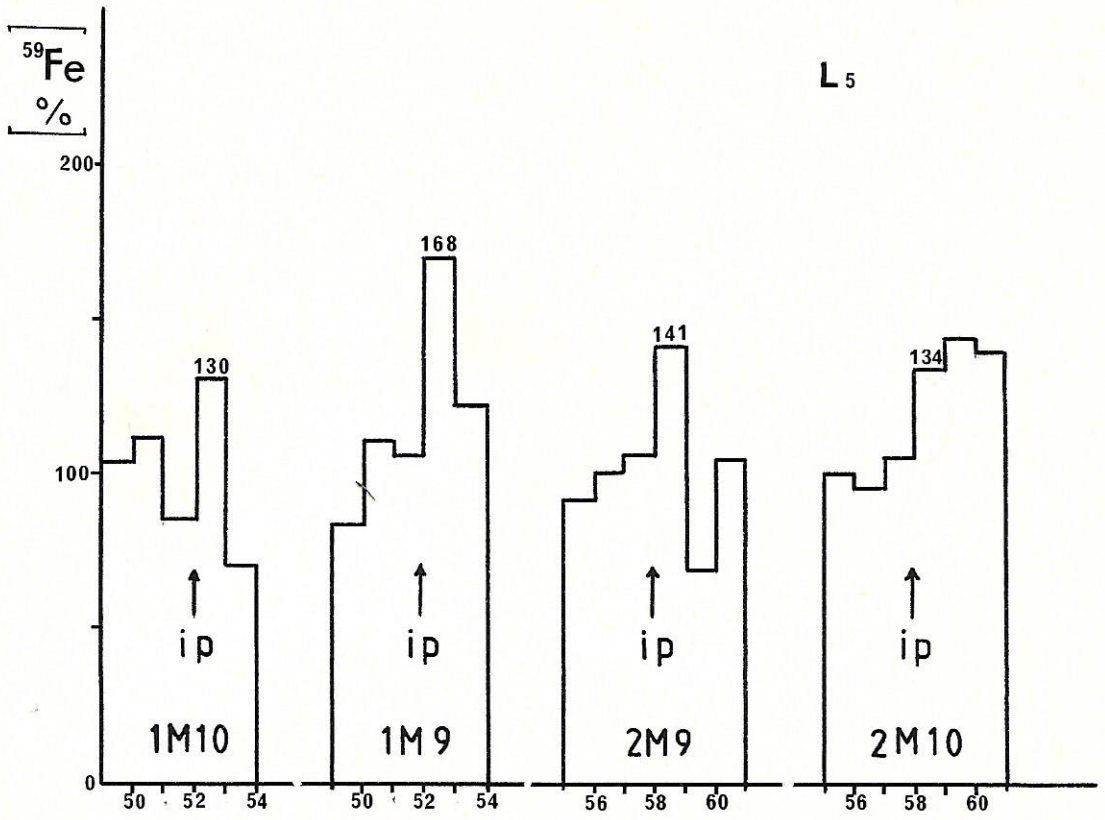
Vertical Axis: Percentage ^{59}iron excretion.

Horizontal Axis: Time/days.

XMn: X is the experiment number, Mn is the mouse number.

Percentage ^{59}iron excretion: is determined on each individual day by taking the mean value (100%) of 3 (Expt. 1 and 2) or 4 (Expt. 3) days before the recorded value of the specified day (indicated by the % value at the head of the column).

ip and ig are intraperitoneal and intragastric administration respectively of 10 mg/mouse.


59Fe
%
L 5
200
100
0
130
168
141
134
ip
ip
ip
ip
1M10
1M 9
2M9
2M10
50 52 54
50 52 54
56 58 60
56 58 60
Time/Days

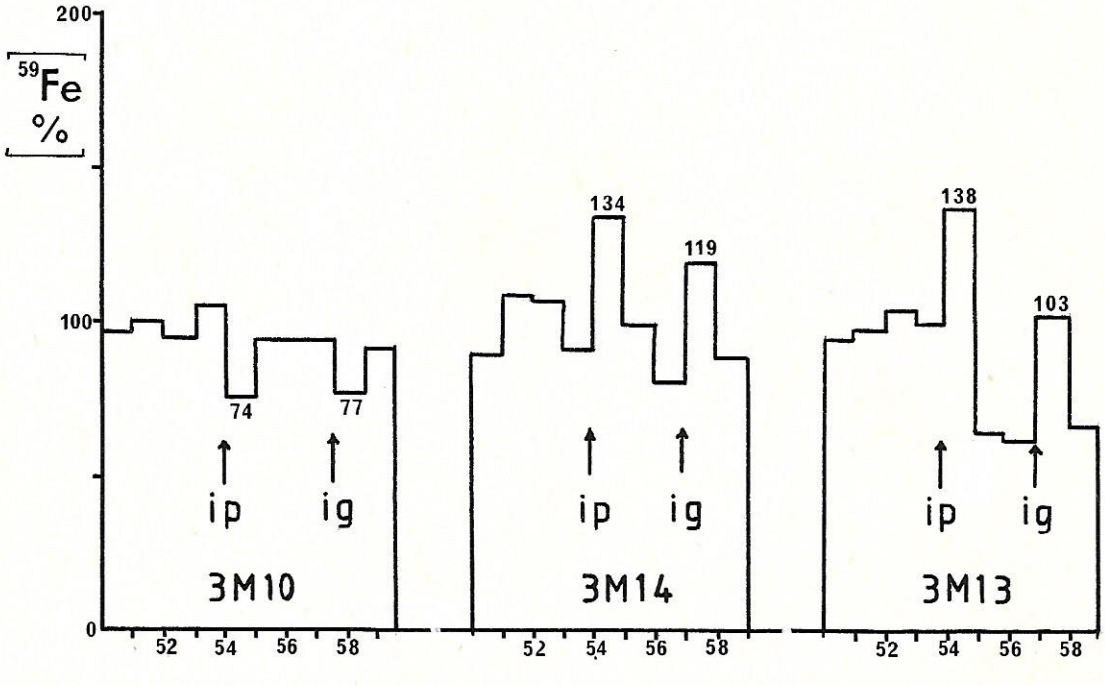

200
59Fe
%
100
0
74
77
134
119
138
103
ip ig
ip ig
ip ig
3M10
3M14
3M13
52 54 56 58
52 54 56 58
52 54 56 58
Time/Days

Fig. 7.7

Profile of percentage ^{59}iron daily excretion from mice treated with DFB.

