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Abstract

Bio-Inorganic Chemistry of Manganese and Titanium

A Thesis
Submitted for the Degree of Doctor of Philosophy by
Shailja Bihari, B.Sc. (Hons.)

Department of Chemistry
Faculty of Science and Engineering
The University of Edinburgh
March 2002
Abstract

A wide range of metals are transported in the body by the protein transferrin, including both essential metal ions and probably also metals used in therapeutic agents. The metal binding sites on transferrin contain tyrosine, histidine and aspartate ligands. This thesis is concerned with studies of the essential metal ion manganese, and with titanium, which is used in anticancer agents.

In order to aid the characterisation of Mn(III) and Ti(IV) transferrins, the Mn(III) and Ti(IV) complexes with the model ligand ethylenebis[(σ-hydroxyphenyl)glycine] (H₄EHPG) have been studied.

The Mn(III) complexes rac-Na[Mn(EHPG)].3H₂O (1) and rac,meso-Na[Mn(EHPG)].H₂O (2), have been prepared and their X-ray crystal structures determined. Complex 1 contains N(S,S)C(R,R) configurations at the N and C stereogenic centres, whilst in the unit cell of complex 2 there are two independent molecules, 2a (meso) and 2b (rac), with N(R,R)C(S,S) and N(R,R)C(S,S) configurations, respectively. Enantiomers of each complex are also present. The Mn(III) centres have Jahn-Teller-distorted octahedral geometry, with two long bonds and four short bonds. ¹H NMR spectra of these high-spin d⁴ paramagnetic complexes are reported. These complexes give rise to similar ligand (phenolate)-to-metal charge-transfer bands as Mn(III)-transferrin. Dissociation of Mn(III) from EHPG occurs below pH 3.4.

The Ti(IV) complex of rac-[Ti(EHPG)(H₂O)].11/3H₂O (3) has also been prepared and the X-ray crystal structure determined. All previously-reported crystalline rac-EHPG metal complexes contain N(S,S)C(R,R), or N(R,R)C(S,S) isomers, whereas 3 unexpectedly contains the N(S,S)C(S,S) and N(R,R)C(R,R) forms. 2D NMR studies indicate that 3 has a similar structure in solution to that in the solid state. A ligand (phenolate)-to-metal charge transfer band was observed at 386 nm, similar to that seen for Ti(IV)-transferrin. Ti(IV)EHPG was stable at pH values down to 1, however, the complex decomposed above pH 7.

Mn(III)-transferrin complexes were prepared by air oxidation of Mn(II) in the presence of transferrin. The oxidation state of manganese bound to transferrin was
Abstract

confirmed by K edge EXAFS. Analysis of the EXAFS data revealed that the metal centre is also Jahn-Teller distorted but with four long bonds and two short bonds, i.e. an inverse distortion to that seen in the Mn(III)EHPG model complexes.

Attempts to prepare other Mn(III) complexes which might be suitable for studies of Mn transfer to proteins are described and include cyclam and bicyclam as ligands. The crystal structure of [Mn(cyclam)Cl_2]Cl_2H_2O was determined, and contained two long axial Mn-Cl bonds of 2.5249 Å. This complex was shown by electronic absorption spectroscopy to undergo a complicated series of reactions in aqueous solution. K edge EXAFS measurements suggested that at least one Cl ligand dissociated from the complex in aqueous solution. The hydrolysis was shown to be inhibited by the presence of fluoride.
Acknowledgements

I would like to thank my supervisor, Professor Peter J. Sadler, for his invaluable support and advice throughout my PhD.

My thanks also go to the many people in the PJS Group, past and present, for their friendship and support during my time at The University of Edinburgh. Many thanks especially to Drs. Michael Weishäupl and Abraha Habtemariam for their help and advice in the laboratory and Dr. Claudia Blindauer for her invaluable support and guidance whilst writing up. Thanks also to my project student Pamela Smith for crystallising one of the complexes.

I am also very grateful to Dr. John Parkinson for his help with NMR experiments. His expertise knowledge of NMR spectroscopy has been invaluable.

Thanks also to Dr. Ian Harvey (Central Laboratory of the Research Councils, Daresbury) for collecting and analysing the EXAFS data, Professor Andrew Harrison for the magnetic susceptibility measurements, and Drs. Simon Parsons and Robert Coxall for the X-ray crystal structure determinations.

I am indebted to my family, for their support, encouragement, patience and understanding, at all times from start to finish, and especially whilst writing up. Thanks all.

Also, a big thank-you to all those, near and far, who believed in me and kept me going with words of encouragement and support.

Finally, I would like to thank the EPSRC for funding.
To my mum
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<td>------------</td>
</tr>
<tr>
<td>acac</td>
<td>acetylacetonate anion</td>
</tr>
<tr>
<td>approx.</td>
<td>approximately</td>
</tr>
<tr>
<td>Arg</td>
<td>L-arginine</td>
</tr>
<tr>
<td>Asn</td>
<td>L-asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>L-aspartate</td>
</tr>
<tr>
<td>bipy</td>
<td>bipyridine</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
</tr>
<tr>
<td>ca.</td>
<td>circa (about)</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jacob disease</td>
</tr>
<tr>
<td>C.N.</td>
<td>coordination number</td>
</tr>
<tr>
<td>COSY</td>
<td>2D correlation spectroscopy</td>
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<td>Cp</td>
<td>cyclopentadienyl, $\eta^5$-C$_5$H$_5$</td>
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<td>Cyclam or cyclam</td>
<td>1,4,8,11-tetra-azacyclotetradecane</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>diars</td>
<td>o-phenylenebisdimethylarsine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>dmpe</td>
<td>1,2-bis(dimethylphosphino)ethane</td>
</tr>
<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
</tr>
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<td>DPDP</td>
<td>$N,N'$-dipyridoxyethylene diamine-$N,N'$-diacetate-5,5'-bis(phosphate)</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethyltriaminepentaacetic acid</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EHPG</td>
<td>$N,N'$-ethylenebis-(o-hydroxyphenylglycine)</td>
</tr>
<tr>
<td>en</td>
<td>ethylenediamine</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>equiv.</td>
<td>equivalent</td>
</tr>
<tr>
<td>EXAFS</td>
<td>extended X-ray absorption fine structure</td>
</tr>
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<td>EXSY</td>
<td>2D exchange spectroscopy</td>
</tr>
<tr>
<td>FBP</td>
<td>ferric ion binding protein</td>
</tr>
<tr>
<td>Glu</td>
<td>L-glutamic acid</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>H$_2$vanpa</td>
<td>1-(3-hydroxysalicylaldeneamino)-3-hydroxypropane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
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<td>H₂BBPEN</td>
<td>N,N'-bis(2-hydroxybenzyl)-N,N'-bis(2-methylpyridyl)ethylenediamine,</td>
</tr>
<tr>
<td>HB(3,5-iPr₂pz)₃</td>
<td>hydrotris(3,5-diisopropylpyrazol-1-yl)borate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>His</td>
<td>L-histidine</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>H.S.</td>
<td>high spin</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
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<td>H₄THPED</td>
<td>N,N,N',N'-tetrakis-(2-hydroxypropyl)-ethylenediamine</td>
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<td>hTf</td>
<td>human serum transferrin</td>
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<tr>
<td>ICP-AES</td>
<td>inductively coupled plasma atomic emission spectroscopy</td>
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<tr>
<td>3,5-iPr₂pzH</td>
<td>3,5-diisopropyl-pyrazole</td>
</tr>
<tr>
<td>IR</td>
<td>infra-red</td>
</tr>
<tr>
<td>Lf</td>
<td>lactoferrin</td>
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<td>LMCT</td>
<td>ligand-to-metal charge-transfer</td>
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<td>L.S.</td>
<td>low spin</td>
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<tr>
<td>Lys</td>
<td>L-lysine</td>
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<tr>
<td>Me</td>
<td>methyl</td>
</tr>
<tr>
<td>Meso, meso</td>
<td>mesomeric</td>
</tr>
<tr>
<td>MLCT</td>
<td>metal-to-ligand charge-transfer</td>
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<tr>
<td>MMT</td>
<td>methylcyclopentadienyl manganese tricarbonyl</td>
</tr>
<tr>
<td>mol.</td>
<td>mole</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NOESY</td>
<td>2D nuclear Overhauser effect spectroscopy</td>
</tr>
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<td>NOE</td>
<td>nuclear Overhauser effect</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
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<tr>
<td>O.S.</td>
<td>oxidation state</td>
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<td>oTf</td>
<td>ovotransferrin</td>
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<td>ox</td>
<td>oxalate anion</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>pmap</td>
<td>bis[2-(2-pyridyl)ethyl]-2-pyridylmethylamine</td>
</tr>
<tr>
<td>R</td>
<td>alkyl or aryl group</td>
</tr>
<tr>
<td>Rac, rac</td>
<td>racemic</td>
</tr>
<tr>
<td>Ref.</td>
<td>reference</td>
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<tr>
<td>RF</td>
<td>radiofrequency</td>
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<td>Definition</td>
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<td>------------</td>
</tr>
<tr>
<td>salen or sal₂en</td>
<td>bis-salicylaldehydeethylenediimine</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SQUID</td>
<td>super quantum interference device</td>
</tr>
<tr>
<td>TBAP</td>
<td>5,10,15,20-tetrakis(4-benzoic acid)-porphyrin</td>
</tr>
<tr>
<td>Tf</td>
<td>transferrin</td>
</tr>
<tr>
<td>Thr</td>
<td>L-threonine</td>
</tr>
<tr>
<td>TMPHPG</td>
<td>(N,N')-trimethylenebis[2-(2-hydroxy-3,5-dimethylphenyl)glycine]</td>
</tr>
<tr>
<td>TMPyP</td>
<td>5,10,15,20-tetrakis(1-methyl-4-pyridyl)-porphyrin</td>
</tr>
<tr>
<td>tren</td>
<td>tris-(2-aminoethyl)amine</td>
</tr>
<tr>
<td>TSP</td>
<td>sodium trimethylsilyl-(d_4)-propionate</td>
</tr>
<tr>
<td>Tyr</td>
<td>L-tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>visible</td>
</tr>
<tr>
<td>X</td>
<td>halogen or pseudohalogen</td>
</tr>
<tr>
<td>XANES</td>
<td>X-ray absorption near edge structure</td>
</tr>
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<td>XAS</td>
<td>X-ray absorption spectroscopy</td>
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</table>
Chapter 1

Introduction: Metal ions in Biological Systems

1.1 Essential Elements

Twenty-four elements are thought to be essential to mammalian life (see Figure 1.1). Apart from C, H, N, O, P and S, numerous metals are necessary for normal function, including the redox-active transition metals V, Mn, Fe, Co, Ni and Cu. These metals play crucial roles in a very large number of biological and biochemical processes. For example, Fe is a vital part of haemoglobin and Cu forms an integral constituent of several metalloenzymes and proteins. Manganese also plays an important role in several physiological processes, and is discussed further in section 1.6.

![Periodic table of the elements](image)

Figure 1.1 Periodic table of the elements. The elements essential for mammalian life are shown in red and the radioactive elements are shown in green.
Some general characteristics of metal ion complexes present in biological systems are given in Table 1.1.

Table 1.1 Some general characteristics of metal ion complexes present in biological systems (adapted from ref. 1).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Na, K</th>
<th>Mg, Ca</th>
<th>Zn, Ni</th>
<th>Fe, Cu, Co, Mo, Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predominant oxidation state</td>
<td>I</td>
<td>II</td>
<td>II</td>
<td>More than one state</td>
</tr>
<tr>
<td>Stability of the complexes</td>
<td>Very low</td>
<td>Low or medium</td>
<td>High</td>
<td>High, but medium in the cases of Mn(II) and Fe(II)</td>
</tr>
<tr>
<td>Preferred donor atoms</td>
<td>O</td>
<td>O</td>
<td>S or N</td>
<td>N or S (and especially O in the cases of some Fe and Mn complexes)</td>
</tr>
<tr>
<td>Mobility in biological media</td>
<td>Very mobile</td>
<td>Semi-mobile</td>
<td>Ni: static, Zn: usually static</td>
<td>Static (semi-mobile in the cases of Mn(II) and Fe(II))</td>
</tr>
</tbody>
</table>

1.2 Metallodrugs

A number of essential metals as well as a variety of non-essential and even radioactive metals have been used in the treatment of disorders as therapeutic or diagnostic agents. A few examples are given below.

Mn(II), Gd(III) and Fe(III) compounds are used as paramagnetic contrast agents in magnetic resonance imaging (MRI) and 99mTc and 67Ga based radiopharmaceuticals are used in clinical diagnosis. Silver and its compounds have long been used as antimicrobial agents in medicine and Sb(V) drugs are used as antiparasitic agents. Bi(III) compounds are used as antiulcer drugs for treating gastrointestinal disorders, and several injectable Au(I) thiolato complexes and one oral Au(I) phosphine complex, auranofin, are used clinically for the treatment of difficult cases of rheumatoid arthritis. Lithium carbonate is used as an antidepressive drug for manic depression, and vanadium compounds (with either vanadate, V(V), or vanadyl, V(IV)) are used as insulin mimetics, since they can stimulate glucose uptake as well as glycogen synthesis. A variety of Mn- and Fe-
based porphyrins and macrocyclic complexes have been reported to exhibit superoxide dismutase (SOD) mimetic activity\textsuperscript{16,17} and macrocyclic bicyclam ligands such as JM3100 and its zinc complex are amongst the most potent inhibitors of the human immunodeficiency virus (HIV)\textsuperscript{18,19}.

Platinum complexes are now amongst the most widely used drugs for the treatment of cancer\textsuperscript{20,21}. So far four injectable Pt(II) compounds have been approved for clinical use and several other cis-diam(m)ine complexes are in clinical trials, including an oral Pt(IV) complex. Currently cisplatin and the second generation drug carboplatin, are two of the most widely used anticancer drugs.

Other metal complexes also exhibit anticancer activity\textsuperscript{22-24}. Two Ti(IV) complexes, titanocene dichloride and budotitane, are currently in clinical trials. The antitumour activity of titanocene dichloride was first recognised in 1979\textsuperscript{25} and since then, the activities of other metalloccenes (V, Nb, Mo, Fe, Ge, Sn) have been reported\textsuperscript{26}. Gallium salts also show anticancer activity\textsuperscript{27,28} and some Ru(III) complexes exhibit anti-metastatic activity\textsuperscript{29-31}.

1.3 Transport of Metal Ions

Metal ions are delivered to cells by a number of biological processes and pathways. Some involve metal transport proteins and others are independent of transport proteins. The transferrins constitute a class of iron binding proteins that form part of a system, which regulates and transports metabolic iron.

1.4 Transferrins

1.4.1 Introduction

The transferrins are a group of proteins that play a regulatory role in iron metabolism\textsuperscript{32}. They contain two homologous metal ion binding sites with high affinities for Fe(III). Transferrins are typically monomeric glycoproteins with a single polypeptide chain containing ca. 670-700 amino acids with molecular weights of ~80 kDa. The characteristic property of transferrins is to bind very tightly but reversibly two Fe(III) ions together with two carbonate anions. Transferrins were
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thought originally to be restricted to vertebrates. However, transferrin-like proteins have since been identified in several invertebrate species where they occur in the hemolymph.\textsuperscript{33-35} In vertebrates, transferrins are found in a variety of body fluids which include the serum transferrins (Tf) present in blood plasma, ovotransferrins (oTf) found in avian egg white, lactoferrins (Lf) found in secretory fluids such as milk, tears, saliva and other secretions as well as in white blood cells, and melanotransferrins,\textsuperscript{36} membrane bound proteins which are present at low levels on the surface of normal cells, but become expressed at high levels in melanoma cells.\textsuperscript{37} Melanotransferrin is found anchored to the membrane surface of melanocytes and other cells \textit{via} a glycosyl-phosphatidylinositol linkage.

Serum transferrin has been found in the blood of all vertebrates examined so far including mammals, birds, reptiles, amphibians, and fish.\textsuperscript{38} It has also been found in many other mammalian fluids, including bile, amniotic fluid, cerebrospinal fluid, lymph, colostrum, and milk.\textsuperscript{39} In human serum, the concentration of transferrin is \textit{ca.} 2.5 mg/ml (35 μM).\textsuperscript{40}

1.4.2 Transferrin as a Metal Ion Mediator

The fundamental role of transferrins,\textsuperscript{32} except melanotransferrin, is to control the levels of free iron in body fluids by binding, sequestering and transporting Fe(III). Transferrin thus prevents the build-up of toxic amounts of excess iron. This helps to maintain the bioavailability of iron while preventing the deposition of insoluble ferric hydroxide aggregates. Under physiological conditions, iron is predominantly in its +3 oxidation state. At concentrations greater than 10\textsuperscript{-17} M, it undergoes rapid hydrolysis and ultimately forms insoluble aggregates of ferric hydroxide.\textsuperscript{41} Moreover, transferrins may also protect against toxic effects of free iron that might otherwise catalyse the formation of free radicals which damage cells.\textsuperscript{42} Ovotransferrin and lactoferrin also have antimicrobial activity. This is probably due to the fact that \textit{in vivo} the proteins are largely present in the apo (iron-free) form and apo-transferrins are able to bind Fe(III) so tightly that it is unavailable for bacterial growth. In addition to these general roles, certain transferrins have more specific functions. Serum transferrin ferries iron between very different cell types, such as the intestinal epithelium, where iron enters the body from the diet, the liver, where it is stored as ferritin, the developing erythroid cells, which need it for haemoglobin biosynthesis, and cells which need iron for cell growth, including tumour cells.
In human serum, only ca. 30% of the metal ion binding sites of transferrin are saturated with iron, and as a consequence there is capacity for the uptake and transport of other metal ions that enter the body. Previous studies have shown that transferrin is able to bind a wide variety of metal ions, other than Fe(III), reversibly, including other transition metals (most of the first row as well as several of the second and third row elements), main group elements, lanthanides and actinides. The natural transferrin cycle for the delivery of iron to cells offers a potential pathway for the transport of other metal ions to cells. Therefore transferrin is thought to play an important role in the transport and delivery of metal ions of physiological and pharmacological importance, including medical diagnostic radioactive isotopes, such as $^{67}$Ga(III) and $^{111}$In(III), therapeutic ions such as Ru(III), and toxic metal ions such as Al(III) (implicated in Alzheimer's disease) and physiologically important metal ions other than Fe(III), such as Mn(III). However, Fe(III) has a higher affinity for the binding site than any of the more than 30 metal ions that have been studied so far, and Fe(III) will readily displace other cations.