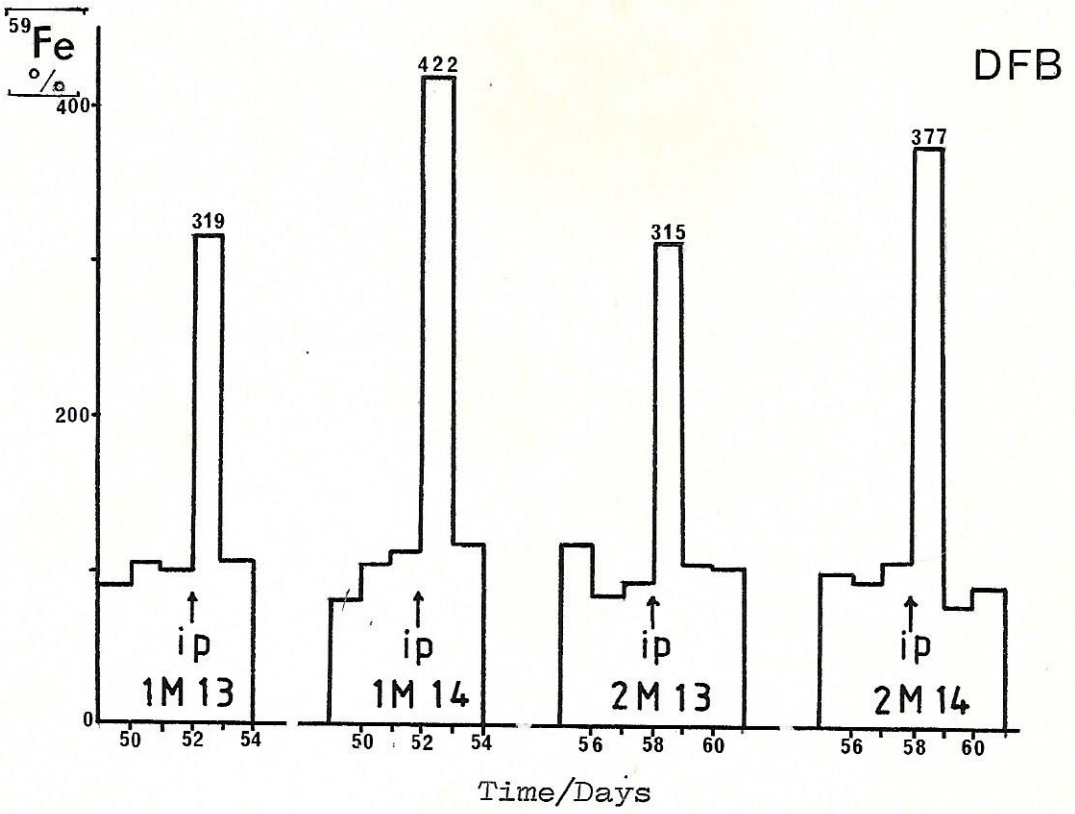
Vertical Axis: Percentage ^{59}iron excretion.

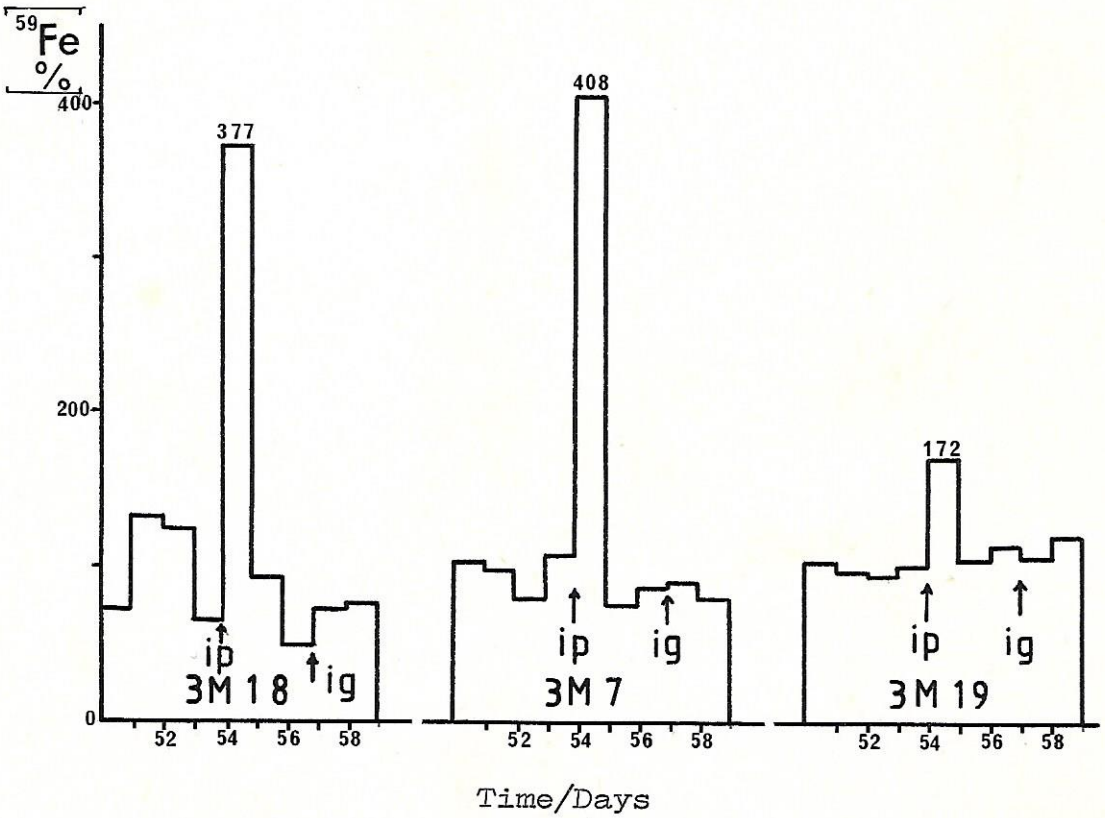
Horizontal Axis: Time/days.

XMn: X is the experiment number, Mn is the mouse number.

Percentage ^{59}iron excretion: is determined on each individual day by taking the mean value (100%) of 3 (Expt. 1 and 2) or 4 (Expt. 3) days before the recorded value of the specified day (indicated by the % value at the head of the column.)

ip and ig are intraperitoneal and intragastric administration respectively of 10 mg/mouse.


59Fe
%
400
200
0
DFB
319
422
315
377
ip
1M 13
ip
1M 14
ip
2M 13
ip
2M 14
50
52
54
50
52
54
56
58
60
56
58
60
Time/Days


59Fe
%
400
200
0
377
408
172
ip
ig
3M 18
ip
ig
3M 7
ip
ig
3M 19
52
54
56
58
52
54
56
58
52
54
56
58
Time/Days

were some variations in the level of ^{59}iron excretion, e.g. mouse $3M_{19}$ had relatively low iron excretion compared to the other mice. DFB failed to remove any iron when it was applied intragastrically to three mice (Fig. 7.7) (91 ± 16% SD).

The mean values of the percentage ^{59}iron excretion induced in mice by the range of drugs and those non-treated by drugs (standards) (Fig. 7.8) was plotted for comparative purposes. This presentation facilitates the comparison of drugs efficacies to remove iron from iron overloaded mice when applied either intraperitoneally or intragastrically.

7.4.2 Other Aspects Studied Related to Iron Excretion

(a) The Duration of Ligand Action

As can be seen from Table 7.4 (expt. 2), excreta collections were carried out at both 12h and 24h intervals. This procedure was adopted with all ligands. Without exception, it was clear that the duration of ligand action occurs within 12h of the ligand administration. Some examples are given in Fig. 7.9. It should therefore be considered that all the expressed 24h period results during the ligand administration are really the sum of the increased percentage ^{59}iron excretion of the first 12h due to ligand administration plus the 12h period when there is no ligand action. Thus if a typical 24h ^{59}iron excretion resulting from ligand administration is 300% the percentage over the first 12h period becomes 500%.