Early studies suggested that the strength of metal ion binding is dependent on the size of the metal ion, and attains a maximum for Fe(III), which has an ionic radius of 0.65 Å. Recent work however, has shown that the strength of binding of metal ions to human serum transferrin correlates with the acidity of metal ions (strength of hydroxide binding), and not with the ionic radius.

1.4.3 Structure of Transferrins

As described in section 1.4.1, transferrins are monomeric glycoproteins with a molecular weight of ca. 80,000 Da, consisting of a single polypeptide chain of about 670-700 amino acid residues. X-ray crystallography has been used to determine the structure of a variety of transferrins, as well as various transferrin half molecules, fragments, mutants, and metal and anion-substituted derivatives. The structures and polypeptide folding patterns of all proteins of the transferrin family are very similar with only slight variations. This is attributed to the high level of sequence identity (~40%) between the proteins.
The X-ray crystal structures of transferrins show that the polypeptide chain is folded into two globular lobes.\textsuperscript{33-66} Transferrins are, thus, bilobal glycoproteins with two structurally similar but not identical lobes, the N-lobe (the amino terminus, first \textit{ca.} 330 residues) and the C-lobe (the carboxy terminus, last \textit{ca.} 330 residues), each containing a metal ion binding site (Figure 1.2).

\textbf{Figure 1.2} X-ray crystal structure of human serum transferrin with one \textit{Fe(III)} bound in the C-lobe (Fe\textsubscript{c-hTf}) (at 2.6 Å).\textsuperscript{53} The N-lobe (yellow) is in the open form and the C-lobe (green) is closed. The Fe(III) is shown as a red ball.

The two lobes are arranged front-to-back (Figure 1.3). The relative orientations of the two lobes vary from one transferrin to another, which may be one of the factors that may contribute to the inequivalence in their functional properties. There is extensive sequence identity between the polypeptide chain of the N- and C-lobes. The N-terminal half of the polypeptide is homologous (level of identity \textasciitilde{}40\%) with the C-terminal half.\textsuperscript{40} Each lobe has a molecular mass of about 40 kDa and the lobes are joined together by a short peptide chain. The peptide varies between different transferrins both in length and in conformation. In human serum transferrin, it is a 14 residue random coil but in lactoferrin it forms a three-turn \(\alpha\)-helix of 12 residues.\textsuperscript{32}
Each lobe is further divided into two domains (N1, N2 and C1, C2) of similar size (Figure 1.3) which consists of alternating α-helical and β-sheet segments (Figure 1.4). This is a common feature for binding proteins of the ‘Venus fly-trap’ family. The domains are of functional significance since the cleft separating the two domains of each lobe is where the metal ion binding site is located.

**Figure 1.3 Domain organisation of transferrins.** The N- and C-terminal lobes are divided into domains N1, N2 and C1, C2, respectively. The two lobes are connected by a peptide, which is helical in lactoferrin (solid line) and less regular in transferrin (dashed line). The black dots represent the two iron sites.

The polypeptide folding pattern for a typical transferrin lobe is shown schematically in **Figure 1.4**. Both lobes have essentially the same folding pattern. Three features of this folding pattern are of particular importance. Firstly, the N-terminus of many helices is directed toward the central binding cleft. The positive charge they carry should help attract anions into the binding cleft. Secondly, the two domains are connected by two extended β-strands running anti-parallel to each other behind the iron site. These two backbone strands form the hinge that allows the lobes to close and open on metal binding and release. This hinge is crucial to the conformational change that occurs during binding and release of metal ions. Thirdly, the Fe(III) ion takes its protein ligands from four different parts of the structure, widely spaced along the polypeptide chain; one from domain 1, one from domain 2, and the other two from the two backbone strands. This is discussed in further detail in section 1.4.4.
The protein thus has the ability to bind two Fe(III) ions. There is one metal and anion binding site per lobe. In Figure 1.2, Fe₃⁻hTf, human serum transferrin has Fe(III) bound in the C-lobe and the N-lobe is unoccupied, i.e. iron-free.

![Figure 1.4 Polypeptide folding pattern and binding of the Fe(III) cation in the N-lobe of human lactoferrin.](image)

A similar folding pattern is found in the C-lobe (as well as in the N- and C-lobes of other transferrins). α-Helices are shown as cylinders and are numbered from 1 to 12, and β-strands are shown as arrows and are labelled from a to k. The interdomain extended backbone strands are shaded and the position of the hinge is also indicated.

All known vertebrate transferrins are glycoproteins except for certain species from fish. However, there is no pattern to the sites of attachment of the carbohydrate chains on different types of transferrins and for the same transferrins from different species. They appear to be almost randomly distributed over the protein surface, and along with their absence in certain species of fish, this suggests that the glycan chains of transferrin are not important and play no direct role in the physiological function of the protein. The carbohydrate chains are not required for receptor recognition either. The chains are attached to the proteins by N-glycosidic linkages.
to asparagine residues. Human serum transferrin has two glycan chains, both in the C-lobe of the protein (Asn413 and Asn611), whereas the rabbit serum transferrin protein has only a single glycan chain bound to the C-lobe (Asn491). In human lactoferrin, there are two carbohydrate chains, one on each lobe, again connected to the protein by N-asparaginyl linkages (Asn137 (N-lobe) and Asn490 (C-lobe)).

1.4.4 Metal Ion Binding Site

Fe(III) is tightly but reversibly bound by the ligands in the metal binding site of transferrin. Transferrin has two high-affinity octahedral binding sites for Fe(III), located in the cleft that separates the two domains in each lobe. In each lobe the ligands for Fe(III) ion are the same. Four of the binding ligands are from four amino acid side-chains of the protein. The Fe(III) binding site in each lobe consists of one nitrogen ligand from the imidazole ring of a histidine residue, and five oxygen ligands, two from the phenolate groups of two tyrosine residues, one from the carboxylate side-chain of an aspartate residue and two others from a synergistically bound bidentate carbonate anion. The iron binding site is shown schematically in Figure 1.5, which is taken from the N-terminal lobe of human serum transferrin. The same organisation is found in both N- and C-terminal sites of both lactoferrin and transferrin.

![Figure 1.5 Fe(III) binding site in the recombinant N-lobe half molecule of human serum transferrin (at 1.6 Å).](image)

Residue numbers are for the N-lobe site, with C-lobe analogues in brackets.
It is of significance that the four amino acid ligands involved in the Fe(III) binding site are from different parts of the protein structure. The aspartate residue, Asp63, is from domain 1, one tyrosine residue, Tyr188, is from domain 2 and the other two ligands, Tyr95 and His249, are from the two interdomain polypeptide backbone β-strands that cross over between the two domains at the back of the iron binding site. Thus the two domains can move apart and adopt a more open conformation, hinged by the backbone strands, allowing the release of Fe(III) from the binding site. The aspartate ligand appears to play a crucial role in the metal site, as it coordinates the metal through one carboxylate oxygen while hydrogen bonding between the two domains (Asp H-bonds to two NH groups from domain 1 and 2) with its other carboxylate oxygen.

1.4.5 Anion Binding Site

Transferrin is unable to bind Fe(III) strongly without the concomitant binding of an anion, normally carbonate. The relationship between the metal ion and the anion is synergistic, i.e. neither is bound strongly in the absence of the other. Thus, there is binding of one mole equivalent of carbonate per metal ion bound.

The carbonate anion occupies a pocket in domain 2 (N2 or C2). The pocket is formed by positively-charged groups: the side-chain of a conserved arginine residue and the N-terminus of α-helix 5. The anion fits perfectly between protein and metal in such a way that the full bonding potential of each oxygen is realised, either in metal coordination or in hydrogen bonding (Figure 1.6). The synergistic anion has an important role in creating the metal binding site. Without this anion, the positive charge of the Arg side-chain and the N-terminus of helix 5 would inhibit metal ions from binding in the specific site. This presumably accounts for the weak, nonspecific metal binding in the absence of anions. Thus, the anion not only neutralises the positive charge but also provides two potential donor atoms for metal coordination.

The synergistic anion may also play a role in iron release. Protonation of the carbonate ion (resulting in bicarbonate) could disrupt the hydrogen bonding pattern and assist in the break-up of the binding site, which is essential to ensure reversibility of metal binding.
Figure 1.6 Schematic diagram showing the characteristic metal and anion binding site in transferrin (adapted from ref. 32). The synergistic bidentate carbonate anion bridges between metal ion and protein, binding directly to the metal ion and hydrogen bonding to a positively-charged region of the protein. Residue numbers are for the N-lobe of human serum transferrin, but the same arrangement of ligands is found in the C-lobe, as well as in the N- and C-lobes of other transferrins. 

In vivo, carbonate is the synergistic anion and has a higher affinity than most other anions. In the absence of carbonate in vitro, a select group of carboxylate anions can fulfil the anion function in transferrin, such as oxalate, glyoxylate and thioglycolate. Both carbonate and oxalate bind to Fe(III) in a bidentate mode. The common features of the synergistic anion are a carboxylate group and a second electron donor group, one or two carbon atoms removed that has potential to act as a metal ligand. However, carbonate is bound most tightly and will displace these anions; only oxalate binds with a comparable strength. This is probably because of its perfect fit between metal ion and protein and the other anions need to displace the Arg side-chain. Therefore the side-chain of Arg124 from the N2 domain of the N-lobe may also play a key role in binding and release of the essential synergistically bound carbonate anion. It is also noteworthy that the affinity of the anion is dependent on the nature of the metal ion.
1.4.6 Metal Induced Conformational Changes

One of the most important structural features of transferrins is that they undergo a large conformational change during Fe(III) uptake and release. Both X-ray crystallographic studies and solution X-ray scattering measurements have shown that the binding of iron and carbonate causes the protein to change its conformation from fully-opened to lobe-closed forms. The metal induced conformational changes in the N-lobe upon Fe(III) binding are shown in Figure 1.7. The protein becomes more compact when the metal ion is bound.

![Figure 1.7 X-ray crystal structures of open (left) and closed (right) forms of the recombinant N-lobe of human serum transferrin (hTf/2N).](image)

The side-chains of the binding amino acids are shown in green, iron and carbonate are shown in red.

The hinging movement of the two domains may have a critical role in the mechanism of iron release, since, when the domains are moved apart to a more open conformation, through a hinge in the extended polypeptide backbone strands, the metal binding site is necessarily pulled apart, leading to iron release. The position of the hinge neatly splits the two ligands provided by the backbone strands, Tyr95 and
His249 (residue numbers for the N-lobe of human serum transferrin), so that in the open conformation Tyr95 remains with the N2 domain (along with Tyr188) and His249 remains with the N1 domain (along with Asp63).

The conformational changes in the N-lobe (N-terminal half molecule) of human serum transferrin involve a large rigid body domain movement of $63^\circ$ of the N2 domain relative to the N1 domain, to give an open binding cleft. Neither of the individual domains shows any significant structural change and the hinge is localised to only a few residues. In addition to this rigid-body movement, several local changes in the iron-binding cleft also occur, i.e. two of the iron ligands, His249 and Asp63, change conformation to form salt bridges with Glu83 and Lys296, respectively, in the apo-form. Both salt bridges do not exist in the holo-form, and need to be broken for iron coordination to occur. The N-lobe of lactoferrin also undergoes similar open-to-closed conformational changes upon iron binding. The N-lobe changes its conformation by rotating the N2 domain $54^\circ$ relative to the N1 domain, by a hinge movement in the two antiparallel extended polypeptide strands that run behind the iron binding site and connect the two domains.

The C-lobe is believed to undergo similar conformational changes as well but to a lesser extent. The reduced flexibility of the C-lobe may arise from the presence of an additional disulfide (-S-S-) bridge, which makes a link between the C1 and C2 domains, and which has no equivalent in the N-lobe of any transferrin. This interdomain disulfide bridge may be of particular importance in modulating the iron binding and release properties of the C-lobe and highlights the structural and functional differences between the two lobes. The structures of hen and duck apo- and holo-ovotransferrin show that iron also induces a C-lobe conformational change from an open to closed form, but the extent of lobe opening in the C-lobe (rotation: hen, $35^\circ$; duck, $49.9^\circ$) is again less than that in the N-lobe (rotation: hen, $53^\circ$; duck, $51.6^\circ$). Solution scattering studies show that the extent of lobe closure can be affected by the particular metal ion bound. The conformational changes are functionally important because they may be crucial for receptor recognition (see section 1.4.8). Binding of other metals such as Cu(II), In(III) or even the large metal ion Sm(III) (ionic radius 0.96 Å), also induce a similar overall conformational change in the protein as Fe(III) binding, although the local changes around the metal binding site are different. This suggests that transferrins carrying metal ions
other than Fe(III), should bind equally well to the specific transferrin receptors on cell surfaces, as those carrying Fe(III).

1.4.7 Receptor-Mediated Endocytosis and the Transferrin Cycle

Receptor-mediated endocytosis of the diferric transferrin complex into an acidic endosome, with subsequent release of Fe(III), is the generally accepted mechanism for Fe(III) delivery to a cell. The general features of this process are now well understood.

Receptor-mediated endocytosis is a natural process which cells utilise for the uptake of proteins or peptides such as low-density lipoproteins, asialoglycoprotein, epidermal growth factor, transferrin, insulin, and small vitamins such as folic acid. The first steps of this process involve binding of the protein to its specific cell membrane receptors, receptor clustering and internalisation through coated vesicles into endosomal acidic compartments. The following steps are strongly dependent on the type of protein-receptor pair; the low endosomal pH may or may not trigger dissociation of receptor and protein, and sorting processes may lead to degradative lysosomal compartments.

The initial event in the uptake of iron from transferrin by cells is the binding of transferrin to a specific receptor protein, i.e. the transferrin receptor, on the cell surface. At the extracellular pH of 7.4, the diferric (iron-saturated) transferrin binds strongly to the specific transferrin receptor. The receptor-transferrin complexes cluster in coated pits and are internalised by the cell. Diferric transferrin is held as a receptor adduct in membrane-bound vesicles (endosomes) which are coated with a protein called clathrin. The clathrin coats are removed and in the endosome, the pH is lowered from the extracellular value of 7.4 to about 5.5, dissociation of the iron from the transferrin occurs and Fe(III) is released inside the cell. The iron free transferrin (apo-transferrin) remains bound to the receptor at low pH with a greater affinity than iron bound (diferric) transferrin. The receptor-apo-transferrin complex is sorted into exocytic vesicles and is recycled back to the surface of the cell. At extracellular pH, apo-transferrin dissociates from its receptor due to its low affinity at pH 7.4, and is released into circulation, ready to bind more Fe(III). The receptor can then bind more diferric transferrin and the cycle continues. This process is shown diagrammatically in Figure 1.8.
Besides diferric transferrin, other metal-transferrin complexes have also been found to interact with transferrin receptors. $^{67}$Ga, a pharmacologically important metal, is also taken up by transferrin receptor-mediated endocytosis. $^{91}$ Ga$_2$-transferrin can inhibit transferrin receptor-mediated cellular uptake of iron. $^{92}$ The toxic effects of aluminium, particularly the neurotoxicity, are probably related to aluminium binding to transferrin, and Al$_2$-transferrin is transported and recognised by the transferrin receptor. $^{93}$ Aluminium uptake is enhanced in a variety of cell lines by addition of apo-transferrin, which has been attributed to receptor-mediated endocytosis of the Al$_2$-transferrin complex. $^{94}$ Several studies have shown that there is little difference between the binding of Fe$_2$-transferrin and Al$_2$-transferrin to transferrin receptor. $^{93,95}$
Thus, receptor-mediated endocytosis may function as a general metal ion transport and regulatory system.

### 1.4.8 Receptor Recognition

As discussed in section 1.4.7, iron is transported into cells via receptor-mediated endocytosis. The receptor imports iron-loaded (diferric) transferrin and recycles apo-transferrin after release of iron in the endosome. Only holo- but not apo-transferrin binds strongly to the transferrin receptor at the cell surface (pH 7.4), while only apo-transferrin binds tightly to the receptor in the endosome (pH ca.5.5), suggesting that conformational changes in transferrin are crucial for receptor recognition.

The major receptor recognition sites on human serum transferrin are thought to be localised on the C-lobe supported by the recent X-ray crystal structure of the human transferrin receptor.

The transferrin receptor of iron-requiring cells is a homodimeric transmembrane protein. It is a dimer of two identical 90 kDa subunits, which are linked by a pair of disulfide bonds. Each subunit is capable of binding one transferrin molecule. Each monomer has a short, NH$_2$-terminal cytoplasmic region (residues 1-67) containing the internalisation motif, a single transmembrane pass region (residues 66-88), and a large extracellular region (ectodomain, residues 89-760) containing a transferrin binding site. The crystal structure of the ectodomain of the human transferrin receptor dimer has been solved recently at 3.2 Å (Figure 1.9). The receptor dimer has a butterfly-like shape with each monomer containing three distinct domains, a protease-like domain (residues 122-188 and 384-606), an apical domain (residues 189-383) and a helical domain (residues 607-760). One transferrin receptor dimer binds two transferrin molecules.

Studies with proteolytically-derived or recombinant N- and C-lobe transferrin show that both lobes of transferrin interact with the receptor but the C-lobe binds more strongly, and thus the primary receptor recognition site in transferrin has been localised to the C-lobe.
Figure 1.9 Structure of the ectodomain of the transferrin receptor. (A) Domain organisation of the transferrin receptor polypeptide chain. The cytoplasmic domain is shown in white, the transmembrane segment is shown in black, the stalk is shown in grey, and the protease-like, apical and helical domains are shown in red, green, and yellow, respectively. Numbers indicate residues at domain boundaries. (B) Ribbon diagram of the transferrin receptor dimer depicted in its likely orientation with respect to the plasma membrane. One monomer is coloured according to the domain, as described above, and the other is shown in blue. The stalk region is shown in grey connected to the putative membrane-spanning helices. Pink spheres in the crystal structure indicate the location of the Sm(III) ions soaked into the crystal during crystallisation.

The transferrin receptor dimer has a convoluted surface (Figure 1.10). There are lateral facing clefts and a bowl-like depression at the top of the molecule. The central bowl-like depression at the top of the molecule is too small to accommodate
two non-interfering transferrin molecules, but the lateral clefts are more likely sites of interaction. Each cleft lies within a single monomer subunit, with all three domains contributing to its surface. There is apparent surface complementarity between transferrin and the lateral clefts of the transferrin receptor, which has allowed a model to be built by docking the two molecules, followed by a rigid body refinement (Figure 1.10).

![Figure 1.10 Proposed model for binding of diferric transferrin (rabbit) to the transferrin receptor showing recognition contacts between the two proteins.](image)

The surface of the transferrin dimer is rendered predominantly in white, with elements of the lateral cleft that are in contact with the docked transferrin molecule coloured according to the domain as in Figure 1.9. The structure of rabbit transferrin is shown as a backbone trace, colour coded by domain. The N-lobe domains, N1 and N2, are shown in orange and red, respectively, and the corresponding domains in the C-lobe, C1 and C2, are shown in blue and purple, respectively. The Fe(III) ions are shown as orange spheres, and the position of human and rabbit N-linked glycosylation sites on transferrin are denoted by black and white asterisks, respectively. White arrows indicate movements of the N2 and C2 domains upon Fe(III) release.
This model suggests that the major contact sites on transferrin are in the C-lobe, and particularly involve the C1 domain, with minor contributions from the N-lobe (N1 domain). It is interesting to note that the glycosylation sites on transferrin point away from the receptor and are not involved in transferrin-receptor interactions. This is consistent with the suggestion that the carbohydrate chains have no role in receptor recognition, as evidenced by the observation that nonglycosylated recombinant transferrin bound to HeLa S3 cells with the same affinity and to the same extent as the glycosylated protein.75

1.5 Models of Transferrins

Low molecular mass compounds have successfully been used as models for the metal ion binding sites of metalloproteins. Much information can be obtained about the nature of metal binding to proteins, e.g. the coordination geometry and stereochemistry, by studying low molecular mass model compounds. Ideally, these should closely resemble the metal ion binding site in the protein, in terms of their donor ligands and have similar physical and chemical properties.

Studies of the stereochemistry and coordination geometry of the donor ligands and electronic and magnetic properties (e.g. absorption bands, magnetic moments, oxidation states) of the specific metal ion binding sites of human serum transferrin have long been aided by studies of metal complexes of the model ligand ethylenebis[(o-hydroxyphenyl)glycine] (EHPG or H4EHPG).100-109

Figure 1.11 Schematic representation of the ligand N,N'-ethylenebis[(o-hydroxyphenyl)glycine] (EHPG or H4EHPG).
EHPG is a hexadentate ligand which contains the same number and similar types of donor groups as are found in the two specific metal ion binding sites of transferrin. EHPG possesses three pairs of chemically equivalent donor atoms: two amine nitrogens, two carboxylate oxygens and two phenolate oxygens (Figure 1.11), and offers an octahedral coordination environment to metal ions.

Previous studies on the Fe(III) complex of EHPG have shown that it shares many similar physical properties with Fe(III)-transferrin. The EHPG ligand has also proved useful in providing insights into the structure and chemistry of a number of other iron-phenolate proteins.

The Fe(III)EHPG complex, rac-Fe(III)EHPG, was recognised as a potential model for Fe(III)-transferrin originally on the basis of its optical and resonance Raman spectra and then later on the basis of its EPR g values and solution chemistry. The Fe(III)EHPG and Fe(III)-transferrin complexes also have similar isomer shifts and quadrupole splitting in the Mössbauer spectra. Also, the charge-transfer bands in the UV-Vis spectra of metal-EHPG complexes display all the characteristic features of the corresponding metallo-transferrin complexes for a wide variety of metal ions, although the charge-transfer bands are often shifted in energy or intensity.

The EHPG ligand itself possesses two asymmetric carbons atoms (Figure 1.11) and chelation to a metal introduces two further chiral centres at the nitrogen atoms. EHPG has been widely used as a ligand in studies of a range of metal ions other than Fe(III) and several X-ray crystal structures of metal-EHPG complexes have been reported.

Trivalent metal complexes of EHPG show diagnostic potential both in radiopharmaceutical applications (\(^{67}\)Ga, \(^{111}\)In) and as MRI contrast agents (Fe(III)). The paramagnetic metal complex [Fe(III)(EHPG)]\(^+\) is an effective hepatobiliary contrast agent for liver enhancement in MRI. The complex has significant hepatocellular uptake and appears to be excreted unaltered into the bile. Hepatobiliary specific paramagnetic metal complexes such as Fe(III)EHPG increase the sensitivity of MRI in the detection of liver disease. Fe(III)EHPG, enhances the liver-to-tumour contrast-to-noise ratio and improves lesion detection which proves promising in the early detection of liver metastases. The five unpaired electrons of
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the high-spin Fe(III) ion in this complex give rise to a high magnetic moment, which is desirable in the paramagnetic enhancement of water proton relaxation rates. Fe(III)EHPG effectively increases the relaxation rates of normal liver tissue and substantially enhances bile and liver signal intensities on magnetic resonance images.

Other substituted EHPG complexes have also been evaluated for use as potential hepatobiliary agents. Ring-substituted Fe(III)EHPG derivatives have been evaluated as paramagnetic contrast agents for the liver and biliary system for image enhancement in MRI.\textsuperscript{119} They increase the spin-lattice relaxation rates of water protons (1/T\_1), yielding image enhancement for detection of lesions and biliary abnormalities. The uptake and excretion by the liver of Fe(III)EHPG derivatives is effectively modulated by substituents at the 5-position on the aromatic ring, which stems from alterations in lipophilicity and protein binding properties. All 5-position ring-substituted Fe(III)EHPG derivatives show higher degree of lipophilicity and albumin binding affinities than the parent compound.

The \textsuperscript{67}Ga and \textsuperscript{111}In complexes of EHPG and its ring-substituted derivatives have been evaluated as potential radiopharmaceuticals for hepatobiliary imaging.\textsuperscript{115,120} The \textsuperscript{99m}Tc(III) complex of the 5-Br-EHPG derivative has also been evaluated \textit{in vivo} for its role as a potential radiopharmaceutical, which is excreted via the bile ducts, for imaging in the liver and gall bladder.\textsuperscript{121}

The \textsuperscript{59}Fe, \textsuperscript{68}Ga, and \textsuperscript{111}In complexes of TMPHPG (N,N'\textsuperscript{-}trimethylenebis[2-\textsuperscript{(2-hydroxy-3,5-dimethylphenyl)glycine}]), a lipophilic derivative of the EHPG ligand, have also been investigated as potential hepatobiliary contrast or imaging agents.\textsuperscript{122} TMPHPG has a longer carbon bridge between the amines than does EHPG and contains methyl substituents at the 3- and 5-positions of the rings.

Both isomers of the Fe(III)(5-Br-EHPG) derivative bind avidly to human serum albumin (HSA).\textsuperscript{123} There are at least two high affinity sites, as well as a larger, indeterminate number of nonspecific, low-affinity sites. HSA transports fatty acids and other lipophilic metabolites and is the most abundant protein in the blood (\textit{in vivo} concentrations are \(-0.6\) mM).\textsuperscript{124} In addition to its binding of endogeneous ligands, this 66.5 kDa protein possesses multiple binding sites for a wide range of anionic and aromatic drugs.
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There appears to be stereospecific binding of rac-Fe(III)(5-Br-EHPG) to the bilirubin site on HSA. Metal complexes that recognise specific sites on biological macromolecules have potential as drugs and molecular probes. The activity of these Fe(III) complexes as hepatobiliary imaging agents most likely stems from hepatocellular binding interactions at sites involved in the transport of bilirubin. These complexes crudely trace the biodistribution of bilirubin, the heme breakdown product. Bilirubin is known to bind to a specific site on HSA in the blood where it is transported into the liver and excreted into bile. Binding of Fe(III)EHPG to albumin slows down the molecular motion and can lead to a further enhancement in relaxivity.

1.6 Chemistry of Manganese

1.6.1 Discovery

Manganese, atomic number 25, atomic weight 54.94, is a first row transition metal, found in Group 7 of the periodic table, along with the elements technetium and rhenium. Manganese was first recognised as an element in 1774 in Stockholm by the Swedish chemists and mineralogists, Carl Wilhelm Scheele (1742–1786) and Torbern Olof Bergman (1735–1784) whilst working with the mineral pyrolusite, a manganese dioxide ore. The element was isolated by their associate Johann Gottlieb Gahn (1745–1818), later in the same year, by the reduction of pyrolusite with charcoal (essentially carbon) by heating.

\[ \text{MnO}_2 + C \rightarrow \text{Mn} + \text{CO}_2 \]

The name manganese is derived from the Latin magnes meaning magnet, so named due to the magnetic properties of pyrolusite. The relative abundance of manganese in the Earth's crust is approx. 0.085%. Manganese is the twelfth most abundant element in the Earth's crust and third most abundant transition metal after iron and titanium. The metal does not occur in the free state, except in meteors, but is most widely distributed over the world in the form of ores, such as rhodochrosite, franklinite, and manganite, amongst many others. The principal ore of manganese is pyrolusite.
Manganese also occurs in a number of substantial deposits, mainly oxides, hydroxides, or carbonates. Large quantities of manganese nodules on the deep ocean floors (e.g. the Pacific seabed) are a potentially rich and extensive source of manganese.\textsuperscript{127} They form when manganese oxides and other metallic salts such as nickel, copper and cobalt precipitate around a rock or shell nucleus. These nodules contain about 24% manganese together with many other elements in lesser abundance.

The most common forms of manganese minerals are oxides, silicates and carbonates. Most of the manganese today is obtained from ores found in Ukraine, Brazil, Australia, Republic of South Africa, Gabon, China and India. Pyrolusite (MnO\textsubscript{2}) and rhodochrosite (MnCO\textsubscript{3}) are among the most common manganese minerals. The pure metal is obtained by the reduction of MnO\textsubscript{2} with sodium, magnesium or aluminium, or by electrolysis.

Manganese is a grey-white metallic element, which resembles iron in its physical and chemical properties but is harder and more brittle. It has a melting point of 1246 ± 3°C, boiling point of 2061°C, and a specific gravity of 7.21 to 7.44. Depending on the allotropic form, manganese can display a valency of 1, 2, 3, 4, 6 or 7. The ground state electronic configuration of manganese is [Ar] 3d\textsuperscript{5}4s\textsuperscript{2}. The pure metal exists in four allotropic forms. The alpha form is stable at ordinary temperatures.

Manganese is chemically reactive. It is quite electropositive, dissolving very readily in dilute non-oxidising acids. The metal also corrodes in moist air and decomposes cold water slowly. Although manganese is relatively unreactive towards non-metals at room temperature, it reacts vigorously with many at elevated temperatures. Manganese combines directly with chlorine, fluorine, nitrogen, oxygen, boron, carbon, sulphur, silicon and phosphorus.

Manganese compounds can display a wide range of oxidation states, varying from −III to +VII, with a variety of coordination geometries. As in the case of titanium, vanadium and chromium, the highest oxidation state of manganese corresponds to the total number of 3d and 4s electrons. Mn(VII) is powerfully oxidising, usually being reduced to Mn(II). Mn(VII) only occurs in the oxo species; permanganate (MnO\textsubscript{4}\textsuperscript{−}), manganese oxide (Mn\textsubscript{2}O\textsubscript{7}) and manganese trioxide fluoride (MnO\textsubscript{3}F).\textsuperscript{126}
As shown in the oxidation state diagram in Figure 1.12, Mn(II) is the most stable and the most common oxidation state, although it is quite readily oxidised in alkaline solution. Mn(II) forms an extensive series of salts with all common anions most of which are soluble in water, although the phosphate and carbonate are only slightly so. Most of the salts crystallise from water as hydrates. Mn(II) has a d⁵ electronic configuration, and with the exception of a few low-spin complexes, the majority of Mn(II) complexes are high-spin. The high-spin d⁵ configuration gives an essentially spin-only, temperature-independent magnetic moment of ~5.9 \( \mu_B \). In octahedral fields this configuration gives spin-forbidden as well as parity-forbidden transitions, thus accounting for the extremely pale colour of such compounds.

In neutral or acid aqueous solution, there is the very pale pink hexaqua ion \([\text{Mn(H}_2\text{O)}_6]^{2+}\), which is resistant to oxidation as shown by the potentials:

\[
\begin{align*}
\text{MnO}_4^- & \quad \text{Mn}^{3+} + 1.5 \quad \text{Mn}^{2+} + 1.18 \quad \text{Mn} \\
+1.51 &
\end{align*}
\]
Chapter 1

Introduction: Metal Ions in Biological Systems

The redox potentials are referenced to the standard hydrogen electrode. In basic media the hydroxide \( \text{Mn(OH)}_2 \) is formed. This is very easily oxidised by air, as shown by the potentials: \(^\text{129}\)

\[
\begin{align*}
\text{MnO}_2 \cdot y\text{H}_2\text{O} & \quad 0.146 \quad \text{Mn}_2\text{O}_3 \cdot x\text{H}_2\text{O} & \quad -0.234 \quad \text{Mn(OH)}_2 \\
\end{align*}
\]

Mn(II) forms complexes with a variety of coordination numbers and geometries. The coordination numbers and geometries of Mn in the most common oxidation states of II, III, IV and VII, are summarised in Table 1.2.

### Table 1.2 Common oxidation states and stereochemistry of manganese. \(^\text{126,128}\)

<table>
<thead>
<tr>
<th>O.S. (^a)</th>
<th>d(^b)</th>
<th>C.N. (^b)</th>
<th>Geometry</th>
<th>Examples (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn(II)</td>
<td>d(^5)</td>
<td>2</td>
<td>Linear</td>
<td>Mn[Cl(C(SiMe(_3))(_2))] (_2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>Tetrahedral</td>
<td>MnCl(_2^2^), MnBr(_2)(OPR(_3)) (_2^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>Square planar</td>
<td>[Mn(H(_2)O)(_4)](_2^2^), H(_2)O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6(^c)</td>
<td>Distorted trigonal bipyramidal</td>
<td>Mn(_2^2^). (_2^2^)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>Octahedral</td>
<td>[Mn(H(_2)O)(_6)](_2^2^).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>Capped trigonal prismatic</td>
<td>Mn(_2^2^). (_2^2^)</td>
</tr>
<tr>
<td>Mn(III)</td>
<td>d(^4)</td>
<td>4</td>
<td>Square planar</td>
<td>MnX (_2).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>Square pyramidal</td>
<td>MnX (_2).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6(^c)</td>
<td>Trigonal bipyramidal</td>
<td>MnX (_2).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>Octahedral</td>
<td>Mn(acac)(_3), [Mn(ox)(_3)](_2^2^), Mn(S(_2)CNR(_2))(_3)</td>
</tr>
<tr>
<td>Mn(IV)</td>
<td>d(^3)</td>
<td>4</td>
<td>Tetrahedral</td>
<td>Mn(1-norbornyl) (_4^2^)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>Octahedral</td>
<td>MnO(_2^2^), MnCl(_6^2^).</td>
</tr>
<tr>
<td>Mn(VII)</td>
<td>d(^0)</td>
<td>3</td>
<td>Planar</td>
<td>\text{MnO}_3^+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4(^c)</td>
<td>Tetrahedral</td>
<td>\text{MnO}_4^2^, \text{MnO}_2^2^F</td>
</tr>
</tbody>
</table>

\(^a\) Oxidation state, \(^b\) Coordination number, \(^c\) Most common states, \(^d\) Key: acac, acetylacetonate anion; bipy, bipyridine; dmpe, 1,2-bis(dimethylphosphino)ethane; EDTA, ethylenediaminetetraacetic acid; Me, methyl; ox, oxalate anion; Ph, phenyl; R, alkyl or aryl group; sal\(_2\)en, bis-salicylaldehydeethylenediamine; tren, tris-(2-aminoethyl)amine; X, halogen or pseudohalogen.
Mn(III) is highly acidic and the very strong acidity of the aqua ion has been attributed to a strong ligand field stabilising a distorted ion, possibly \([\text{Mn(OH)}(\text{H}_2\text{O})_3]^{2+}\). The Mn(III) aqua ion\(^\text{130}\) can be obtained by electrolytic or peroxosulfate oxidation of Mn(II) solutions, or by reduction of MnO\(_4^-\). The ion plays a central role in the complex redox reactions of the higher oxidation states of manganese in aqueous solutions. It is most stable in acid solutions, since it is very readily hydrolysed:

\[
\text{Mn}^{3+} + \text{H}_2\text{O} = \text{Mn(OH)}^{2+} + \text{H}^+ \quad K = 0.93
\]

the initial monomer slowly polymerizing.\(^\text{131}\)

Under suitable conditions, the \(\text{Mn}^{3+}-\text{Mn}^{2+}\) couple is reversible (\(E^0 = 1.559\) V in 3 \(M\) LiCl\(_4\)). The Mn(III) ion is slowly reduced by water:

\[
2\text{Mn}^{3+} + \text{H}_2\text{O} = 2\text{Mn}^{2+} + 2\text{H}^+ + \frac{1}{2}\text{O}_2
\]

The Mn(III) state can be stabilised in aqueous solution by complexing anions such as \(\text{C}_2\text{O}_4^{2-}\), \(\text{SO}_4^{2-}\), and EDTA\(^4-\), but even the most stable species \([\text{Mn(EDTA)}(\text{H}_2\text{O})]^-\) undergoes decomposition because of slow oxidation of the ligand.

The \(5E_g\) (\(t_{2g}^1e_g^2\)) state for octahedral Mn(III) is subject to Jahn-Teller distortion. Because of the odd number of \(e_g\) electrons, this distortion should be appreciable. Indeed, a considerable elongation of two \textit{trans} bonds with little change in the lengths of the other four has been observed in many Mn(III) compounds. Other forms of high-spin Mn(III) complexes show a moderate tetragonal compression.

Mn(III) has only one spin-allowed absorption band (\(5E_g \rightarrow 5T_{2g}\)), which is expected to be in the visible region. The red-brown colours of high-spin Mn(III) compounds can thus be attributed to such absorption bands. However, the spectra of some six-coordinate Mn(III) complexes are not so simple, and they are difficult to interpret in all their detail, presumably because static and dynamic Jahn-Teller effects perturb the simple picture based on \(O_h\) symmetry.
1.6.2 Uses of Manganese

Manganese is widely used in a number of alloys with iron, aluminium, bronze, nickel-silver, and nickel-chromium. It is also an important component of steel. In steel, manganese improves the rolling and forging qualities, strength, toughness, stiffness, wear resistance, hardness and hardenability. The use of manganese is essential in the manufacture of steel as it is a unique deoxidising and desulfurising agent and no substitute exists for it.