Fig. 7.8

Comparison of the efficacy of ligands
to mobilise iron from iron overloaded mice.

Plot of the Mean $\pm$ Standard Deviation of percentage ^{59}iron excretion.

Vertical Axis: Percentage ^{59}iron excretion.
Closed columns represent the mean percentage of ^{59}iron excretion.
Open columns represent the standard deviation of this mean.

Horizontal Axis: Lx-mice treated with an X ligand.
St-mice used as standards where no ligand was administered.
n-number of mice studied.
ip,ig: intraperitoneal and intragastric administration.

Percentage ^{59}iron excretion: is determined on each individual day by taking the mean value (100%) of 3 (Expt. 1 and 2) or 4 (Expt. 3) days before the recorded value of the specified day (indicated by the % value at the head of the column , Figs. 7.2 – 7.7)

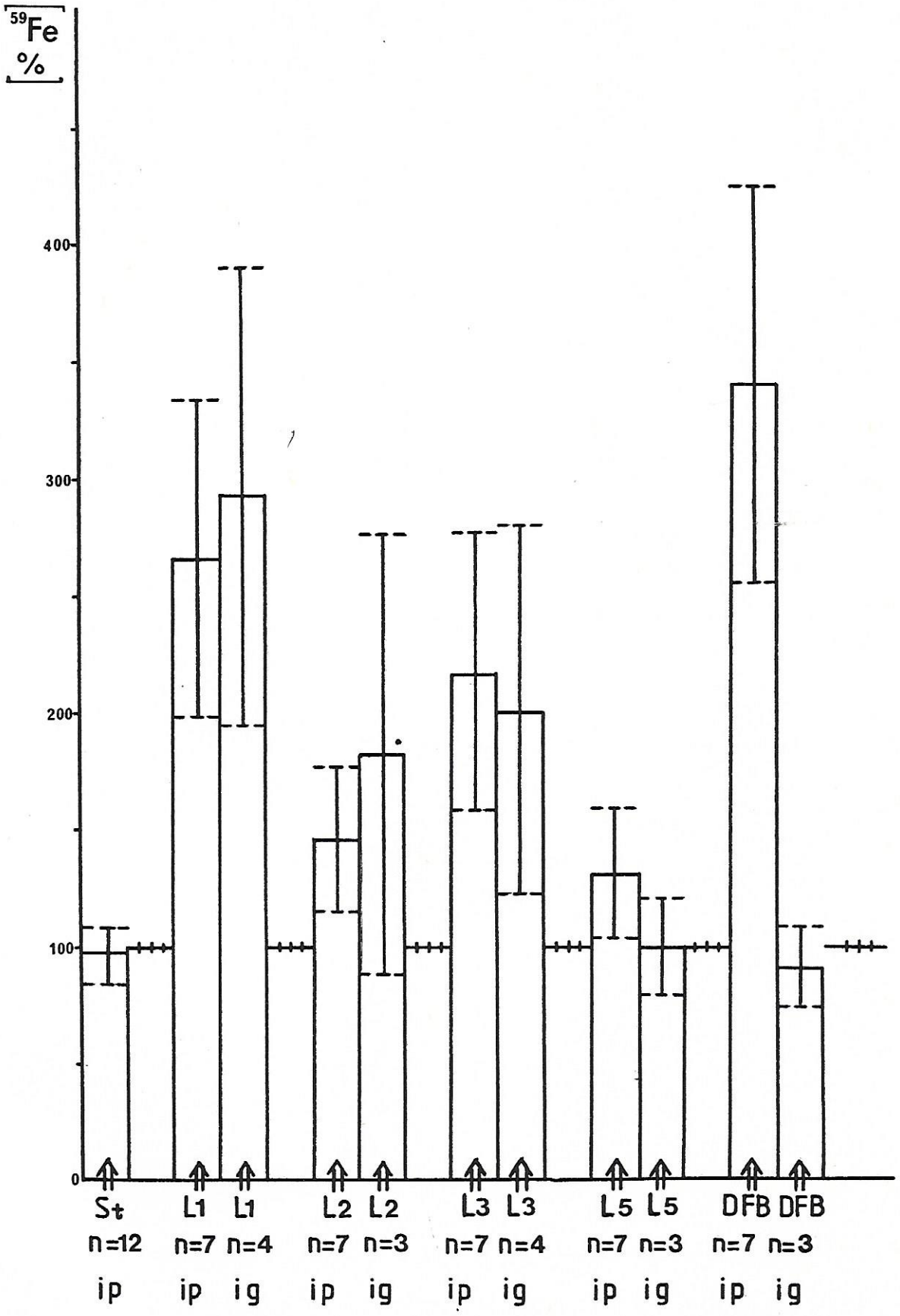

59Fe
%
400
300
200
100
0
St
L1 L1
L2 L2
L3 L3
L5 L5
DFB DFB
n=12
n=7 n=4
n=7 n=3
n=7 n=4
n=7 n=3
n=7 n=3
ip
ip ig
ip ig
ip ig
ip ig
ip ig

Fig. 7.9

Duration of ligand action.

Excretion profile of 4 mice, at 12 h intervals after the ligand administration (time = oh).

Vertical Axis: Percentage ^{59}iron excretion.

Horizontal Axis: Time/h.