Manganese dioxide ($\text{Mn}_2\text{O}_3$) occurs natively as pyrolusite and is prepared artificially by heating manganese nitrate; it is used in dry-cell batteries as a depolariser, in paint and varnish oils, for colouring glass and ceramics, and in the preparation of oxygen, chlorine and iodine. $\text{Mn}_2\text{O}_3$ is also used to “decolourise” glass that is coloured green by impurities of iron, and in drying black paints. Manganese by itself colours glass an amethyst colour, and is responsible for the colour of true amethyst. Manganese sulphate ($\text{MnSO}_4$), a pink crystalline solid, is prepared by the action of sulphuric acid on manganese dioxide and is used in dyeing cotton. Sodium and potassium permanganate ($\text{NaMnO}_4$ and $\text{KMnO}_4$) are dark purple crystals, formed by the oxidation of acidified manganese salts, which are used as oxidisers and disinfectants. Permanganate is a powerful oxidising agent and is used in quantitative analysis and in medicine.

Manganese as the organometallic compound, methylcyclopentadienyl manganese tricarbonyl (MMT) is used as an octane-enhancing additive (antiknock agent) in petrol and manganese ethylenebis(dithiocarbamate) is used as a fungicide. Manganese is also used in fertilisers.

1.6.3 Manganese in Biological Systems

Manganese is widely distributed throughout the animal kingdom. It is an essential trace element in living systems. It is vital to plant and animal life and is essential to reproduction in animals.
While manganese is widely distributed in the biosphere, it is present in only trace concentrations in animal tissues. Serum concentrations of manganese are typically less than 10 nM, whilst tissue concentrations are generally less than 4 μM. The average human body contains between 200 and 400 μmol of manganese, which is distributed uniformly throughout the body. There is little variation among species with regard to tissue manganese concentrations, reflecting in part the absence of manganese storage proteins. Tissues with high levels of mitochondria tend to have high manganese concentrations, as the concentration of manganese in mitochondria is higher than in the cytoplasm. Hair can accumulate high amounts of manganese and pigmented structures such as retina, dark skin, and melanin granules contain high concentrations of manganese. Bone, liver, pancreas, and kidney typically have higher concentrations of manganese (20-50 nmol/g) than other tissues. Brain, heart, lung, and muscle typically contain less than 20 nmol/g of manganese and blood and serum concentrations are in the order of 200 and 20 nmol/L, respectively. Bone can account for up to 25% of total body manganese because of its mass; however, bone manganese concentrations are not homeostatically controlled, and bone manganese is not readily mobilizable.

Manganese Enzymes

Manganese has some 20 identified functions in enzymes and proteins. It is one of several first-row transition elements that have been employed by biological systems to assist in varied metabolic and structural roles. Manganese is used to give structural support to proteins and is a cofactor in chemical transformations that include hydrolytic and redox reactions. It is an essential constituent of a number of enzymes, some of which have been structurally characterised by X-ray crystallography and are listed in Table 1.3.
Table 1.3 Examples of manganese enzymes and their functions.\textsuperscript{133}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>O.S.\textsuperscript{a}</th>
<th>Ligands for Mn</th>
<th>Function of Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase (SOD)</td>
<td>II/III</td>
<td>Asp, His, His, His, water</td>
<td>$\text{O}_2^-$ dismutation</td>
</tr>
<tr>
<td>Arginase\textsuperscript{b}</td>
<td>II</td>
<td>Mn\textsubscript{A}: His, Asp, {bridging OH, Asp, Asp}, Mn\textsubscript{B}: Asp, His</td>
<td>Hydrolysis of L-Arg to give urea and L-ornithine</td>
</tr>
<tr>
<td>Xylose isomerase\textsuperscript{b}</td>
<td>II</td>
<td>Mn\textsubscript{A}: Asp, Asp, Glu, {bridging Glu}, Mn\textsubscript{B}: His, Asp</td>
<td>D-xylose $\rightarrow$ D-xylulose (isomerisation)</td>
</tr>
<tr>
<td>Phosphatase\textsuperscript{b}</td>
<td>II</td>
<td>Mn\textsubscript{A}: Gly carbonyl (backbone), 3H\textsubscript{2}O, {bridging OH, Asp, Asp}, Mn\textsubscript{B}: Asp, 2H\textsubscript{2}O</td>
<td>Phosphate removal</td>
</tr>
<tr>
<td>Aminopeptidase\textsuperscript{b}</td>
<td>II</td>
<td>Mn\textsubscript{A}: His, Glu, H\textsubscript{2}O, {bridging OH, Asp, Glu}, Mn\textsubscript{B}: Asp, H\textsubscript{2}O</td>
<td>Cleaves N-terminal amino acid from peptide</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Oxidation State, \textsuperscript{b}Dinuclear.

**Manganese Neurochemistry**

Manganese is essential for normal brain function. It is abundant in the brain in enzymes such as glutamine synthase and superoxide dismutase. The control of metal neurochemistry appears to be vital for the prevention of neuronal degradation, and for the understanding, perhaps also effective treatment, of conditions such as Parkinson’s disease, senile dementia, Alzheimer’s disease and even Creutzfeldt-Jacob disease (CJD). Miners and workers in some industries are at risk of toxicity from inhaling manganese. Chronic inhalation can lead to symptoms of Parkinson’s disease. Manganese is also thought to play a major role in CJD and bovine spongiform encephalopathy (BSE). It is reported that Mn(II) binds to the Cu-prion protein and is then converted into Mn(III) which is the complex which cause CJD and BSE.\textsuperscript{135-137}
1.6.4 Manganese Diagnostic and Therapeutic Agents

Superoxide Dismutase Mimics

Superoxide dismutase (SOD) is a redox active metalloenzyme which catalyses the dismutation of the cell-poisoning superoxide radical, \( \text{O}_2^- \), into the non-radical products oxygen, \( \text{O}_2 \), and hydrogen peroxide, \( \text{H}_2\text{O}_2 \):\(^{138}\)

\[
\begin{align*}
\text{M}^{n+1} + \text{O}_2^- & \rightarrow \text{M}^{n+} + \text{O}_2 \\
\text{M}^{n+} + \text{O}_2^- + 2\text{H}^+ & \rightarrow \text{M}^{n+1} + \text{H}_2\text{O}_2
\end{align*}
\]

where \( \text{M}^{n+} \) is the metalloenzyme in the reduced state and \( \text{M}^{n+1} \) is the enzyme in the oxidised state.

Superoxide radical anions, \( \text{O}_2^- \), are a product of cellular respiration, activated polymorphonuclear leukocytes and endothelial cells.\(^{139}\) \( \text{O}_2^- \) has been postulated to be a mediator of ischemia reperfusion injury as well as inflammatory and vascular diseases,\(^{140}\) and can react with NO to form damaging peroxynitrite, \( \text{ONOO}^- \).\(^{141}\) SOD is the main enzyme that is responsible for controlling the presence of \( \text{O}_2^- \). The role of SOD is to regulate the lifetime of superoxide and protect against superoxide-mediated cytotoxicity.

The extracellular enzymes Cu- and Zn-SOD are found in the cytoplasm of eukaryotic cells, and the intracellular Mn-SOD enzyme in the mitochondria. Mn-SOD is also found in prokaryotic cells along with Fe-SOD. The Cu- and Zn-containing enzyme\(^{142,143}\) catalyses the dismutation of \( \text{O}_2^- \) with pH independent rate constants measured to be in excess of \( 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \) while the Mn enzyme\(^{144,145}\) is approximately 10-fold less active.

The use of SOD in therapy is limited by its short plasma half-life (clearance by the kidney) and inability to penetrate cell membranes (i.e. extracellular activity only). Other problems associated with the use of an enzyme as a pharmaceutical agent are the cost, bioavailability, stability and immunogenicity. Low molecular mass SOD mimics are therefore of much interest as potential pharmaceuticals.
A variety of Mn- and Fe-based porphyrins and macrocyclic complexes exhibit SOD mimetic activity and these are potentially useful for the treatment of diseases such as ischemia-reperfusion injury.\textsuperscript{146-149} Mn(II) and Mn(III) macrocycles appear to be particularly promising.\textsuperscript{150,151} The macrocyclic ligand effect has been employed in these Mn complexes to enhance the kinetic and thermodynamic stability.\textsuperscript{152} Thus, manganese superoxide dismutase mimics have clinical potential in SOD therapy.

**Pentaaza Macrocyclic Complexes**

The Mn(II) complex of 1,4,7,10,13-pentaazacyclopentadecane, Mn([15]aneN\textsubscript{5})Cl\textsubscript{2} (SC-52608, \textbf{Figure 1.13A}), and derivatives of this complex which bear substituents on the carbon backbone of the macrocycle (\textbf{Figure 1.13B}) are highly active and stable SOD mimics.\textsuperscript{150,153} These complexes catalytically decompose superoxide with $k_{\text{cat}}$ values ranging from 1.4 to $9.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4 (the value of $k_{\text{cat}}$ at any pH is obtained from the slope of the plot of $k_{\text{obs}}$ versus [Mn(II) complex]).

![Figure 1.13](image-url)

\textbf{Figure 1.13} (A) The pentaaza macrocyclic Mn(II) complex, SC-52608, and (B-D) derivatives of this complex which bear substituents on the carbon backbone. (C) The 2,3-(R,R)-8,9-(R,R)-bis(trans-cyclohexano) substituted Mn(II) complex, SC55858 and (D) the 2,3-(R,R)-8,9-(S,S)-bis(trans-cyclohexano) substituted derivative.
The complex SC-52608 is able to scavenge superoxide and therefore effectively protect the regionally ischemic and reperfused myocardium from injury.\textsuperscript{154-156} It also inhibits neutrophil-mediated injury to aortic endothelial cells,\textsuperscript{157} potentiates the levels of nitric oxide (a potent vasorelaxant),\textsuperscript{158} and is a potent anti-inflammatory agent.\textsuperscript{159}

The \textit{trans}-2,3-cyclohexano group as a substituent is particularly effective at increasing both SOD activity and kinetic stability of the Mn complex.\textsuperscript{151,153,157} The $k_{\text{cat}}$ value for the dismutation of superoxide by the 2,3-(\textit{R},\textit{R})-\textit{trans}-cyclohexano substituted complex, SC-54417,\textsuperscript{153} is $9.09 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, which is more than twice that of the unsubstituted parent compound, SC-52608 ($k_{\text{cat}} = 4.13 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$).\textsuperscript{150} The catalytic rate constant increases as additional \textit{trans}-cyclohexano groups are added, with SC-55858 ($k_{\text{cat}} = 1.2 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$) having three times more SOD activity than the unsubstituted SOD mimic SC-52608.\textsuperscript{151} However, simply increasing the number of substituents on the carbon backbone of the macrocyclic ring does not necessarily imply that the resultant Mn(II) complex will possess any SOD activity. This is shown by the differences in catalytic activity of the Mn(II) complexes of two isomeric bis(\textit{trans}\textendash fused cyclohexano) substituted 1,4,7,10,13-pentaaazacyclopentadecane ligands, complexes C (SC-55858) and D in Figure 1.13, respectively.\textsuperscript{151} The two complexes incorporate two \textit{trans}-cyclohexano fused backbones on the macrocyclic ring ligands; a structural motif which enhances both stability and activity. SC-55858, the 2,3-(\textit{R},\textit{R})-8,9-(\textit{R},\textit{R})-bis(\textit{trans}-cyclohexano) substituted Mn(II) complex (complex C), was found to be an excellent SOD catalyst with a second-order rate constant at pH = 7.4 of $1.2 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$. In contrast, the isomeric (2,3-(\textit{R},\textit{R})-8,9-(\textit{S},\textit{S})-bis(\textit{trans}-cyclohexano) substituted derivative, complex D, was found to have virtually no detectable catalytic SOD activity. This implies that the position, number and stereochemistry of the substituents on the carbon atoms of the macrocyclic ring exert an effect on the catalytic rate. The crystal structure of complex D was determined and reveals that the Mn(II) ion is coordinated in a pentagonal bipyramidal array of the five nitrogens of the macrocyclic ligand and capped by two \textit{trans}-chloro ligands.

Mechanistic studies with SC-55858 (complex C) and the pentamethyl substituted complex, including D$_2$O rates, have been reported to be consistent with the existence of two pathways for the electron-transfer from Mn(II) to superoxide, each of which possesses a rate-determining step which involves oxidation of Mn(II) to Mn(III).\textsuperscript{151} The major pathway is proton dependent and occurs via an outer-sphere mechanism;
hydrogen atom transfer from a bound water on the Mn(II) to HO$_2^*$ to yield a Mn(III) hydroxo intermediate. The second pathway is pH independent, and involves the oxidation of Mn(II) by an inner-sphere dissociative pathway, in which the superoxide anion binds to a vacant coordination site on Mn(II) followed by protonation/oxidation to yield a Mn(III)hydroperoxo species. Subsequent reduction of the intermediate Mn(III) with the superoxide anion completes the catalytic cycle.

Substituent effects on the rates and relative contributions of the two pathways to the overall rate of SOD activity is ascribed to the propensity of the ligand to fold around Mn(II) forming a pseudo-octahedral complex similar in geometry to the oxidised Mn(III) complex. Folding of the pentaaza macrocyclic ligand has been confirmed as a relevant structural motif for this series of Mn(II) complexes by the X-ray structure determination of the bis(nitrate)derivative of complex A in Figure 1.13, [Mn-(C$_{10}$H$_{25}$N$_5$)NO$_3$]NO$_3$, which reveals a six coordinate structure with a folded conformation of the macrocyclic ligand. Increasing the number of substituents on the carbon atoms of the macrocycle invariably increases stability (both kinetic and thermodynamic) of the Mn(II) complexes derived from SC-52608 (complex A, Figure 1.13).

The Mn(II) complex with a derivative of the macrocyclic ligand, 1,4,7,10,13-pentaazacyclopentadecane, containing the added bis(cyclohexylpyridine) functionalities, M40403 (Figure 1.15), catalyses the dismutation of superoxide with rates approaching that of the native Mn-SOD enzyme. M40403 has a catalytic SOD rate $> 2 \times 10^7$ M$^{-1}$ s$^{-1}$, which is comparable to that of Mn-SODs at a pH of ~6. M40403 inhibits inflammation, vascular thickening and chronic reperfusion injury in a number of in vitro and in vivo animal models.

![Figure 1.15 Structure of the SOD mimic M40403.](image-url)
Porphyrin Complexes

The SOD activity of a manganese porphyrin complex was first reported over 20 years ago by Pasternack et al.\textsuperscript{162} The complex Mn(III)-5,10,15,20-tetrakis(1-methyl-4-pyridyl)-porphyrin, (Mn(III)TMPyP, Figure 1.15), catalyses the dismutation of superoxide with an apparent rate constant of \(1 \times 10^7 \text{ M}^{-1} \text{s}^{-1}\) at pH 7.4-7.8.\textsuperscript{16} The complex is reversibly reduced by one electron in aqueous media and retains its activity in the presence of EDTA, suggesting that the complex is stable.\textsuperscript{146} Mn(III)TMPyP also possesses catalase activity and is also capable of decomposing peroxynitrate.\textsuperscript{163-165}

![Figure 1.15 Mn(III)-5,10,15,20-tetrakis(1-methyl-4-pyridyl)-porphyrin, (Mn(III)TMPyP).](image)

The complex Mn(III)-5,10,15,20-tetrakis(4-benzoic acid)-porphyrin (MnTBAP) exhibits both SOD activity and catalytic activity for the destruction of peroxynitrate. Thus, the anti-inflammatory effects of the complex could be due to multiple scavenging roles, namely superoxide dismutation and peroxynitrate destruction.\textsuperscript{166}
MnTBAP can protect against neurodegeneration and is therefore of potential interest for the treatment of brain diseases such as Parkinson’s and Alzheimer’s diseases.\textsuperscript{167}

Two five-coordinate monomeric (benzoato)Mn(II) complexes, Mn(Obz)(HB(3,5-iPr₂Pz)₃) and Mn(Obz)(3,5-iPr₂pzH)(HB(3,5-iPr₂Pz)₃) where HB(3,5-iPr₂Pz)₃ is the ligand hydrotris(3,5-diisopropylpyrazol-1-yl)borate and 3,5-iPr₂pzH is 3,5-diisopropyl-pyrazole, have also been implicated as SOD mimics.\textsuperscript{168}

\textbf{Magnetic Resonance Imaging Contrast Agents}

Magnetic resonance imaging (MRI) is now a powerful tool in clinical diagnosis.\textsuperscript{169,170} Diseases can be detected from differences in \(^1\)H NMR resonances (mainly due to H₂O) between normal and abnormal tissues \textit{via} administration of external paramagnetic contrast agents. MRI contrast agents shorten proton relaxation times and therefore enhance the image contrast between normal and diseased tissue.\textsuperscript{171} The design of the ligand allows paramagnetic ions to be targeted to specific organs. The effect of the paramagnetic contrast agent in MRI is to increase the signal intensity of the tissue containing the agent.

Manganese (high spin Mn(II)), gadolinium (Gd(III)) and iron (Fe(III)) compounds have been used as MRI contrast agents. These ions have a large number of unpaired electrons (5, 7 and 5, respectively) and long electron spin relaxation times.\textsuperscript{172,173} Examples of manganese-based MRI contrast agents are Teslascan\textsuperscript{174} and Mn(II)-tetrasulfonated phthalocyanine.\textsuperscript{175}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{Caption}
\caption{Mn(II)-N,N’-dipyridoxylethylenediamine-N,N’-diacetate-5,5’-bis(phosphate) (MnDPDP, Teslascan).}
\end{figure}
Teslascan, the mangafodipir trisodium salt (Figure 1.16), (Mn(II)-N,N'-dipyridoxylethlenediamine-N',N'-diacetate-5,5'-bis(phosphate), MnDPDP) is an intravenously administered manganese chelate in clinical use for enhancing contrast in the liver for the detection of hepatocellular carcinomas. Some dissociation of Mn(II) occurs in the liver, and enhancement can also be obtained in functional adrenal tissues. The relaxivity of this complex is about 35% greater than that of the Mn complexes of DTPA and DOTA, which also do not contain directly coordinated water molecules, unlike their gadolinium analogues. Mn(II)-tetrasulfonated phthalocyanine also shows tumour localisation properties and is a more efficient relaxation agent than the analogous Gd(III) complexes.