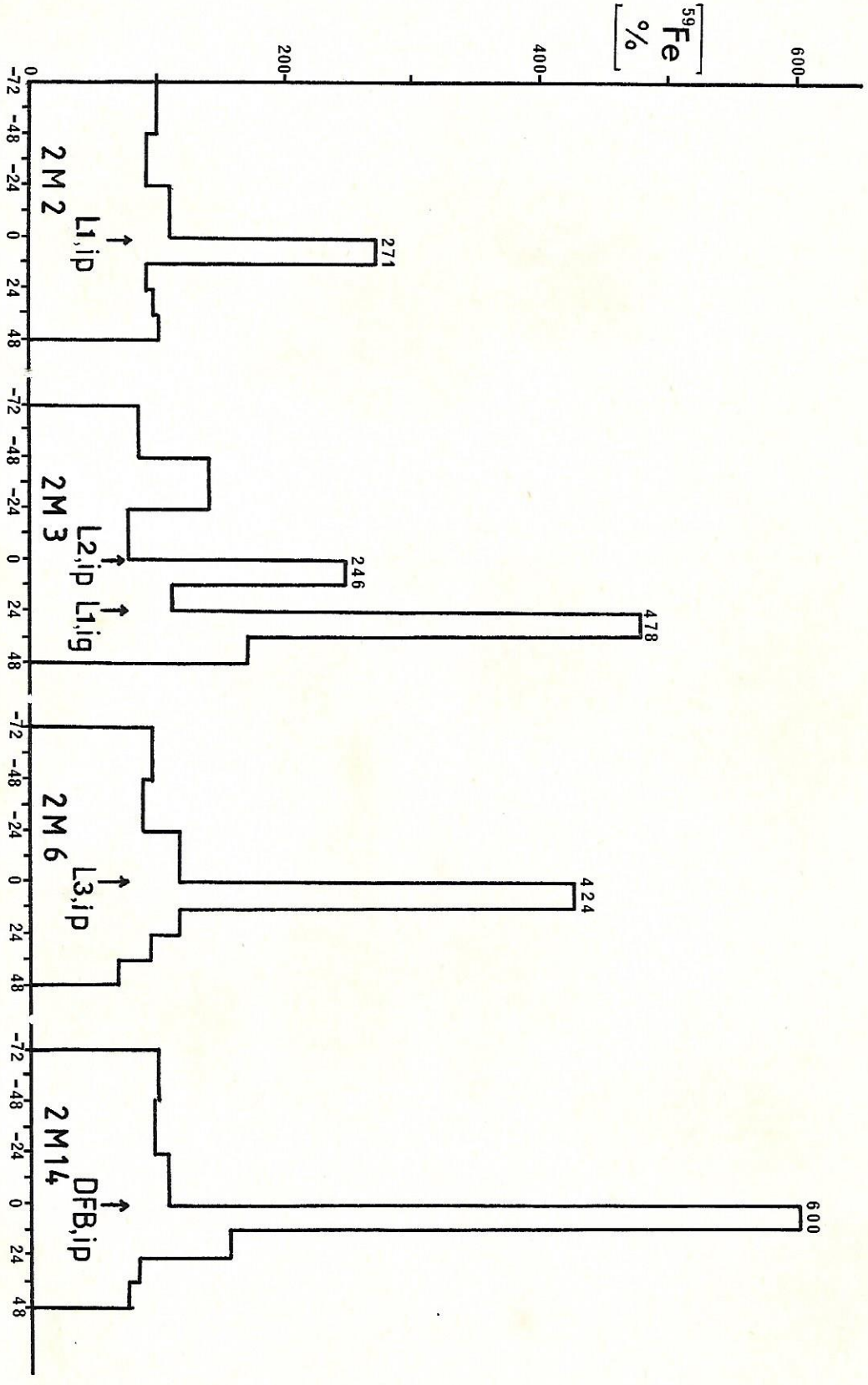
Lx - Number of ligand.

ip, ig are intraperitoneal and intragastric administration respectively, of 10 mg/mouse.

XMn - X is the experimental number, Mn is the mouse number.

Percentage ^{59}iron excretion: Is determined at 12 h periods in comparison to the mean value (100%) of 3 days* before the ligand administration. The % value at the head of the column indicates the percentage excretion of the 12 h period immediately after ligand administration.

* The mean value was estimated from the average of the half of the daily excretions.


^{59}Fe %
600
400
200
0
-72
-48
-24
0
24
48
2M 2
L1,ip
271
2M 3
L2,ip
L1,ig
246
478
2M 6
L3,ip
424
2M 14
DFB,ip
600
Time/Hours

(b) Repetitive Administration of Ligands

In another small experiment (Table 7.4, expt. 4), L_2 was first administered intraperitoneally to mice $2M_3$ and $2M_4$ and after 24h L_1 intragastrically to the same mice (Fig. 7.10). The mouse $4M_3$ responded moderately to L_2 when applied intraperitoneally (178%) and very well to L_1 on the following day when applied intragastrically (303%). The other mouse failed to respond to L_2 when applied intraperitoneally (107%) but again intragastric administration of L_1 caused high ^{59}iron excretion (204%). Thus repetitive administration of ligands caused an increase in ^{59}iron excretion and mice responded better to L_1 compared to L_2.

(c) The Effect of Overnight Starvation

Two main observations can be deduced from the effect of overnight starvation prior to ligand administration (Table 7.4, expt. 2). First there was a general decrease in the rate of excretion of faeces and also a decrease of ^{59}iron excretion in the four mice studied (Fig. 7.11), compared to non-starved mice. However, ^{59}iron excretion increased by approximately 70% after the intragastric administration of L_3 to mice $5M_{12}$(290%) and $5M_{11}$(222%) compared to non-starved mice $3M_{21}$ and $3M_{16}$, which received the same intragastric dose of L_3, causing increase of ^{59}iron excretion of 113% and 171% respectively.

Fig. 7.10

The Repetitive Administration of Ligands.

Vertical Axis: Percentage ^{59}iron excretion.
Horizontal Axis: Time/days
XMn: X is the experiment number, Mn is the mouse number.
Percentage ^{59}iron excretion is determined on each individual day by taking the mean value (100%) of 3 days (56-58) before the ligand administration.
ip and ig are intraperitoneal and intragastric administration respectively of 10 mg/mouse.

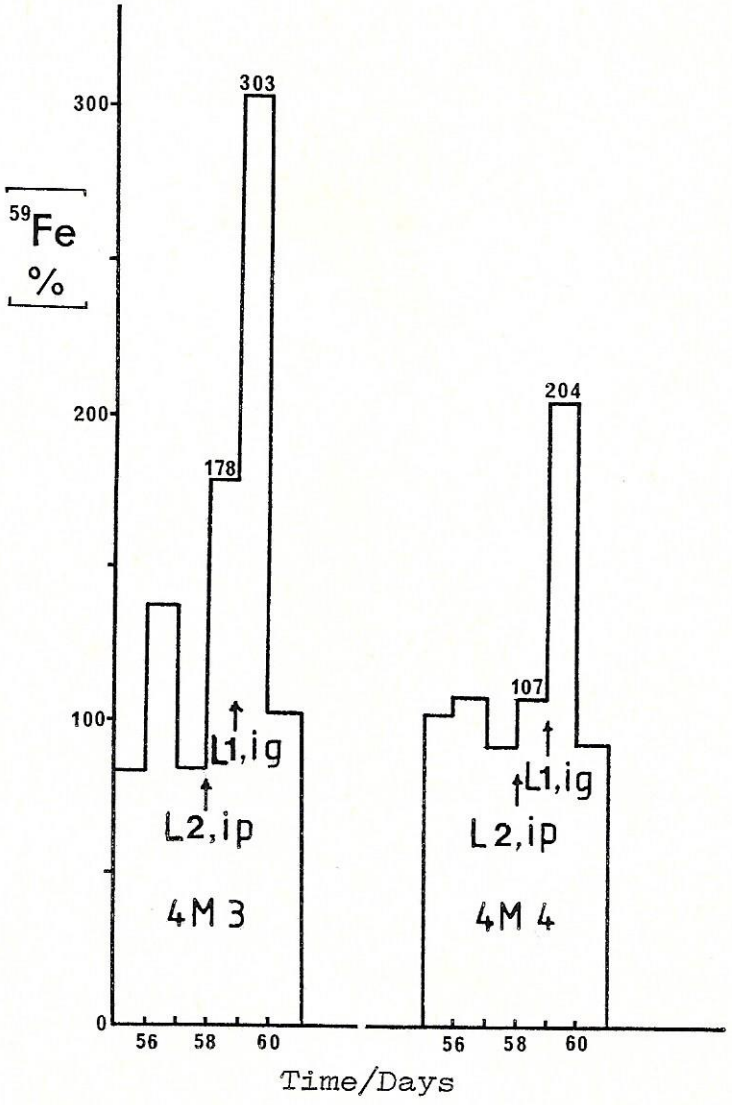


Fig. 7.11

The effect of starvation.