1.7 Chemistry of Titanium

1.7.1 Discovery

Titanium, atomic number 22, atomic weight 47.88, is a first row transition metal, found in Group 4 of the periodic table, along with the elements zirconium and hafnium. Titanium was first discovered in 1791 in Cornwall, England, by the British mineralogist and clergyman William Gregor (1761-1817). It was four years later in 1795 that titanium was rediscovered, by the German chemist Martin Heinrich Klaproth (1743-1817), and identified as an element. Klaproth gave titanium its present name, which is derived from the Latin Titans, (the first sons of the Earth, in Greek mythology). Impure titanium was prepared by Nilson and Pettersson in 1887; however, the pure metal (99.9%) was not made until 1910 by Hunter by heating TiCl₄ with sodium in a steel bomb.

Titanium is the ninth most abundant element in the Earth’s crust with a relative abundance of approx. 0.6%. Titanium compounds are widely distributed in nature but the element is never found in a pure state. The main ores of titanium are ilmenite (FeTiO₃) and rutile, one of the several crystalline varieties of TiO₂. Titanium is almost always present in igneous rocks and in the sediments derived from them. It also occurs in titanates and in many iron ores. Deposits of ilmenite and rutile are found in Florida, California, Tennessee, and New York. Australia, Norway, Malaysia, India and China are also large suppliers of titanium minerals. Because of
the difficulty and expense of separating titanium from the ores with which it is found, it is an expensive metal. Titanium is present in the ash of coal, in plants, and in the human body. Titanium is also present in meteorites, on the moon and in the sun and other stars.

Titanium, when pure, is a lightweight, lustrous, white metal. It has a melting point of 1668°C and a boiling point of 3287°C and a specific gravity of 4.54. Titanium can display a valence of +2, +3, or +4. The ground state electronic configuration of titanium is \([\text{Ar}]\, 3d^24s^2\). Natural titanium consists of five isotopes with atomic masses from 46 to 50. All are stable. Eighteen other unstable isotopes are also known. The metal is dimorphic. The hexagonal alpha form changes to the cubic beta form very slowly at about 880°C. Its chemical properties resemble those of zirconium, the element below it in group 4 of the periodic table. The metal is strong, has a low density, is easily fabricated, and has excellent corrosion resistance. It is ductile only when it is free of oxygen. The metal when heated ignites and burns in air and is the only element that burns in nitrogen. Titanium is resistant to dilute sulfuric and hydrochloric acid, most organic acids, moist chlorine gas, and chloride solutions. The metal is not attacked by mineral acids at room temperature or even by hot aqueous alkali. The metal combines with oxygen at red heat, and with chlorine at 550°C.

The metal has a hexagonal close-packed lattice and resembles other transition metals such as iron and nickel in being hard, refractory, and a good conductor of heat and electricity. It is however, quite light in comparison to other metals of similar mechanical and thermal properties and unusually resistant to certain kinds of corrosion.

Although unreactive at ordinary temperatures, titanium combines directly with most nonmetals, for example, hydrogen, the halogens, oxygen, nitrogen, carbon, boron, silicon, and sulfur, at elevated temperatures. The resulting nitride (TiN), carbide (TiC), and borides (TiB and TiB\(_2\)) are interstitial compounds that are very stable, hard, and refractory.

Titanium metal can be produced by the Kroll processes which involves the reduction of titanium tetrachloride with magnesium. Extremely pure metal can be obtained by decomposing the iodide. The metal cannot be obtained by the common method of
reduction with carbon because a very stable carbide is produced; moreover, titanium metal is very reactive towards both oxygen and nitrogen at elevated temperatures. Titanium metal is considered to be physiologically inert; however, titanium powder may be a carcinogenic hazard.

Titanium has five common oxidation states, -I, 0, II, III, IV. The most stable and the most common oxidation state is Ti(IV); compounds in lower oxidation states are quite readily oxidised to Ti(IV) by air, water, or other reagents. The Ti(IV) ion does not exist in isolation, since the energy for removal of four electrons is high and Ti(IV) compounds are generally covalent. The spherical symmetry of the d<sup>0</sup> configuration allows a variety of stereochemistries for Ti(IV). The coordination number of Ti(IV) can vary from 4, 5, 6, 7, to 8, common geometries being tetrahedral (TiCl<sub>4</sub>), distorted tetrahedral (Cp<sub>2</sub>TiCl<sub>2</sub>), trigonal bipyramidal (TiCl<sub>5</sub>), square pyramidal ([TiOCl<sub>4</sub>]<sup>2-</sup>, [TiO(porphyrin)]), octahedral (TiF<sub>6</sub><sup>2-</sup>, Ti(acac)<sub>2</sub>Cl<sub>2</sub>, TiO<sub>2</sub>), pentagonal bipyramidal ([TiCl(S<sub>2</sub>CNMe<sub>2</sub>)<sub>3</sub>]), capped trigonal prismatic ([Ti(O<sub>2</sub>)F<sub>3</sub>]<sup>3-</sup>), dodecahedral ([Ti(ClO<sub>4</sub>)<sub>4</sub>]) and distorted dodecahedral ([[TiCl<sub>4</sub>(diars)]).

Ti(IV) is a hard metal ion in the hard-soft classification. Ti(IV) compounds characteristically undergo hydrolysis to give species with Ti-O bonds, in many of which it is octahedrally coordinated by oxygen ligands. Polymeric species with -OH- or -O- bridges are common for Ti(IV) in aqueous solution. No aquated Ti(IV) salts can be isolated, but Ti(IV) possibly exists in solution though the main species are hydrolysed. Many studies in different media show that species such as Ti(OH)<sub>2</sub><sup>2+</sup>, Ti(OH)Cl<sup>2+</sup>, or possibly Ti<sub>2</sub>(OH)<sub>6</sub><sup>2+</sup> may be present.

1.7.2 Uses of Titanium

Titanium is important as an alloying agent with aluminium, molybdenum, manganese, iron, and other metals. Titanium metal and its alloys are light in weight and have very high tensile strength, even at high temperatures. Titanium is as strong as steel, but 45% lighter and is 60% heavier than aluminium, but twice as strong. Titanium has potential use in desalination plants for converting seawater into fresh water. Titanium resists seawater and sea-air corrosion or rust as well as platinum and better than stainless steel. When pure, titanium dioxide has an extremely high index of refraction with an optical dispersion higher than diamond, although its not as hard.
Pure crystalline titanium dioxide is produced artificially for use as a gemstone, but it is relatively soft. Star sapphires and rubies exhibit their asterism as a result of the presence of TiO$_2$. Titanium dioxide is extensively used as a pigment in paint, as it is permanent and has good covering power. Titanium oxide pigment accounts for the largest use of the element. Titanium paint is an excellent reflector of infrared, and is extensively used in solar observatories where heat causes poor seeing conditions. Titanium tetrachloride, a liquid, is used to iridize glass. This compound fumes strongly in moist air and has been used to produce smoke screens.

Titanium is also an important catalyst in the polymerisation of olefins. Titanium esters, formed by the reaction of the tetrachloride with alcohols, are used as waterproofing agents on fabrics. Titanium sulfate is used as a textile mordant.

1.7.3 Medicinal Chemistry of Titanium

A number of complexes with titanium have shown to be active against cancer. Two such complexes, which have reached clinical trials, are titanocene dichloride and budotitane.$^{182,183}$

![Titanocene dichloride and Budotitane](image)

Figure 1.17 (A) Titanocene dichloride, and (B) Budotitane.

Titanocene dichloride is a bent sandwich complex. The central metal ion is in a distorted tetrahedral coordination geometry with two $\pi$-bonded cyclopentadienyl
(Cp) ligands and two chloride ligands bound in a cis configuration (Figure 1.17A). The bis(β-diketonato)-Ti(IV) complex, budotitane, is octahedral, coordinated by the four oxygen atoms from the two diketonato units and two other oxygen atoms from the two cis-ethoxide ligands (Figure 1.17B).

Both complexes undergo rapid hydrolysis in water, which causes problems with their formulation. A precipitate of TiO₂ is formed.
1.8 Aims of Thesis

(i) To prepare manganese serum transferrin and investigate the nature of the manganese binding sites using electronic absorption spectroscopy and X-ray absorption spectroscopy.

(ii) To prepare and characterise manganese complexes of EHPG as a model for manganese transferrin.

(iii) To study the binding of EHPG to Ti(IV) as an aid to elucidation of the uptake of titanium by transferrin from the anticancer drug titanocene dichloride.

(iv) To investigate the stabilisation of Mn(III) by other ligands such as cyclam.
1.9 References


Chapter 1

Introduction: Metal Ions in Biological Systems


Chapter 1

Introduction: Metal Ions in Biological Systems


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Chapter 2

Materials and Methods

In this Chapter, the sources of materials and some specific techniques used in this thesis are briefly described. These include NMR, UV-Vis, SQUID, EPR, ICP-AES, EXAFS and XANES.

2.1 Materials

2.1.1 Reagents

The reagents used in this work, and their sources, are listed in Table 2.1.

Table 2.1 Reagents and sources.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Chemical Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-transferrin, human serum</td>
<td>Sigma</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Citric acid, monosodium salt</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Cyclam</td>
<td>Lancaster</td>
</tr>
<tr>
<td>Dibromo-(p)-xylene</td>
<td>Acros</td>
</tr>
<tr>
<td>Ethylenebis((\alpha)-hydroxyphenyl)glycine</td>
<td>Aldrich and Sigma</td>
</tr>
<tr>
<td>(\text{MnCl}_2.4\text{H}_2\text{O})</td>
<td>Aldrich</td>
</tr>
<tr>
<td>(\text{Mn(II)acetate.4H}_2\text{O})</td>
<td>Aldrich</td>
</tr>
<tr>
<td>(\text{Mn(III)acetate.2H}_2\text{O})</td>
<td>Aldrich</td>
</tr>
<tr>
<td>Nitritriacetic acid</td>
<td>Aldrich</td>
</tr>
<tr>
<td>(\text{P(NMe}_2)_3)</td>
<td>Acros</td>
</tr>
<tr>
<td>(\text{Ti(III)Cl}_3)</td>
<td>Aldrich</td>
</tr>
<tr>
<td>(\text{N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES)})</td>
<td>Aldrich</td>
</tr>
</tbody>
</table>
All chemicals were used as received without further purification, except for apo-transferrin which was purified according to the method described in section 3.2.1.

### 2.1.2 Solvents

**Anhydrous Solvents**

When anhydrous solvent is specified, the solvent was dried in the manner described in Table 2.2, using standard procedures.\(^1\)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Drying Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>Distilled over Mg/l₂</td>
</tr>
<tr>
<td>N,N-Dimethylformamide (DMF)</td>
<td>Dried over KOH or CaH₂, then distilled over MgSO₄</td>
</tr>
<tr>
<td>Toluene</td>
<td>Distilled over Na</td>
</tr>
</tbody>
</table>

**Purification of Water**

A Millipore Elix 5 system was used for the deionisation of water used for synthesis.

All water for protein work was purified using a Millipore Elix 5 system, followed by an Elgastat UHQ II deioniser. The purified water obtained using this procedure is referred to in this thesis as ultrapure water.

### 2.2 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a very powerful and versatile technique for investigating the structure and dynamics of molecules. The first NMR signals were recorded independently by two separate physicists, Felix Bloch and Edward Mills Purcell, in 1945, for which they were jointly awarded the Nobel Prize for Physics in 1952. Their preliminary findings were published, almost simultaneously, in 1946.\(^2,3\)
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The physical basis of NMR spectroscopy is nuclear magnetism. Nuclear magnetic moments are very sensitive to their environment and yet interact only very weakly with them, exerting a negligible influence on the molecular properties, thus making them ideal tools for analysis.

A number of textbooks have been published describing the theory, applications and practical aspects of NMR spectroscopy and can be referred to for a more in-depth description.4-8

2.2.1 Nuclear Magnetism

Spin Angular Momentum

Magnetic nuclei possess an intrinsic angular momentum known as spin, which can be characterised by the spin quantum number, $I$. The spin quantum number of a nucleus is determined largely by the number of unpaired protons and neutrons. Protons, neutrons (and electrons) all have a spin of $I = \frac{1}{2}$. Consequently, for a given nucleus, $I$ can have one of the following values: $I = 0, \frac{1}{2}, 1, \frac{3}{2}, 2, \ldots$, with $I > 4$ being very rare.

Nuclear Magnetisation

The spinning of a nucleus (a charged body) generates a magnetic moment. Every nucleus with spin angular momentum ($I$) therefore also possesses a nuclear magnetic moment ($\mu$). Both $\mu$ and $I$ are vector quantities and therefore have direction as well as magnitude, which according to quantum theory are quantized. Thus the magnetic energy of the nucleus is restricted to certain discrete values, and the number of allowed orientations for the angular momentum is also restricted. The angular momentum ($I$) of a spin-$I$ nucleus has $2I + 1$ possible states. These energy states (and therefore the direction of $\mu$) can be characterised by the magnetic spin quantum number, $m_I$, which has $2I + 1$ values in integral steps between $+I$ and $-I$. Thus the angular momentum of a nucleus with a spin, $I = \frac{1}{2}$, (e.g. $^1$H) has only two allowed states, whereas a nucleus with $I = 1$, has three possible states.

The magnetic moment of a nucleus, $\mu$, is directly proportional to its spin angular momentum, $I$, and is related by a proportionality constant $\gamma$, known as the
gyromagnetic ratio. \( \gamma \) is a characteristic constant of each type of nucleus. Isotopes have different characteristic values of \( \gamma \), however not all isotopes are NMR active.

\[ \mu = \gamma I \]

### 2.2.2 Effect of a Magnetic Field

The number of \( m_I \) states (energy levels) for a nucleus with spin \( I \) is \( 2I + 1 \). In the absence of a magnetic field, the \( m_I \) states are all degenerate. When a nucleus is placed in a magnetic field, the degeneracy is removed, and the different states will have different energies depending on how the nuclear magnetic moment is orientated relative to the applied field. For a spin-\( 1/2 \) nucleus, such as \( ^1H \), there are two different allowed orientations, which correspond to nuclei having their spins aligned with or opposed to the magnetic field. The lower energy state, in which the nuclear magnetic moment is parallel to the applied magnetic field, \( B_0 \), corresponds to \( m_I = +\frac{1}{2} \). The higher energy state in which the magnetic moment is antiparallel to \( B_0 \), corresponds to \( m_I = -\frac{1}{2} \).

The energies \( (E) \) of the allowed orientations of the nuclear magnetic moment depend on the strength of the applied field \( (B_0) \), the size of the nuclear magnetic moment \( (i.e. \) the gyromagnetic ratio, \( \gamma \) \), and on the orientation of the moment in the applied field \( (m_I) \):

\[ E = m_I \gamma B_0 \hbar \]

The \( m_I \) states are separated by an energy \( \Delta E \), which depends on the strength of the interaction between the nucleus and the field, \( i.e. \) on the size of the nuclear magnetic moment and the strength of the magnetic field.

\[ \Delta E = \hbar \gamma B_0 \]

### 2.2.3 Resonance Frequencies

The energy difference, \( \Delta E \), between two energy levels \( (m_I \) states) corresponds to a particular, precise electromagnetic frequency. Transitions of nuclei between
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different levels are possible if the resonance condition $\Delta E = h\nu$ is satisfied, *i.e.* by irradiation with electromagnetic radiation of frequency $\nu$, known as the resonance frequency. Since the separation of energy levels is also dependent on the strength of the magnetic field and the gyromagnetic ratio, each different isotope will resonate at a characteristic, distinct frequency.

$$\Delta E = h\nu = h\gamma B_0 = h\gamma B_0 / 2\pi$$
$$\nu = \gamma B_0 / 2\pi$$

The resonance frequency of a magnetic nucleus is found to vary in direct proportion to the applied magnetic field. For a given field strength, the resonance frequency of a nucleus, is determined principally by its gyromagnetic ratio, $\gamma$.

The $2I + 1$ states for a spin $I$ nucleus are equally spaced. The selection rule for NMR is $\Delta m_I = \pm 1$, *i.e.* transitions are allowed only between adjacent levels. Since all energy levels are equally spaced, all $2I$ allowed transitions for a spin $I$ nucleus have the same energy, and only a single resonance will be observed in the spectrum.

NMR signals can be routinely detected for almost any magnetic nucleus and most elements have at least one naturally occurring magnetic isotope. The magnetic properties of some common NMR active nuclei are given in Table 2.3.

**Table 2.3 Selected NMR properties of some common nuclei.**

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Spin Quantum Number/ $I$</th>
<th>Natural Abundance ($%$)</th>
<th>Magnetic Moment/ $\mu$</th>
<th>Gyromagnetic ratio/ $\gamma$ ($10^8$ rad T$^{-1}$ s$^{-1}$)</th>
<th>Resonance Frequency ($\nu$ MHz)</th>
<th>Relative Sensitivity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H</td>
<td>$\frac{1}{2}$</td>
<td>99.985</td>
<td>2.798</td>
<td>2.675</td>
<td>42.577</td>
<td>1.000</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>$\frac{1}{2}$</td>
<td>1.108</td>
<td>0.702</td>
<td>0.673</td>
<td>10.705</td>
<td>0.016</td>
</tr>
<tr>
<td>$^{15}$N</td>
<td>$\frac{1}{2}$</td>
<td>0.365</td>
<td>-0.283</td>
<td>-0.271</td>
<td>4.315</td>
<td>0.001</td>
</tr>
</tbody>
</table>

$^a$ In a 1 T magnetic field, $^b$ Versus $^1$H at constant field.

Nuclei with spins $> \frac{1}{2}$ are quadrupolar and normally give rise to broad NMR signals making them difficult to study.
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2.2.4 Chemical Shifts and Coupling Constants

Nuclear Shielding and Chemical Shift

The resonance frequency of a nucleus in a molecule is determined principally by its gyromagnetic ratio, $\gamma$, and the strength of the applied magnetic field, $B_0$. However, the resonance frequency also depends, to a lesser extent, on the position of the nucleus in the molecule, i.e. on the local electron distribution within the molecule, such that the exact resonance frequency is characteristic of the chemical environment of the nucleus. This effect is known as the chemical shift. The number of different chemical shift resonances in a proton NMR spectrum derives from the number of non-equivalent protons present in a molecule.