Vertical Axis: Percentage ^{59}Fe excretion.

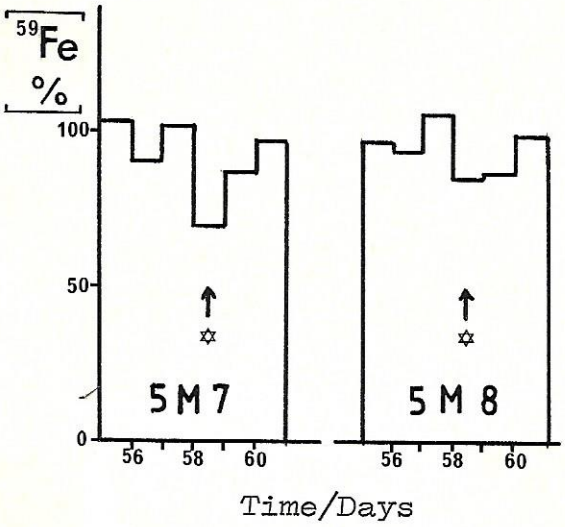
Horizontal Axis: Time/days

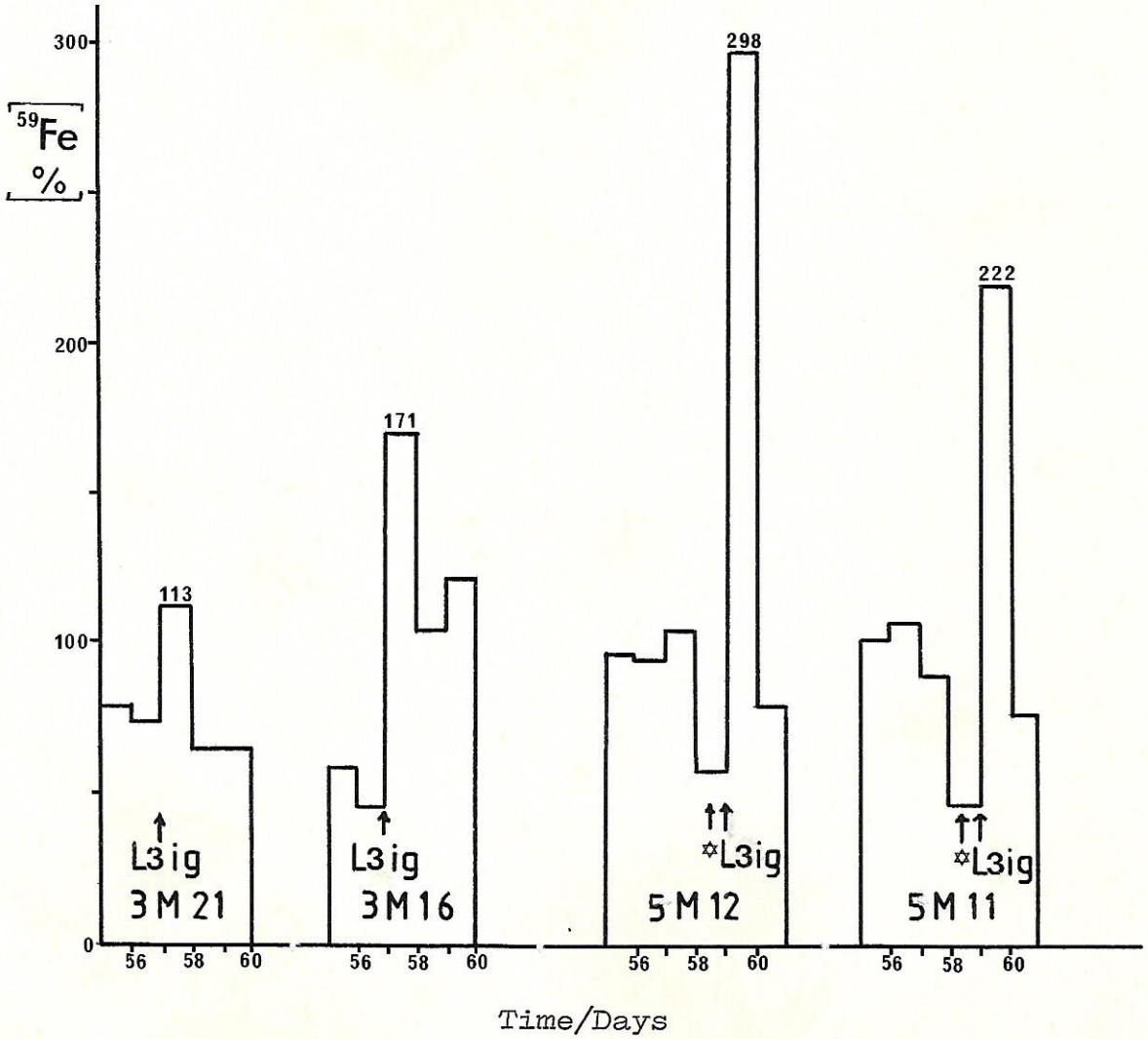
XMn: X is the experiment number, Mn is the mouse number.

✡ :overnight starvation

ig: intragastric administration.

Percentage ^{59}Fe excretion: Is determined on each particular day in comparison to the mean value (100%) of 3 days (56-58 day) or 4 days (51-54 day) for experiments 5 and 3 respectively, before the individual specified day (indicated by the % value at the head of the column.)


59Fe
%
100
50
0
5 M 7
5 M 8
56
58
60
Time/Days


300
200
100
0
59Fe
%
113
171
298
222
L3ig
3 M 21
L3ig
3 M 16
L3ig
5 M 12
L3ig
5 M 11
56
58
60
Time/Days

7.5 Discussion of the in vivo Experiments

7.5.1 Toxicity of Ligands

Although there cannot be any conclusions regarding the long-term toxicity effects imposed on mice by the ligands used in this study at doses of 500 mg kg^{-1}, it seems that L_1, L_2 and L_3 are not lethal at these doses. Similarly it was confirmed that maltol (L_5) is not toxic at this dose.[27] In contrast, L_4 and L_6 caused death to mice at 500 mg kg^{-1} in a relatively short time. There was no apparent toxicity when mice were injected with L_4 and L_6 at approximately one tenth of the same dose, the mice surviving more than four months. A broad toxicological study is required in order to establish LD_{50} values and the long term effects of the compounds. In particular the possible interference with iron dependent enzymatic and biological processes should be investigated. However, in this study, such work was not pursued as it was intended to establish whether or not the compounds had any ability to remove iron from iron overloaded animals.

7.5.2 In vivo Removal of ^{59}iron by Ligands

In addition to their in vitro ability to sequester iron from ferritin and transferrin, the group of ligands which form the focus of this study also possess the ability to sequester iron from iron overloaded mice in vivo.

The method used to label the mice with ^{59}iron was satisfactory. Thus the profile of ^{59}iron excretion of

iron overloaded mice, which were treated with ^{59}Fe-lactoferrin is similar to that of ^{59}Fe-citrate labelling used by Young.[157] Although the absolute ^{59}iron excretion level is not the same between the mice studied, the ^{59}iron excretion in each individual mouse reaches a steady state before the ligand administration. Thus ligand induced increased in ^{59}iron excretion is not expected to be due to any other experimental condition, other than the ability of these ligands to mobilise iron from mice.

1,2-Dimethyl-3-hydroxy-pyrid-4-one (L_1) would appear to be the most promising new iron chelator of this preliminary study. It was effective in removing iron from all iron overloaded mice when it was applied intraperitoneally and more importantly intragastrically. Bearing in mind that L_1 is a bidentate ligand and DFB a hexadentate one, it was unexpected to find that the levels of ^{59}iron excretion caused by L_1 were comparable to that of DFB when both ligands were applied intraperitoneally.

Although during the course of this study there was no quantitative measurement of iron excretion and also no organ evaluation to examine the site of iron removal, the deep red wine colour of the urine of the mice treated with L_1 is an indication that the iron complex of L_1 is cleared at least partly through the kidneys.

2,4-Dihydroxy-pyridine-1-oxide (L_3) was the second most potent synthetic iron chelator used in this study. Although its efficacy in removing iron from iron overloaded mice is

lower than that of L_1, it effectively caused an increase in ^{59}iron excretion in all the mice treated either intraperitoneally or intragastrically. This ligand resembles an aromatic hydroxamic acid in contrast to L_1 where nitrogen is not involved in the chelating ring, thus it should be appreciated that L_1 and L_3 belong to completely different groups with regard to chelation.

1-methyl-3-hydroxy-pyrid-2-one (L_2) appears to be a less potent iron chelator in vivo when compared to L_1 and L_3. Indeed in a few cases there was no increase in ^{59}iron excretion after its administration. More elaborate studies are needed to indicate whether L_2 could be a useful iron chelator in vivo.

2-methyl-3-hydroxy-pyr-4-one (L_5) has been previously shown to be unable to remove iron from iron overloaded rats when administered orally.[27] However, the resemblance to the α-keto hydroxy-pyridones and its efficacy in removing iron from ferritin prompted the re-examination of its effects on iron overloaded mice. In this study it is confirmed that maltol is orally ineffective. However, when applied intraperitoneally it caused an overall increase in ^{59}iron excretion and although small when compared to the other ligands, it was nevertheless quite significant. This ligand is cheap, non-toxic and has been consumed by humans as a food additive.[158] If in a dose response study, the iron excretion is increased and it proves to be effective in its own right or in a

synergistic role with the conventional application of DFB it could substantially reduce the present cost of treatment.

The efficacy of desferrioxamine (DFB) in removing iron from iron overloaded animals and humans is well established. As a result of this extensive documentation it was included in the present study for comparative purposes. Thus it is established that the experimental design adopted in this study is reliable for the preliminary screening of iron chelators.

When DFB was applied intraperitoneally it caused a large increase in ^{59}iron excretion in all the mice studied. In contrast, intragastric administration of DFB triggered no observable increase in ^{59}iron excretion. Thus confirming the well established fact that DFB is orally ineffective and further confirming the suitability of the system adopted in this study.

7.5.3 Comparison of the Efficacy of Ligands

The efficacy of the ligands in mobilising iron from iron overloaded mice when applied intraperitoneally and intragastrically is depicted in Fig. 7.8 for comparison purposes. To summarise up the efficacy of the ligands was as follows:

$DFB \, , \, L_1 > L_3 > L_2 > L_5$ (intraperitoneally)

$L_1 > L_3 > L_2 > L_5 \, , \, DFB$ (intragastrically)

It should be emphasised, however, that what is important

in the treatment of iron overload diseases, is whether a negative iron balance can be achieved in a controllable manner using an iron chelator which is basically cheap and not toxic.

Furthermore, excessive iron removal from non-stored non-transient subcellular compartments could be proved to be damaging. Thus in the future, studies should be directed to examine the usefulness of iron chelators within this context in addition to the quantitative aspect of iron removal.

7.5.4 Other Factors Influencing Iron Excretion

The duration of ligand action was briefly studied in this work. It was observed that all the ligands appear to function in their capacity as iron chelators, within 12h of the intraperitoneal or intragastric administration. There was no further increase in ^{59}iron excretion after this period. Whether this is due to the metabolism of the ligands, their rapid excretion, or the depletion of the chelatable iron pool remains to be examined. This is an important aspect and it will be essential to study the effect of a repeated 12h or shorter period administration in future experiments.

The influence of starvation on the efficacy of orally administered ligands is another factor briefly examined. Since all the ligands (L_1 - L_7) in this study form stable, water soluble iron complexes, it is conceivable that iron in the gastrointestinal tract derived from the diet could

be sequestered and taken into the body by these ligands. The probable fate of such a complex would be excretion without loss of iron, thus decreasing the efficacy of the drug dose. Overall there was 70% increase in ^{59}iron excretion in the mice deprived of food compared to those which were not starved. Further studies are needed to substantiate this statement, including the examination of the eating habits of the animals under investigation.

It is hoped that this study heralds a new chapter in the development of clinically useful iron chelators. It should be stressed, however, that this work is far from complete. Thus, further toxicological and other animal studies, including monitoring of tissue and cellular level iron mobilisation as described in the introduction, should be carried out, in order to develop a clear understanding of the mode of action of these ligands in vivo.

The preliminary animal studies suggest that ligands L_1, L_2 and L_3 have potential as orally active chelators in the treatment of iron overload diseases. This use will not only alleviate many psychological problems associated with infusion pumps and painful injections

currently favoured, but the cheaper production cost of the compounds should, in principle, render it possible for Third World countries to successfully treat thalassaemia and related diseases.

APPENDIX

The means of different groups of mice are compared in pairs by the t test of the form:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{SD^2}{n_1} + \frac{SD^2}{n_2}}}$$

The results are extracted from (Fig. 8). If the degree of probability is smaller than 5% ($P < 0.05$) the null hypothesis is discounted.

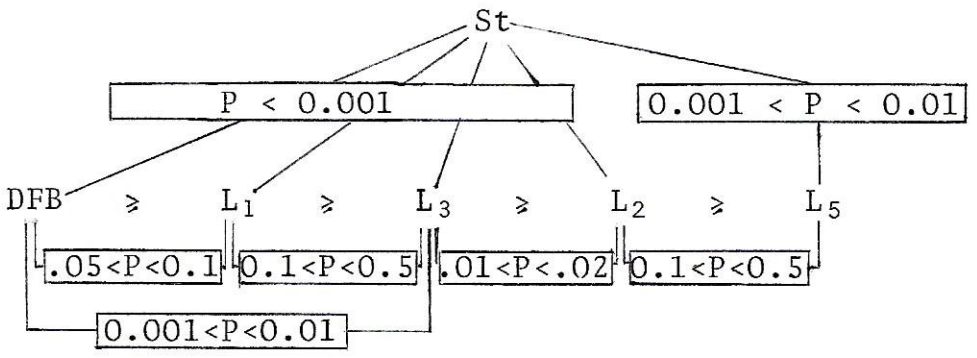
n = the number of mice in a group.