Chemical shifts arise because the magnetic field $B$ actually experienced by a nucleus in an atom or molecule differs slightly from the external field $B_0$, i.e. the field that would be felt by a bare nucleus stripped of its electrons. The magnetic field experienced by a nucleus in a molecule is influenced by the local electron distribution in its vicinity. The motion of electrons in a molecule in an applied magnetic field induces a small local magnetic field at the nucleus. The induced local field can either augment or oppose the external field, thus resulting in nuclear shielding or deshielding, i.e. upfield or downfield shifts, respectively. The extent of the chemical shift depends upon the degree to which a magnetic nucleus is shielded from the applied magnetic field $B_0$ by its surrounding electrons. The more dense the electron cloud the less effective is the magnetic field experienced by the nucleus, the more shielded it is, which leads to a lower chemical shift value. Any influence which reduces the electron density will reduce the shielding effect and the nucleus is said to be deshielded. Deshielding leads to a higher value of chemical shift. The resonance frequency of a nucleus is therefore characteristic of its environment.

The chemical shift, $\delta$, is defined in terms of the difference in resonance frequencies between the nucleus of interest ($v$) and a reference nucleus ($v_{\text{ref}}$), and is given as a fraction of the applied magnetic field, in parts per million, on the $\delta$ scale which is then independent of the applied field.

$$\delta = 10^6 \frac{(v - v_{\text{ref}})}{v_{\text{ref}}}$$
δ increases as the amount of shielding decreases. Conventionally the δ scale increases from right to left in an NMR spectrum. The left-hand side is known as the downfield (high frequency) end, and the right-hand side is the upfield (low frequency) end. Thus more heavily shielded nuclei (smaller ν, smaller δ) appear towards the right-hand side of the spectrum. Chemical shifts are referred to as upfield or downfield, meaning more shielded or less shielded, respectively. ¹H signals of diamagnetic compounds are usually observed between about 0 and 12 ppm.

Spin-Spin Coupling

Although each distinct nuclear environment has a distinct resonance, the magnetic interactions between different NMR active nuclei in a molecule causes NMR lines to split into a small number of components with characteristic relative intensities and spacings. This is known as spin-spin coupling, scalar coupling or J-coupling. The splitting pattern depends upon the number and nature of the interacting nuclei.

The splitting arises because the spins of neighbouring nuclei interact with one another. The nuclear magnetic moments of other NMR active nuclei surrounding the nucleus under observation produce small magnetic fields in addition to the applied magnetic field and the small induced local magnetic field which is characterised by the chemical shift. The small magnetic field of one nucleus affects the other. Thus the observed nucleus sees not one resultant field but several depending on the number and nature of the surrounding NMR active nuclei.

For the most simple case of two spin-½ nuclei interacting, two lines are observed for each nucleus. If the spin of the neighbouring nucleus is aligned with the applied magnetic field, the total effective field at the nucleus under observation will be larger than the applied field causing the resonance frequency to be higher. Conversely if the spin of the neighbouring nucleus is aligned opposed to the applied magnetic field, the effective field will be reduced and the resonance will be shifted to a lower frequency. The consequence of this coupling is that the nucleus comes into resonance at two slightly different values of applied magnetic field. Since there is an almost equal probability of the nucleus being in either spin state, this results in two lines of equal intensity in the NMR spectrum instead of a single resonance. The same principle of splitting can be applied to larger numbers of nuclei in the same
molecule resulting in multiplet patterns of resonances. When the signal from any
given nucleus is split, then the observed multiplicity depends on the number of nuclei
to which it is coupled.

The difference between resonance frequencies of the split lines is called the coupling
constant \( (J) \). It is independent of the spectrometer field used and is measured in
Hertz (Hz). Spin-spin coupling is mediated by the electrons in the chemical bonds
connecting the coupled nuclei. Therefore, the size of \( J \) diminishes as the number of
bonds between the two NMR active nuclei increases.

**Integration**

The intensity of an NMR signal is proportional to the number of nuclei resonating at
that frequency with the result that the area under the peak is proportional to the
number of protons being detected. Thus, the relative numbers of nuclei in different
chemical environments can be quantified by comparing the areas under peaks, a
process known as integration.

**Natural Abundance**

Elements can exist as more than one isotope. These isotopes may have different \( I \)
values and therefore different spin and they will not necessarily all be NMR active.
Some common nuclei, such as \(^{12}\text{C}\), have a value of \( I = 0 \), *i.e.* no angular momentum,
no magnetic moment and consequently do not give NMR spectra. Even the isotopes
which are active will have different gyromagnetic ratios and hence different
resonance frequencies. The different isotopes will therefore show different coupling
constants and different splitting patterns.

**2.2.5 Relaxation Processes**

Once a physical system is perturbed from its equilibrium condition, and then the
perturbing influence is removed, the system will return to its original equilibrium
condition in a finite time. Thus the system is said to relax. Spin-lattice relaxation
and spin-spin relaxation are the two relaxation processes that allow nuclear spins to
return to equilibrium following some disturbance, *e.g.* by a radiofrequency pulse.
In spin-lattice relaxation as the nuclei approach equilibrium, the energy released is dissipated in the surroundings, i.e. the lattice, and there is a net loss of energy from the system. The change in population is characterised by a time $T_1$, known as the spin-lattice relaxation time. A very effective pathway for spin-lattice relaxation occurs when unpaired electrons are present, and will be discussed later.

Spin-spin relaxation arises from the transfer of magnetisation between the various nuclei of a spin system. Accordingly, this process does not result in a net loss of energy from the system. The spin-spin relaxation time is termed $T_2$ ($T_2 \leq T_1$).

Since both processes lead to the shortening of the life-time of individual excited spin states, they give rise to line broadening.

### 2.2.6 2D NMR Spectroscopy

2D NMR allows for the collection and presentation of much more information than 1D spectra. A 2D spectrum is essentially a contour map, with two axes, for example, being J-resolved or shift correlated. J-Resolved spectra allow the separate presentation of chemical shift and coupling information. The chemical shift is plotted on one axis and the multiplicity on the other. A shift-correlated 2D spectrum allows you to establish which nuclei are coupled, either through bonds, space, or even by chemical exchange processes. The coupling can be either homonuclear or heteronuclear.

Application of a suitable pulse sequence and processing results in a 2D plot, with the conventional 1D spectrum along the diagonal, and crosspeaks identifying nuclei that are interacting with each other.

**Correlation Spectroscopy (COSY)**

In proton NMR spectroscopy it is often valuable to identify all the couplings and connections present in a molecule. COSY is used to identify pairs of nuclei, (e.g. protons), that have a mutual scalar coupling. Through-bond connectivities (i.e. spin-spin couplings) between protons separated by two or three bonds can be established by COSY experiments.
2.2.7 2D Exchange Spectroscopy: EXSY/NOESY

Dynamic processes such as chemical exchange, cross relaxation and NOE can be investigated by 2D EXSY/NOESY NMR spectroscopy. A NOESY spectrum identifies all pairs of nuclei with significant NOEs.

**Nuclear Overhauser Effect Spectroscopy (NOESY)**

Through-space connectivities between protons close in space can be established by NOESY experiments. NOESY yields correlation signals, which are caused by dipolar cross-relaxation between nuclei (e.g. $^1$H-1H) in a close spatial relationship. The intensities of the cross-peaks are inversely proportional to the sixth power of the distance between two nuclei, $i$ and $j$, $\propto 1/r_{ij}^6$.

**Exchange Spectroscopy (EXSY)**

EXSY is used to identify pairs of protons that are undergoing chemical exchange. Thus the cross-peaks in a 2D EXSY spectrum are due to chemical exchange between nuclei (e.g. $^1$H-1H). The corresponding exchange rates, $k_{ex}$, can be calculated from:

$$k_{ex} = \frac{1}{2T_m} \ln \frac{1 + (I_c/I_d)}{1 - (I_c/I_d)}$$

This relates the intensities of cross ($I_c$) and diagonal ($I_d$) peaks derived from EXSY spectra to the exchange rate at the temperature at which the experiment was conducted. $T_m$ is the mixing time, a crucial 2D NMR parameter, which corresponds to the time the nuclei are allowed to interact with each other.

This equation is applicable to systems which exhibit first order exchange between two sites in the same molecule.9-11

2.2.8 Paramagnetic NMR Spectroscopy

Transition metals with unpaired electrons are paramagnetic and a variety of effects is associated with their presence in a sample whose NMR spectrum is being recorded.
Peaks can experience large chemical shifts and can be highly broadened. These shifts are observed for a nucleus, generally in a ligand attached to a paramagnetic metal ion or in a stable radical such as a nitroxide.\textsuperscript{12}

### 2.2.9 Paramagnetic Shifts

Paramagnetic shifts are produced by unpaired electrons. Unpaired electrons give rise to large dipolar magnetic fields. The gyromagnetic ratio of the electron is 660 times that of a proton, which can result in substantial nuclear shielding/deshielding. Changes in nuclear shielding due to interaction with unpaired electron spin density are called paramagnetic shifts. There are two types of paramagnetic shifts: dipolar (pseudocontact) shifts, which result from an interaction between the nucleus and electron through space, and contact shifts, which are due to the delocalisation of electron spin density through bonds (i.e. direct delocalisation and spin polarization). The interaction between a magnetic nucleus and an unpaired electron is called hyperfine coupling.

#### The Contact Shift

A direct coupling between the unpaired electron on the metal ion and the nuclear spin of the proton causes changes in NMR resonance frequency of the proton. This is known as a contact shift or a Fermi contact shift. The contact shift arises from the presence of unpaired electron spin density at the resonating nucleus, and is proportional to the amount of spin density.

The spin density at the nucleus of an atom in a ligand is a small fraction of an electron, which, in the presence of an external magnetic field, gives rise to a permanent, time-averaged, additional magnetic field that adds to the applied magnetic field. Therefore, the nucleus experiences a further shift in resonance frequency, which depends on the amount of unpaired electron delocalised onto the ligand.

The contact shift ($\delta_{\text{con}}$) of a nucleus in a molecule with electron spin $S$ and an isotropic $g$ tensor are given by:\textsuperscript{13}

$$
\delta_{\text{con}} = \frac{A g_e H_y S (S+1)}{h} \frac{1}{3 \gamma \kappa T}
$$
The contact shift is proportional to the hyperfine interaction constant, $A$, which depends on the net electron spin density at the observed nucleus, $\rho$ ($A \propto \rho$). Thus a very small $A$ value can give rise to a large isotropic shift. The linewidth is also proportional to $A$, so if $A$ is very large (high electron spin density) the NMR line will be too broad to observe. The sign of the contact shift usually alternates along a chain if the spin-delocalisation is mainly \textit{via} $\pi$ orbitals; $\sigma$ delocalisation gives like signs, and sharper attenuation with distance from the spin source.\textsuperscript{14}

\textit{The Dipolar Shift}

A dipolar or pseudocontact shift arises from a through-space interaction between the unpaired electrons on the metal ion and the nucleus. It falls off inversely with $r^3$, where $r$ is the distance between the metal ion and the nucleus. Dipolar contributions are observed only in complexes which are magnetically anisotropic (having unequal magnetic susceptibility tensor components). When the magnetic susceptibility of the molecule is axially symmetric:\textsuperscript{13}

$$\delta^{pc} = \frac{\mu_0}{4\pi} \frac{\mu_B^2 S(S+1)}{9kT} \left( g_\parallel^2 - g_\perp^2 \right) \frac{1}{r^3} \left( 3\cos^2 \theta - 1 \right)$$

where $r$ is the distance between the proton and the metal centre, and $\theta$ is the angle formed by the metal-nucleus vector of distance $r$ and the applied magnetic field $B_0$, as shown in \textbf{Figure 2.1}.

\begin{figure}[h]
\centering
\includegraphics[width=0.3\textwidth]{figure2.1.png}
\caption{Definition of the angle ($\theta$) between the metal-nucleus (proton, H) vector of length ($r$) and the symmetry axis of the complex.}
\end{figure}

The above equation is valid when the nucleus interacts with the unpaired electron as a point dipole (\textit{i.e.} when delocalisation can be neglected and the nucleus is relatively
far from the metal). The significant delocalisation of unpaired electrons, at least within the coordination sphere, can prevent the quantitative use of the above equation, for donor atom nuclei or for nuclei close to the donor atoms.

2.2.10 Relaxation and Line Broadening

Most of the first-row paramagnetic transition metals cause some line broadening. The line broadening varies greatly and may be offset by the spectral expansion: for example, up to 730 ppm is reported for the range of proton shifts in vanadocene derivatives. Both contact and dipolar paramagnetic interactions result in line-broadening of the resonances. The linewidth at half the maximum height of the resonance ($\Delta\nu_{\text{1/2}}$) is given by:

$$\Delta\nu_{\text{1/2}} = \frac{1}{\pi T_2}$$

where $T_2$ is the spin-spin relaxation time.

Nuclear relaxation due to dipolar coupling with unpaired electrons is given by:

$$R_{1M} = R_{2M} = R_{1pM} = \frac{4}{3} \left( \frac{\mu_0}{4\pi} \right)^2 \frac{\gamma_1^2 \gamma_2^2 \mu_p^2 S(S+1)}{r^6} T_{1e}$$

where $R_{1M} = 1/T_1$ and $R_{2M} = 1/T_2$. $T_1$ and $T_2$ are spin-lattice and spin-spin relaxation times, respectively.

Thus the shorter the electronic relaxation time ($T_{1e}$), the smaller the paramagnetic effects on nuclear relaxation. The broadening due to dipolar interactions is inversely proportional to the sixth power of the distance ($\propto 1/r^6$).

The contribution of contact interactions to relaxivity is given by:

$$R_{1M} = R_{2M} = \frac{2}{3} S(S+1) \left( \frac{A}{\hbar} \right)^2 T_{1e}$$

For systems with an orbitally non-degenerate ground state, such as octahedral Ni(II) and tetrahedral Co(II) complexes, the application of the above equation for the contact shift is valid; otherwise the above equation represents an approximation.
Quadrupolar interactions also cause spin relaxation. Many $I > \frac{1}{2}$ nuclei in low symmetry environments have a large quadrupolar interaction and consequently very efficient spin relaxation. The most obvious manifestation of this is large linewidths (small $T_2$). The other major consequence of quadrupolar relaxation is the loss of multiplet structure for spins that are scalar-coupled to quadrupolar nuclei. Thus efficient spin-lattice relaxation results in only a single line being observed at the mean resonance frequency.

2.2.11 Temperature Dependence

Upon measuring the temperature dependence of the chemical shifts, a plot of chemical shift vs. $1/T$ should produce a straight line with a slope proportional to $A$ for systems exhibiting Curie law behaviour. Thus the paramagnetic shifts for such systems are predominantly contact in origin.

2.2.12 Experimental

NMR spectra were recorded on either a Bruker DMX 500 MHz spectrometer or a Varian Inova 600 MHz spectrometer using 5 mm tubes. NMR spectra were also recorded by the NMR service, The University of Edinburgh, Department of Chemistry, at 250 and 200 MHz. The $^1$H chemical shifts were internally referenced to either TSP (sodium 3-trimethylsilylpropionic acid, 0 ppm) or dioxane (3.767 ppm to TSP at 298 K). Water was suppressed by presaturation.

NMR signals are described as singlets (s), doublets (d), triplets (t), multiplets (m) and broad (br).

2.3 Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy is a technique used for the analysis of paramagnetic molecules. A brief description of the theory and EPR relative to the metal ions studied in this thesis is given below. A detailed discussion of the theory and practical aspects of EPR can be found in other texts.  

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Electron paramagnetic resonance, also known as electron spin resonance (ESR), is the name given to the process of resonant absorption of microwave radiation by paramagnetic ions or molecules, with at least one unpaired electron spin, and in the presence of a static magnetic field. EPR is used to investigate the electronic structure of transition metal complexes.

### 2.3.1 EPR Theory

An electron has both spin and charge and therefore possesses a magnetic moment. EPR spectroscopy involves the interaction of the magnetic moment of an electron with an external magnetic field. Because of the quantization of spin only a discrete number of energy levels exist. An electron can be characterised by the spin quantum number $S = \frac{1}{2}$. When placed in a magnetic field $B$ there are two different energy states, characterised by the magnetic quantum numbers $m_s = +\frac{1}{2}$ and $-\frac{1}{2}$, for the electron (Figure 2.2), the separation ($\Delta E$) between them being $g_e \beta_e B$, where $g_e = 2.0023$ (the Landé splitting factor) and $\beta_e$ is the Bohr magneton. In the lower energy state, which corresponds to $m_s = -\frac{1}{2}$, the magnetic moment of the electron is aligned along the magnetic field. The higher energy state in which the magnetic moment of the electron is aligned against the magnetic field, corresponds to $m_s = +\frac{1}{2}$.

![Energy level diagram for a free (isolated) electron in a magnetic field](image)

**Figure 2.2** Energy level diagram for a free (isolated) electron in a magnetic field. There are two different energy states for the electron, $m_s = +\frac{1}{2}$ and $m_s = -\frac{1}{2}$. The lower energy state, $m_s = -\frac{1}{2}$, corresponds to the magnetic moment of the electron being aligned with the magnetic field and the higher energy state, $m_s = +\frac{1}{2}$, corresponds to the magnetic moment of the electron being opposed to the magnetic field. The energies of the two spin states diverge linearly as the magnetic field increases. No EPR transition occurs until the separation between the energy levels equals the microwave energy.
In the EPR spectrum with the magnetic component of the microwave field perpendicular to the main magnetic field, B, there will be absorption of microwave energy when the selection rule $\Delta m_s = \pm 1$ is obeyed and when the microwave energy is equal to the energy separation between the two states, $\Delta E = h \nu = g_e \beta_e B$, where $h$ is Planck's constant and $\nu$ is the frequency of radiation. Thus, the energy difference between the two spin states depends upon the strength of the magnetic field. In EPR spectroscopy the electromagnetic radiation frequency is kept constant and the magnetic field is scanned, so that the energy difference between the two spin states matches the energy of radiation. Thus, electromagnetic radiation will only be absorbed by the electron at distinct values of magnetic field. The magnetic field is swept until it reaches a value at which the sample absorbs some of the microwave energy. The absorption of energy causes a transition from the lower energy to the higher energy state. At this point a change via the intensity of the reflected microwave energy is detected. For instrumental reasons a first derivative of the absorption is usually recorded.