$\bar{X}$ = the mean ^{59}Fe excretion of a group.

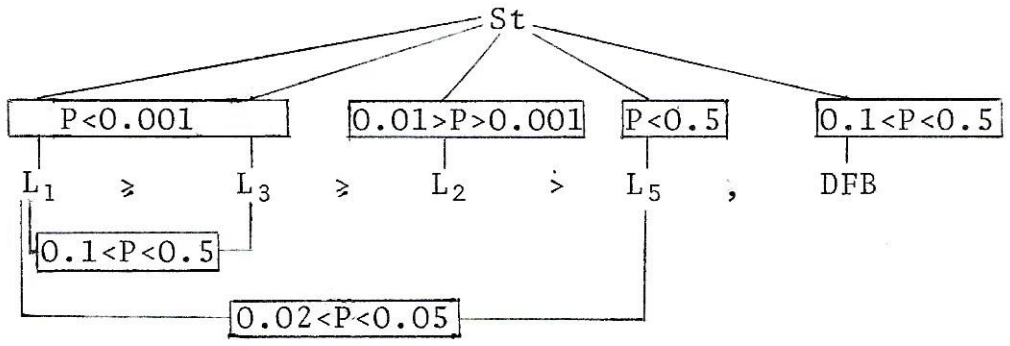
SD = the standard deviation of the two groups combined.

Suffix 1, 2 denote different groups of mice

Intraperitoneal administration



Intragastric administration



CHAPTER EIGHT

GENERAL DISCUSSION

The results of this work are of great significance to the study of iron metabolism in general and the treatment of iron overload in thalassaemia in particular. Some of the new chelators have a number of properties which have never been shown in other iron chelators before, such as iron mobilisation from transferrin and ferritin *in vitro* at physiological pH and from mice *in vivo*.

8.1 Design of New Chelators

Four different types of chelators have been studied, based on the following parent molecules:

(a) 3-hydroxy-pyr-4-one, e.g. L_5;

(b) 1-methyl-3-hydroxy-pyrid-4-one, e.g. L_1;

(c) 1-methyl-3-hydroxy-pyrid-2-one, e.g. L_2;

(d) 2-hydroxy-pyridine-1-oxide, e.g. L_4, L_6, L_7.

However, the number of ligands which can be synthesised from these molecules including their trimers could be infinite, if one considers the number of substituents which can be introduced to the heteroaromatic ring, as explained in Chapter Two. The limitations which could be imposed on the number and type of substituents on a chelator should be considered within the context of the following properties:

(1) Size: long alphatic chain substituents as well as bulky hexadentate ligands will influence cell permeability and mobilisation of iron from ferritin;

(2) Steric effects: may interfere with the chelation;

(3) Electron releasing and electron withdrawing effects: will influence the charge of the ligand at physiological pH and the stability of its iron(III) complex;

(4) Charge of the ligand and its complex: will influence membrane permeability;

(5) Water/lipid solubility of the ligand and its iron(III) complex: will influence rate of cell permeation and membrane partition;

(6) Stability in acid and neutral conditions;

(7) Stability to oxidation;

(8) Sites of absorbance and excretion in the body;

(9) Pharmacological or toxicological effects;

(10) Metabolic effects;

(11) Affinity for iron(III) and other metals;

(12) Ability to mobilise iron(III) from transferrin and ferritin;

(13) Ability to mobilise iron(III) <u>in vivo</u>;

(14) Ability to prevent the accumulation of iron in the tissues.

8.2 Evaluation of New Iron Chelators

The theoretical assumptions on the ligand properties are useful in a broad sense, what is more important, however, is to test them using an appropriate series of experiments and improve them until the required properties are met, if this is possible. This was the approach in this project. The pKa studies of the nitro derivatives of 2-hydroxy-pyridine-1-oxide and their iron studies prompted the search for electron releasing substituents, furthermore, extensive literature survey on compounds of similar chemical structure led to the discovery of 2- and 4-pyridones with an adjacent hydroxyl group.

The *in vitro* iron(III) studies at physiological pH revealed that almost all the ligands (L_1 - L_7) formed soluble neutral complexes. The partition coefficient results together with the RBC experiments indicated a wide variation in lipophilicity and RBC permeability. Although L_1 and L_3 formed 3:1 neutral iron complexes, they failed to diffuse across the RBC membrane, in contrast to the other ligand iron(III) complexes, which did so at different rates depending on their lipophilicity.

It was estimated that partition coefficients of approx. P = 0.3 will be sufficient for this type of ligand iron(III) complexes to diffuse across the RBC membrane. However, if intracellular iron mobilisation is desirable, the ligand in question would be expected to interfere with enzymes and proteins whose metabolic pathways are associated with iron.

Furthermore, its biotransformation, if any, would be more feasible intracellularly. Even if ligand iron(III) complexes could not diffuse through membranes, it is possible that other mechanisms could be involved in intracellular iron mobilisation. Thus the ligands could diffuse through the cell membrane, chelate iron and diffuse out, a mechanism presently attributed to the mode of action of DFB(see general introduction). Another aspect of iron metabolism related to membrane permeability is the ability of ligand iron(III) complexes to diffuse through the gastrointestinal lumen. If these complexes can donate their iron to the liver or to transferrin, assuming that they are in equilibrium with this protein in the blood stream, they could be useful in the treatment of iron deficiency anaemia.

Iron mobilisation from transferrin is an important screening test for iron chelators in vitro. It is conceivable that even if chelators could not diffuse through membranes to sequester intracellular iron from iron overloaded tissues, iron mobilisation from transferrin itself at certain levels could be sufficiently fast to maintain iron balance in the body and furthermore, would be a less hazardous process since the site of action would be extracellular. In this work it was shown that iron could be mobilised rapidly from transferrin by certain ligands in a biphasic reaction. The existence of two kinetically distinct sites of iron in the mobilisation process from transferrin is in agreement with the "heterogeneity hypothesis" in relation to the functional aspect of

transferrin. Furthermore, although all ligands did react with transferrin, the dissociation of the iron(III) ligand complex from the iron(III) ligand-transferrin ternary complex took place at different rates which suggests that although certain ligands, like DFB, for example, have the thermodynamic capability to sequester iron from transferrin, the kinetic parameters of the reaction are such that iron mobilisation could be insignificant in physiological terms. However, in the presence of a mediator, such as NTA which is less capable thermodynamically in removing iron from transferrin, but which can exchange iron faster with this protein, the efficacy of ligands like DFB significantly increases.[108]

Mobilisation of storage iron from ferritin and haemosiderin in vivo would be the most efficient mechanism in relieving iron overloaded tissues, since these two intracellular proteins contain the highest amount of inorganic iron in the body. The new chelators were shown to be more effective in this respect compared to other clinically tested chelators. The slow rates of these reactions, however, is an indication of the difficulties of solubilising iron(III) polynuclear complexes. In this respect small molecular weight chelators at high concentrations and with high iron(III) stability constants look promising, furthermore, reducing agents may enhance this process.