In real chemical systems the unpaired electron will be associated with at least one atom. One consequence of this is that $g$ may no longer be equal to 2.0023 (the $g$ value of a free electron) and it may vary with the angle which the system presents to the applied magnetic field, i.e. the system may be anisotropic. The $g$ value is a proportionality constant approximately equal to 2 for most samples but varies depending on the electronic configuration of the ion. For $g > 2.0023$ the resonance position will move to lower magnetic field, whilst for $g < 2.0023$ it moves to higher field. For d-transition metal systems deviations of $g$ values from 2.0023 may be considerable and they may also be very anisotropic. The anisotropy in $g$ may be classified into one of three types: isotropic, axial or rhombic.

**Isotropic g values**

A paramagnet has isotropic $g$ values when $g_x = g_y = g_z$. For d-transition metal compounds, this would only occur for perfectly cubic, octahedral, or tetrahedral geometries, a very rare occurrence. An isotropic spectrum could also arise from a paramagnet with lower symmetry in solution, provided it is tumbling much more rapidly than the time scale of the EPR experiment. Any anisotropy is averaged, and $g_{iso} = g_{av} = \frac{1}{3}(g_x + g_y + g_z)$. 


Axial and Rhombic g values

Jahn-Teller distorted molecules are most likely to have axial g values, as the four bonds in the equatorial plane are similar and short whereas the two axial bonds are elongated giving rise to an additional g value. A molecule is said to have axial g values when two of the principal g values are equal. By convention the unique value is usually designated as $g_\parallel$, "g parallel", ($= g_z$ say), whilst the other value is $g_\perp$, "g perpendicular", ($= g_x = g_y$). Since $g_{av} = \frac{1}{3}(g_x + g_y + g_z)$, therefore $g_{av} = \frac{1}{3}(2g_\perp + g_\parallel)$. For a single isolated paramagnet the resonance magnetic field now depends upon the angle between the applied magnetic field, B, and the principal axes of the g tensor for axial symmetry, $g_\parallel$.

Paramagnets are said to have rhombic g values when $g_x \neq g_y \neq g_z$. The resonance fields for a single isolated paramagnet depend upon the orientation of the applied magnetic field with respect to all three axes of the g tensor.

2.3.2 Electron Spin Relaxation Times

Table 2.4 shows that transition metal ions with long electron spin relaxation times give rise to EPR spectra at room temperature but lead to broadening of NMR resonances, whereas metal ions with short electron spin relaxation times give EPR spectra only at low temperature and cause less broadening of NMR peaks.

Table 2.4 Electron spin relaxation times ($\tau_S$) of some common paramagnetic metal ions and relative line broadening effects.¹⁸

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>$d^a$</th>
<th>$S$</th>
<th>$\tau_S$ (s)</th>
<th>Line Broadening¹ (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti(III)</td>
<td>$d^1$</td>
<td>$\frac{1}{2}$</td>
<td>$10^{10}$-$10^{11}$</td>
<td>20-200</td>
</tr>
<tr>
<td>Mn(III)</td>
<td>$d^4$</td>
<td>2</td>
<td>$10^{10}$-$10^{11}$</td>
<td>150-1500</td>
</tr>
<tr>
<td>Mn(II)</td>
<td>$d^5$</td>
<td>$\frac{3}{2}$</td>
<td>$\sim10^{-8}$</td>
<td>100000</td>
</tr>
<tr>
<td>Fe(III), H.S.</td>
<td>$d^5$</td>
<td>$\frac{3}{2}$</td>
<td>$10^{9}$-$10^{11}$</td>
<td>200-12000</td>
</tr>
<tr>
<td>Fe(III), L.S.</td>
<td>$d^5$</td>
<td>$\frac{1}{2}$</td>
<td>$10^{-11}$-$10^{-13}$</td>
<td>0.5-20</td>
</tr>
<tr>
<td>Fe(II), H.S.</td>
<td>$d^6$</td>
<td>2</td>
<td>$10^{-12}$-$10^{-13}$</td>
<td>5-20</td>
</tr>
</tbody>
</table>

¹ For a proton at 5 Å from the metal; 500 MHz $^1$H resonance frequency.
2.3.3 Effect of Nuclear Coupling on EPR Spectra

The nuclei of the atoms in a molecule or complex often have a magnetic moment, which produces a local magnetic field at the electron. The interaction between an unpaired electron and a nucleus with a non-zero nuclear spin can give rise to certain additional features in an EPR spectrum. The interaction between the unpaired electron and the metal nuclear spin is termed the metal hyperfine interaction (A).

The major effect in the EPR spectrum of electron spin-nuclear spin interaction can be illustrated by considering an isolated atom with one unpaired electron \( S = \frac{1}{2} \) and a nucleus with a nuclear spin \( I = \frac{1}{2} \). Each energy state of the electron in the magnetic field (dashed lines) is split symmetrically into two components (solid lines), see Figure 2.3. The EPR spectrum is controlled by the selection rules \( \Delta m_S = \pm 1, \Delta m_I = 0 \), which results in two resonances of equal intensity (see Figure 2.3). These resonances are displaced symmetrically about the original g value. Their separation is equal to the metal hyperfine coupling constant A.

![Figure 2.3 Energy level diagram for a paramagnet with \( S = \frac{1}{2} \) and \( I = \frac{1}{2} \). The electron interacts with the nuclear spin of the metal ion, which causes splitting of the energy levels.](image-url)

The above considerations can be generalised for any metal. For any metal nucleus with nuclear spin \( I \), each electronic spin level, \( m_S \), will be split into \( 2I+1 \) new energy levels. The result is that the EPR spectrum consists also of \( 2I+1 \) resonances of equal...
relative intensity. Although all the lines in an EPR spectrum have equal relative intensity, for a d-transition metal compound in solution the lines in the spectrum may have unequal relative amplitudes which are due to changes in peak-peak linewidths. Such changes in linewidths are fairly common in solution spectra of d-transition metal compounds.

2.3.4 Paramagnets with $S > \frac{1}{2}$

Multiple unpaired electron systems can arise for transition metal compounds where the metal ion is in a particular oxidation state and ligand field, e.g. octahedral Mn(II) has a $S = \frac{3}{2}$ ground state with five unpaired electrons. The EPR spectra arising from a spin triplet ($S = 1$) and a spin sextet ($S = \frac{5}{2}$), examples of integral and non-integral electron spin systems, is discussed below.

When placed in a magnetic field a paramagnet with spin $S$ has $2S+1$ different energy states. Thus a spin triplet paramagnet ($S = 1$) has three different energy states, $M_S = 1, 0, -1$. There are two allowed ($\Delta M_S = \pm 1$) EPR transitions which are coincident because the $M_S = 1$ and $M_S = -1$ levels diverge with equal magnitude from the $M_S = 0$ level, the energy of which is unaffected by the magnetic field. A spin sextet ($S = \frac{5}{2}$) has six different energy states, $M_S = \frac{5}{2}, \frac{3}{2}, \frac{1}{2}, -\frac{1}{2}, -\frac{3}{2}, -\frac{5}{2}$. There are now five allowed ($\Delta M_S = \pm 1$) EPR transitions which are coincident. All six energy levels vary with the magnetic field. Thus the EPR spectrum of each of these cases would consist of a single resonance at a magnetic field given by $B = \hbar v / g \beta_e$.

**Zero-Field Splitting**

In centres with $S > \frac{1}{2}$ the combined action of low symmetry ligand field and spin-orbit coupling usually leads to zero-field splittings. Zero-field splitting consists of removing some of the degeneracy of the $M_S$ components in the absence of an applied magnetic field. The larger the zero-field splitting the greater the admixture of excited states via spin-orbit coupling and hence a shortening of relaxation times. Short relaxation times cause the resonances to broaden with the consequence that EPR spectra may be difficult to observe at ambient temperatures. For this reason it is often necessary to cool complexes to liquid helium temperatures in order to observe an EPR spectrum.
EPR spectra of transition metal ions with an even number of unpaired electrons are very difficult to obtain, because of the phenomenon of zero-field splitting. The ground state spin multiplet is split in a zero magnetic field by the low symmetry components of the crystal field. The small number of spectra reported for systems with integer-spin ground states are confined to a handful of metal ions.

The observation of a spectrum may critically depend upon the microwave frequency and magnetic fields which are available. If the separation between the energy levels due to zero-field splitting effects is larger than the microwave quantum used in the spectrometer then no EPR signals are observed. This is a particularly severe problem when $S$ is an integer. Thus despite the fact that the systems contains unpaired electrons, we will be unable to detect an EPR signal. Such systems are EPR silent because there is either not enough energy to achieve resonance or the magnetic field required to reach the resonance condition exceeds that available at conventional fields and frequencies. For such systems, high-frequency and high-field EPR spectroscopy (HF-EPR, $\nu > 90$ GHz) can be employed to obtain EPR spectra. HF-EPR employs microwave frequencies up to $\sim 550$ GHz and magnetic fields up to $\sim 15$ T which allows the detection of spectra of integer spin multiplets with very large zero-field splittings. HF-EPR spectroscopy has been very successfully applied to the investigation of a variety of mononuclear transition metal ions with integer-spin states such as Fe(II),$^{19}$ Cr(II),$^{20}$ Mn(III),$^{21-26}$ and Ni(II).$^{27-29}$

### 2.3.5 EPR Spectra of Mn(II) and Mn(III)

High-spin Mn(III) has a $d^4$ electron configuration with four unpaired electrons resulting in a $S = 2$ spin ground state. An $S = 2$ spin system has five degenerate energy levels ($M_S = \pm 2, \pm 1, 0$) in the absence of zero-field splitting effects. However, large zero-field splittings are expected for Mn(III) complexes as a result of the low symmetry due to Jahn-Teller distortions from octahedral geometry. A combination of large zero field splitting and unfavourable (fast) spin relaxation effects makes the observation of EPR spectra at conventional microwave frequencies (X-band: $\sim 9$ GHz; Q-band: $\sim 35$ GHz) of $S = 2$ systems relatively rare. The adverse relaxation effects usually mean that temperatures near that of liquid helium are required in order to observe spectra. The observation of EPR signals from Mn(III) is extremely rare and to date only a few EPR spectra of Mn(III) have been reported with most of them referring to forbidden transitions.$^{30-33}$
Materials and Methods

2.3.6 Experimental

An X-band (ca. 9.25 GHz) Bruker ER200D-SRC electron spin resonance spectrometer was used. Flat cells made of high purity fused quartz were used for aqueous solutions. This minimised the absorption of microwave radiation by aqueous solutions.

2.4 Ultraviolet and Visible Electronic Absorption Spectroscopy

Optical spectroscopy is based on the Bohr-Einstein frequency relationship, which is given by:

\[ \Delta E = E_2 - E_1 = h\nu \]

This relationship connects the discrete atomic or molecular energy states \( E_n \) with the frequency \( \nu \) of the electromagnetic radiation. The proportionality constant \( h \) is Planck's constant which has a value of \( 6.626 \times 10^{-34} \text{ Js} \).

Absorbed or emitted radiation of frequency \( \nu \) can therefore be assigned to energy differences \( (\Delta E) \) between two specific energy levels in an atom or molecule. Absorption spectroscopy involves transitions from the lower energy ground state to the higher energy excited states. For absorption spectroscopy in the ultraviolet (UV) and visible (Vis) region, the wavelength range is between 200 and 800 nm. Above 800 nm lies the near IR region and below 200 nm the N2-UV and vacuum UV regions.

Absorption of UV-Vis radiation in organic molecules is restricted to certain functional groups (chromophores) that contain valence electrons of low excitation...
Electronic transitions are normally between a bonding or lone pair orbital and an unfilled non-bonding or anti-bonding orbital. Transitions for metal complexes are discussed in sections 2.4.2 and 2.4.3.

Each electronic state has associated with it a number of vibrational and rotational levels of also discrete energy. The molecule can undergo a transition from and to any number of sub-vibrational levels and as a result broad absorption bands are observed, which are made up of numerous closely packed but discrete lines.

### 2.4.1 The Beer-Lambert Law

If a beam of monochromatic light of intensity $I_0$ passes through a solution of path length $l$ (cm) containing solute of molar concentration $c$, then the transmitted light of intensity $I$, is related to the incident light intensity, $I_0$, by:

$$T = \frac{I}{I_0} = 10^{-εcl}$$

or

$$A = \log_{10}(I_0/I) = \varepsilon cl$$

where $ε$ is the molar extinction coefficient (in units of M$^{-1}$ cm$^{-1}$), $A$ is the absorbance and $T$ is the transmittance (both dimensionless). The above equation is known as the Beer-Lambert law which states that the absorbance ($A$) at a particular wavelength is related to the concentration of the sample ($c$) and the path length of the light beam ($l$) through the sample cell by the molar extinction coefficient, $ε$, at that wavelength. The molar extinction coefficient, $ε_{λ}$, at a particular wavelength $λ$ (nm) is a quantity characteristic of the substance. The concentration, $c$, can be determined if the extinction coefficient, $ε_{λ}$, of the substance to be determined is known.

The Beer-Lambert law applies to dilute solutions, *i.e.* the extinction coefficient $ε$ is independent of the concentration of a substance at the given wavelength $λ$ only for dilute solutions. The majority of systems obey the Beer-Lambert law.

### 2.4.2 Transition Metal Compounds

Many metal ions and their complexes, in particular first and second row transition metal ions, absorb radiation in the visible region. The absorption bands can, in
principle, arise from three different types of transitions: d→d, charge-transfer and intraligand. Charge-transfer transitions can be further sub-divided into two categories; ligand-to-metal and metal-to-ligand, each of which will be discussed briefly. Detailed descriptions can be found in other texts.\textsuperscript{35,36}

\textit{d→d transitions}

According to crystal field theory, in an octahedral field, the d-orbitals of a metal split into two sets of energetically different orbitals; the d\textsubscript{xy}, d\textsubscript{xz}, and d\textsubscript{yz} orbitals (the t\textsubscript{2g} set) decrease in energy and the energy of the d\textsubscript{x\textsuperscript{2}-y\textsuperscript{2}} and d\textsubscript{z\textsuperscript{2}} orbitals (the e\textsubscript{g} set) increases. The energy differences between the t\textsubscript{2g} and the e\textsubscript{g} orbitals are often such that transitions between them result in absorption bands in the visible region. d→d transitions occur due to the presence of partially-filled d-orbitals.

According to the selection rules, electronic transitions involving a change in spin are forbidden, \textit{i.e.} $\Delta S = 0$ for spin-allowed transitions. However, whether spin-allowed or spin-forbidden, d-d transitions are generally very weak and usually give rise to relatively weak bands in the UV-Vis spectrum. This is due to the Laporte Rule which states that electronic transitions between states of equal parity are forbidden.

For octahedral complexes all d-d transitions are Laporte forbidden. Nevertheless transition metal complexes can be coloured. The parity selection rule forbidding d-d transitions is relaxed under certain circumstances, including the situation where the molecules do not possess a centre of symmetry. The observed intensities of the d-d transitions in octahedral coordination result from distortions which remove the centre of symmetry. There are two common mechanisms by which the parity selection rule is relaxed, namely static or dynamic removal of the centre of symmetry. If any distortion occurs which permanently or temporarily removes the centre of symmetry, then the effective group to which the molecule belongs is no longer O\textsubscript{h}. In general such effects do not relax the selection rules greatly, but they do provide a pathway for some absorption intensity.

\textbf{2.4.3 Charge-Transfer Bands}

Many complexes exhibit charge-transfer absorption bands. These complexes consist of an electron-donor group bonded to an electron acceptor group. When such a
complex absorbs radiation, an electron from the donor is transferred to an orbital that is largely associated with the acceptor. In metal complexes either the ligand or the metal ion can act as the electron donor.

If an electron is transferred from orbitals that are predominantly ligand in character to orbitals that are predominantly metal in character, such charge-transfer processes are termed ligand-to-metal charge-transfer (LMCT) transitions. Conversely, an electron transition from orbitals primarily associated with the metal to orbitals primarily associated with the ligand is known as a metal-to-ligand charge-transfer (MLCT) transition.

LMCT transitions occur when the metal has empty valence shell orbitals and the ligand has electrons to donate. Most charge-transfer transitions are of this type. Examples include metal complexes with phenolate ligands and high oxidation state metal ions, e.g. Fe(III).

MLCT transitions are expected when the ligand has low-lying empty orbitals; unsaturated ligands with empty $\pi$ anti-bonding orbitals (e.g. 2,2'-bipyridine) are prime candidates for MLCT transitions. Complexes of such ligands with the more readily oxidisable transition metal ions (e.g. Ti(III), Fe(II)) frequently generate fairly low energy charge-transfer absorption of the MLCT type.

### 2.4.4 Extinction Co-efficients

Fully allowed electronic transitions have high molar extinction coefficients of the order of $10^4 - 10^6 \text{ M}^{-1} \text{ cm}^{-1}$. At the other extreme, Laporte and spin forbidden d-d transitions in centrosymmetric molecules, such as the crystal field spectra of octahedral Mn(II) species (very pale pink or yellow in colour) may have extinction coefficients of the order of $10^{-2} - 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$. Charge-transfer bands often have extinction coefficients between 500 and 20,000 $\text{ M}^{-1} \text{ cm}^{-1}$. The intensities of the different types of electronic transitions of 3d transition metal complexes is summarised in Table 2.5.
Table 2.5 Intensities of spectroscopic bands in 3d transition metal complexes.\textsuperscript{36}

<table>
<thead>
<tr>
<th>Band Type</th>
<th>$E_{\text{max}} / (\text{M}^{-1} \text{cm}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spin-forbidden</td>
<td>$&lt; 1$</td>
</tr>
<tr>
<td>Laporte-forbidden d-d</td>
<td>20-100</td>
</tr>
<tr>
<td>Laporte-allowed d-d</td>
<td>ca. 500</td>
</tr>
<tr>
<td>Symmetry-allowed (e.g. charge-transfer)</td>
<td>1000-50000</td>
</tr>
</tbody>
</table>

Charge-transfer processes frequently lie at the blue end of the visible region, or in the ultraviolet region. However if the metal is easily oxidisable, and the ligand reducible, or vice versa, then charge-transfer transitions may occur at quite low energies, even in the near infrared. Charge-transfer bands are often broad and intense and may obscure weaker crystal field transitions in the same region.

2.4.5 Absorption Characteristics of Proteins

The near-UV (250-350 nm) absorbance spectrum of a protein is almost entirely generated from its aromatic amino acids (tyrosine, tryptophan and phenylalanine) with a small contribution from disulfide bonds. Peptide bonds absorb strongly below 230 nm. Because each protein has a unique amino acid sequence, the particular aromatic amino acid content of each protein results in a unique spectrum in the near-UV region. Protein concentrations can be calculated by applying the Beer-Lambert law to the spectral intensity at 280 nm: $c = \frac{A_{280}}{l \varepsilon}$, where $c$ is the protein concentration (M), $\varepsilon$ is the molar extinction coefficient (M$^{-1}$ cm$^{-1}$) at 280 nm, $l$ is the path length (cm).