Even if the site of iron mobilisation of many chelators is mainly the transient (or labile) iron pool, mainly present in the parenchymal liver cells, the new iron chelators prepared not only would mobilise this iron if they could diffuse through these cells, but since this iron is thought to be in equilibrium between this pool and the ferritin, haemosiderin and transferrin pools, they would do so more efficiently since all the other chelators have not been shown to mobilise significant amounts of iron from transferrin or ferritin *in vitro*.

Although all the *in vitro* studies were successful and the indications were such that theoretically all the new chelators would be capable of mobilising iron from iron-overloaded animals, the next screening program was planned to study exactly that. The conditions *in vivo*, however, are more complex and in most cases unpredictable. In the simple toxicity study two of the ligands (L_4 and L_6) were found to be toxic at a high dose to normal animals and were excluded from the iron-overloaded animal studies for the time being. Two other ligands L_2 and L_5 increased iron excretion moderately and the last two L_1 and L_3 cause substantial iron excretion when they were administered intraperitoneally and more important, orally. Although there is not any evidence yet to suggest a mode of action for all the ligands *in vivo*, their animal study results reflect to a great extent, their results *in vitro* in relation to transferrin and ferritin. The animal experiments re-established the high potential of these new chelators and increased their prospects of being clinically

useful in iron mobilisation from iron-overloaded patients.

8.3 The General Properties of the Ligands

L_1 seems to be the most promising ligand used in the study. It is capable of mobilising iron(III) from transferrin rapidly and quantitatively, and furthermore, from ferritin, thus being a better iron chelator than DFB in this respect. In the *in vivo* experiments L_1 increased iron excretion from iron-overloaded mice at a level similar to that of DFB but more importantly, it did so when it was administered orally. Rather surprisingly, its iron(III) complex did not diffuse through the red blood cell membrane. Whether or not this property is essential to the clinical applications outlined in earlier chapters is not clear. This question should be further examined because neither the DFB-iron(III) complex nor that of L_3 did diffuse through the red blood cell membranes and yet from the animal experiments these three ligands (L_1, L_3 and DFB) were the most successful in iron mobilisation. Is this a coincidence or a significant result? Further work is needed to answer this question, but it should be borne in mind that the ligands L_1 and L_3 are quite different in their charge at physiological pH, the former being essentially neutral and the latter being negatively charged.

L_3 was the second most successful ligand with similar results to that of L_1 . Regarding iron mobilisation from transferrin, L_3 was less efficient than L_1, but in the ferritin experiments L_3 was proven to be the most successful.

This is a significant point because if the site of iron mobilisation *in vivo* is ferritin iron, a continuous administration of L_3 will be more efficient than other chelators in directly relieving iron from iron loaded tissues.

L_2 and L_5 have similar properties. They both seemed to react with transferrin but their rate of iron removal was slow. However, over long periods of time, L_2 was shown to be a better chelator than L_5.

In the ferritin and animal experiments L_2's efficacy in iron mobilisation was slightly higher than that of L_5. Although both ligand iron(III) complexes were successful in diffusing through the RBC membrane, their rates were different, L_5's being much faster than L_2's. It is again difficult to correlate iron mobilisation efficacy with membrane permeability. In contrast their *in vitro* ferritin and transferrin iron mobilisation results are in agreement with those of the animal experiments; thus L_2 is more effective than L_5, but both are less effective than L_1 and L_3.

L_4 and L_6 were found to be toxic to normal mice, probably because of their high lipophilicity. Both ligands are capable of mobilising substantial amounts of iron from both transferrin and ferritin. It could be useful to carry out further animal experiments in order to establish whether their toxicity rises, from iron chelation followed by cell membrane permeability of their

iron complex and subsequent decompartmentalisation of iron into other tissues, which are more prone to the toxic effects of this metal, e.g. heart.

8.4 General Considerations

Several other aspects in relation to the ligands will now be discussed. It was mentioned in Chapter Seven that when L_1 was administered to iron overloaded mice, the colour of their urine, 6 h after the administration was red, an indication that the clearance of some of L_1-iron(III) complex is probably through the kidneys. Ethyl maltol[158] an analog of L_5 was rapidly and extensively absorbed when applied orally to dogs and also both these compounds were rapidly and extensively metabolised in the same animal as glucuronides and ethereal sulphates, the common pathway of phenolic compounds. When the Zn and Na salts of 2-mercapto-pyridine-1-oxide[159] were administered orally to rats, rabbits and monkeys, they were absorbed to a great extent (60-80%) from the gastrointestinal tract and rapidly excreted in the urine, except from some 15% which was exreted in the bile. When the same compound was applied intraveneously to swine[160] it was excreted in the urine mainly as its S-glucuronide.

From the above discussion one may expect that the new ligands and their iron complexes will have similar metabolic fates, i.e. it seems likely that their metabolites will be excreted through the urine.

Tropolone and some compounds of similar chemical structure to L_1 and L_2 [161] were found to inhibit catechol -O-methyl transferase, an enzyme which inactivates catecholamines and also metabolise many xenobiotic catechols. It would be useful to study the effect of the new chelators and some of their analogs on this enzyme, not only from a metabolic point of view, but also for possible clinical application to diseases [162] associated with this enzyme.

Iron status and infection is another area related to iron chelators [163] In recent years there has been a great interest in this subject, which is based on the observation that on the one hand iron deprivation by the transferrins of invading microbes is an important defence mechanism in animals [164] and that on the other hand, iron deficiency impairs the animal's immunity against these microbes. [165] "Optimal iron nutrition" is therefore essential for a viable defence mechanism. [166] In this respect, iron chelators can increase or decrease microbial growth depending on their ability to donate or withhold iron. [163]

Many other physiological regulations depend on the "iron status" of the organism. This, however, is not within the scope of this thesis, but at the same time it is important to realise that iron chelation in vivo is not a simple process but rather a part of a complex system which may be difficult to evaluate.

The Possible Uses of the New Iron Chelators

The possible applications and uses of the iron chelators of this work can be summarised as follows:

(a) treatment of iron overload;

(b) treatment of iron deficiencies;

(c) antimicrobial activity;

(d) catecholamine analogs;

(e) analytical iron reagents;

(f) treatment of plant "chlorosis" in agriculture;

(g) iron free cleaning of equipment and rust proofing;

(h) chelation and excretion of other toxic metals including the radioactive ones like Pu;

(i) treatment of chronic inflammatory diseases;

(j) study of iron metabolism in general.

8.6 Conclusion

It has been shown that all the new chelators prepared in this work are capable of mobilising iron from transferrin and ferritin *in vitro*. Furthermore, some of these chelators were found to remove iron from iron overloaded mice when they were applied intraperitoneally and orally. These significant results render the possibility of them being used for the treatment of iron overload in β thalassaemia in particular, and also for the study of different aspects of iron metabolism in general.

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