In addition to absorption in the UV region, many metalloproteins exhibit d-d transitions and charge-transfer bands in the visible region due to the presence of the metal centre(s). UV-Vis spectroscopy provides a method for analysing metal sites if the metal ion is coloured and attempts have been made to deduce environments from d-d and charge-transfer spectra. Most of the charge-transfer transitions involve a metal ion which serves as an electron acceptor and side-chains from proteins which serve as electron donors. An example is Fe$_2$-transferrin which has a LMCT band at ca. 465 nm, arising from charge-transfer from Tyr to Fe(III).\textsuperscript{37} This provides a
convenient way to detect specific metal-protein binding and release by UV-Vis spectroscopy.

2.4.6 Experimental

UV-Vis spectra were recorded on a Shimadzu UV-2501PC UV/Vis spectrophotometer using 1 cm path length quartz cuvettes. The measurements were made at ambient temperature unless otherwise stated, in which case the temperature was controlled by a Linkam Peltier stage controller PE60.

Spectra were normally referenced to solvent alone and were processed with UVPC software.

2.5 Inductively Coupled Plasma Atomic Emission Spectroscopy

Inductively coupled plasma atomic emission spectroscopy (ICP-AES) is a spectroscopic method for elemental analysis. It can be used for both qualitative and quantitative determinations. A number of textbooks have been published describing ICP-AES and can be referred to for a more detailed description. 38-42

ICP-AES is a multi-element analytical technique which can be used for the simultaneous determination of major, minor and trace constituents.

2.5.1 Theory of Atomic Emission Spectroscopy

Atomic emission spectroscopy is based on the spontaneous emission of radiation by an atomic species (free atoms or ions), when the excitation is performed by thermal or electrical energy.

Each atom has a number of possible energy states. The emission (or absorption) of radiation originates from the transition of electrons between particular pairs of these energy levels. When an atom or ion in the ground state absorbs energy the electrons are excited from the lower energy level to a higher energy state. The lifetime of an excited atom or ion is brief, and its return to the lower energy ground state is
accompanied by the spontaneous emission of a photon of radiation. The emitted radiation has a frequency \( v \), which is characteristic of that particular transition. The emission of radiation occurs in near-UV (180–300 nm) and visible (300–770 nm) regions of the electromagnetic spectrum. In ICP-AES the energy source used to excite the atom or ion is a plasma.

Atomic emission spectra are much simpler than the corresponding molecular spectra because there are no vibrational and rotational states and the transition occurs between distinct atomic energy levels. Atomic emission spectra are therefore made up of a limited number of narrow peaks or emission lines at distinct wavelengths. Since every element has a characteristic emission spectrum, the wavelength of the emission lines are characteristic of the elements present in the plasma source and hence in the sample. The detection of radiation at particular wavelengths can be applied to the qualitative analysis of the sample and the intensities measured at these wavelengths to the quantitative analysis of the analyte elements.

**Atomization**

When the sample is introduced into a plasma source, it is first evaporated and dissociated into free atoms and ions, and then further energy is supplied to excite the free atoms and ions to higher energy states. In ICP-AES, a high energy argon plasma is used to atomise the sample under analysis. Atomization is a process in which the sample is converted into gaseous atoms or elementary ions, i.e. the sample is volatilised and decomposed to produce atomic gas. The atomised particles collide with the various constituents of the plasma which causes ionisation and excitation.

**Plasma**

A plasma is an electrically neutral, partially ionised gas composed of ions, electrons and neutral species (atoms and molecules) of an inert gas which is maintained by an external field, and has sufficiently high temperature to atomise, ionise and excite most elements. A variety of inert gases such as argon, helium and nitrogen can be used to form analytically useful plasmas. Plasmas have significantly higher gas temperatures and less reactive chemical environments than ordinary combustion flames. Typically quoted plasma temperatures range from 7000 to 10000 K. A
typical plasma has the overall appearance of a very intense, brilliant white, non-transparent core, topped by a flame-like tail.

The high temperature of the plasma and the dissociation of the sample into atoms and ions and their excitation are produced by collisions with other particles, mainly with free electrons. The argon ions, once formed in the plasma, are capable of absorbing sufficient power from an external source to maintain the temperature at a level at which further ionisation sustains the plasma indefinitely. In ICP-AES, the plasma is sustained by high frequency electromagnetic fields.

2.5.2 ICP Torch

A schematic of an ICP torch is shown in Figure 2.4. It consists of three concentric glass tubes (mostly made of quartz for greater thermal resistance) through which an inert gas, usually argon, flows at a total rate of ~30 l min⁻¹. The tubes are designated 'outer or coolant tube', 'intermediate tube', and 'inner or carrier gas tube'. The diameter of the largest tube is about 2.5 cm.

Each tube has an entry point, with those of the intermediate and outer tubes being arranged tangentially to that of the inner tube. The inner tube is typically 1 mm in diameter and is the tube through which the sample is introduced. The intermediate tube carries the main argon supply for the plasma and the outer tube carries the third flow of gas which acts as a thermal shield. This flow of argon thermally isolates the plasma from the quartz tube and protects the quartz from extreme heat. Argon flows in a tangential manner through the outer and intermediate tubes at a relatively high flow rate of ~10 l min⁻¹.

Located around the top of the outer glass tube is a two or three turn water-cooled copper induction coil. The induction coil which is connected to a radiofrequency generator, delivers the power to the plasma, typically in the range 0.5–1.5 kW, at a frequency of 27–40 MHz. The radiofrequency generator produces a high-frequency oscillating current in the induction coil, which induces an intense oscillating magnetic field around the coil in which the lines of force are axially orientated inside the plasma torch and follow elliptical closed paths outside the induction coil (Figure 2.4). The plasma is generated on top of the torch.
Figure 2.4 Schematic diagram of an inductively coupled plasma torch.

The plasma is initiated by a spark from a Tesla coil. The spark is a source of 'seed' electrons. The seed electrons start oscillating within the magnetic field and accelerate and as a result reach energies sufficient to ionise gaseous atoms in the field. The resulting ions and their associated electrons, then interact with the oscillating magnetic field produced by the induction coil. The magnetic field causes the ions and electrons (charged particles within the coil) to flow in the horizontal plane of the coil in the closed annular (circular) paths depicted in Figure 2.4. The electrons and ions meet resistance to their flow, Joule or Ohmic heating is the consequence of their resistance to this movement. These ions and electrons transfer energy to other atoms in the plasma gas by collisions to create a very high temperature plasma. This occurs almost instantaneously. Subsequent collisions with other gaseous atoms causes further ionisation and so on, so the plasma becomes self-sustaining.

Sample Introduction

Because the outer and intermediate gases flow tangentially the plasma continually revolves and has a weak spot at the centre of its base, through which the inner gas flow containing the sample may be introduced.
The sample aerosol is carried into the hot plasma by a flow of inert gas, usually argon flowing at about 1 l/min through the central quartz tube. The gas flow exiting the central quartz tube has sufficient velocity to punch a hole through the centre of the plasma, thereby forming the characteristic toroidal, annular or doughnut-shaped plasma. Sample atoms in solution are aspirated into the plasma where the aerosol is successively desolvated, vaporised, decomposed and atomised. The atoms formed are subsequently ionised and excited in the plasma which consequently causes it to emit characteristic radiation. The atoms in the plasma are promoted to excited electronic states by collisions with other atoms, and these excited atoms emit characteristic radiation as they return to the ground state.

**Nebulization**

The sample solution is introduced into the plasma in the form of a fine argon aerosol using a pneumatic nebulizer. The basic function of a nebulizer is to convert a solution of the sample into an aerosol by the action of a carrier gas. The sample is pumped into a stream of argon carrier gas to produce an aerosol. A spray chamber further reduces the aerosol particle size towards the ideal size for desolvation and ionisation/excitation in the plasma source of ~10 μm.

**2.5.3 Emission Properties**

The elements studied in this thesis and their emission properties are given in Table 2.5.

The difference in energy between two specific energy levels corresponds to a discrete frequency of radiation, v. The electronic transitions from the excited state to the ground state occur at specific wavelengths. For each element there are a number of wavelengths at which the emission can be detected, due to the excitation of electrons to more than one excited state. The frequency at which the emission occurs corresponds to the energy differences of the two states.
Table 2.5 Emission properties of manganese and iron.

<table>
<thead>
<tr>
<th>Element</th>
<th>Sensitivity (ppb)</th>
<th>Emission Wavelengths/λ (nm)</th>
<th>Relative Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manganese</td>
<td>0.4</td>
<td>257.610*</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>260.569</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td></td>
<td>293.306</td>
<td>89</td>
</tr>
<tr>
<td>Iron</td>
<td>3</td>
<td>238.204*</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>239.562</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td></td>
<td>259.940</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>271.441</td>
<td>95</td>
</tr>
</tbody>
</table>

* Emission wavelengths used.

Emission wavelengths that are very close in energy may cause line overlap which may lead to spectral interferences. The emission intensity at a characteristic wavelength of an element is proportional to the concentration of the element in the sample.

2.5.4 Experimental

ICP-AES measurements were performed on aqueous samples using a Thermo Jarrell Ash IRIS inductively coupled plasma atomic emission spectrometer equipped with a concentric Meinhard nebulizer with a cyclonic spray chamber and a CID camera detector. In order to ensure steady sample uptake, a peristaltic pump was used to transport the aqueous sample solution to the nebulizer.

The ICP-AES spectrophotometer was calibrated using freshly-prepared dilute aqueous solutions of a 1000 ppm manganese and iron ICP, plasma emission standards (ARISTAR®), purchased from BDH Laboratory Supplies.

Elemental analyses were carried out without digestion of the sample and ultrapure water was used for the dilution of samples and standards. All data were interpreted
using thermospec/CID IRIS software. Wavelengths used to calculate concentrations were free of spectral interferences (Table 2.5).

2.6 X-ray Absorption Spectroscopy

X-ray absorption spectroscopy (XAS) is a well established technique for examining the immediate environment of any specific type of atom in physical, chemical and biological systems. It can be used to determine the local structure around an atom in amorphous solids, liquids, complex crystals and biological molecules, such as metalloproteins. The atom of interest is selectively excited by an appropriate X-ray wavelength. The exact energy of the X-ray is dependent on the particular atom being studied. A detailed discussion of the theory and applications of XAS can be found in various texts.43-46

![Schematic X-ray absorption spectrum showing the different regions.](image)

**Figure 2.5** Schematic X-ray absorption spectrum showing the different regions.

In principle, the profile of a particular X-ray absorption edge can provide useful information, concerning the electronic structure and the immediate environment of a specific atom. The X-ray absorption spectrum at an absorption edge (K, L, M, etc.) is divided into two parts, the X-ray Absorption Near Edge Structure (XANES) and the Extended X-ray Absorption Fine Structure (EXAFS) (Figure 2.5). The XANES referring to the structure in the X-ray absorption spectrum near an absorption edge while the EXAFS refers to the oscillatory structure that can extend for many hundreds of volts above the absorption edge.47-49
The finite electron mean free path limits the probing sphere to less than 10 Å. The local nature of the process gives the technique its major strength, in that the structure around a specific atom can be determined very accurately. The XANES region contains information regarding the oxidation state of the element whose absorption edge is studied. The EXAFS region yields accurate bond distances (± 0.01 Å), element types (Z ± 2) and coordination numbers (20%) of the nearest neighbours up to 6 Å from the excited atom.43

2.6.1 Basic Principles of XAS

The theory of photoelectron scattering provides an essentially complete description of the processes leading to EXAFS and XANES.

When the energy of the incident X-rays is equal to the threshold energy necessary to eject a core electron from the absorbing atom (Figure 2.6) then a large increase in absorption occurs known as the absorption edge (Figure 2.5).

![Energy-level diagram for XAS.](image)

**Figure 2.6 Energy-level diagram for XAS.** An X-ray photon of sufficient energy can remove a strongly bound inner electron of an atom corresponding to a particular absorption edge (K, L, M, etc.).

The energy of the X-rays is gradually increased such that it traverses an absorption edge (K, L, M, etc.) of the element of interest. At certain values of energy, specific to each element, a sudden increase in the amount of energy absorbed is observed.
The energy of the absorption edge corresponds to the energy required to eject an electron from an inner shell of the absorbing atom.

The energy of the X-ray photon \((hv)\) is totally transferred to the electron. After overcoming the electron binding energy \((E_b)\), the ejected photoelectron carries the excess energy in the form of kinetic energy which is given by \(E_e = (hv - E_b)\). This ejected photoelectron will interact with the surrounding atoms.

The ejected electron can be considered as a wave, travelling outwards from the central absorbing atom, *i.e.* the electron propagates between atoms as a free spherical wave.\(^5\) For an isolated atom, the photoelectron wave propagates away indefinitely without further interaction with the excited atom, the tail-off is smooth and there is a gradual monotonic decrease in the absorption coefficient with increasing energy away from the absorption edge. In a real system, there are many scattering atoms. The propagating spherical wave is scattered back when it encounters neighbouring atoms. This is illustrated, in **Figure 2.7**, using a simplified 1D plane wave model.

![Figure 2.7](image)

**Figure 2.7** A schematic diagram of the outgoing photoelectron wave (solid lines) being backscattered by the neighbouring atoms (backscattered waves—dashed lines).

This results in constructive and destructive interference of the photoelectron waves between the outgoing waves from the excited central atom and the back-scattered waves from neighbouring atoms. The interference depends on the interatomic distance between the atom that ejected the photoelectron and the nearest neighbours.
This gives rise to a wave modulation superimposed on top of the smooth X-ray absorption spectrum of the isolated counterpart. These oscillatory features are observed in the EXAFS region of the X-ray absorption spectrum. Thus the structure of the post-edge region of the X-ray absorption spectrum is related to the radial distribution of atoms in the sample. Since backscattering amplitude and phase are dependent on the type of atom doing the backscattering and the distance it is from the central atom, by analysing the EXAFS, *i.e.* the frequency and amplitude of the oscillations, information regarding the coordination environment of the absorbing atom can be obtained.

Although EXAFS is recorded as a function of energy it is conventional to plot the data as a function of $k$, the photoelectron wave vector. In terms of its wave property, the photoelectron travels outwards, away from the atom with wave vector $k$ given by the de Broglie momentum relation:

$$k = \frac{2\pi}{h} \sqrt{2m_e E_e}$$

where $h = $ Planck’s constant ($6.6 \times 10^{-34}$ Js)

$m_e = $ Rest mass of the electron

$E_e = $ Kinetic energy of the ejected electron

The result of a Fourier transform is a series of peaks, one corresponding to each shell of atoms contributing to the EXAFS.

### 2.6.2 EXAFS Theory

XANES is dominated by multiple scattering events, whilst EXAFS is primarily due to single scattering events. The interpretation of the EXAFS in terms of the local chemistry of a compound was shown to be possible by Sayers *et al.* They formulated a semi-empirical single scattering short-range order theory for the X-ray absorption fine structure which could adequately account for the observed oscillatory structure in the X-ray absorption coefficient beyond 50 eV from the absorption edge and described the potential of EXAFS in the determination of local atomic geometries. They showed that the frequency and the amplitude of the EXAFS could be related to the interatomic distances and the coordination number around the photoexcited atom.
They also pointed out that the experimental EXAFS data can be converted into a radial structure function containing inter-atomic distances, number of atoms, and widths of coordination shell around a specific atom in the absorbing material. Thus the local atomic environment of the excited atom could be determined by analysing the measured oscillatory structure.

Ashley and Doniach\textsuperscript{52} and Lee and Pendry\textsuperscript{50} independently formulated the modern EXAFS theory, known as the spherical wave method, and can be referred to for a full derivation. The spherical curved wave description of the EXAFS phenomenon includes analysis of low energy parts of the EXAFS spectrum and takes into account the curvature of the electron wave. They showed that a complete quantitative description of the EXAFS process was possible and that, except for the energies very close to the absorption edge, the single scattering formalism is usually sufficient to describe the observed data. They showed that when the energy of the photoelectron is sufficiently high, the curvature of the electron wave can be neglected and therefore the theory can be greatly simplified as the plane-wave approximation. This approximation results in an expression equivalent to that derived by Stern semi-empirically. Stern's original expression for the EXAFS function $\chi(k)$ describes the oscillatory part of the $K$ absorption coefficient.\textsuperscript{53} In this high energy approximation, the oscillatory EXAFS function, $\chi(k)$, associated with a $K$-absorption edge may be written as:

$$
\chi(k) = -\sum_{j} \frac{N_j}{kR_j^2} |f_j(\pi)| \cdot \sin(2kR_j + 2\delta_j + \psi_j) \cdot \exp(-2\sigma_j^2k^2) \cdot \exp(-2R_j/\lambda)
$$

The EXAFS function $\chi(k)$ is dependent on the number of scattering atoms $N_j$, the distance of the scattering atoms $R_j$ from the primary absorber, and on the type of scattering atom through the characteristic energy dependence of the backscattering amplitude $|f_j(\pi)|$. $2\delta_j$ is the phase shift due to the potential of the emitting atom which an electron experiences on leaving and re-entering the excited atom and $\psi_j$ is the phase of the backscattering factor. The mean square variation in $R_j$ is represented by the Debye-Waller factor, $\sigma_j^2$, which assumes a harmonic distribution. $\lambda$ is the elastic mean-free path of the photoelectron and only near neighbours contribute because of the presence of the mean free path factor $[\exp(-2R_j/\lambda)$, i.e. it is the damping term $[\exp(-2R_j/\lambda)$ which invariably limits the backscattering contribution to $\leq 6$ Å from the excited atom.
A knowledge of the phase shift \((2\delta_1 + \psi_j)\) is required in the quantitative analysis of EXAFS data in order to obtain the correct interatomic distances, \(R_j\). A Fourier transform without allowing for the phase shift correction results in an error of up to 20% in the bond distance.

This equation assumes that the photoelectron is scattered off only one neighbouring atom, i.e. EXAFS results from only a single scattering event. At lower photoelectron energies the plane wave approximation breaks down.

As indicated by the damping term, \(\exp(-2\alpha_1^2 k^2)\), the amplitude of the EXAFS oscillation decays with \(k^2\). In order to maintain a similar level of signal to noise ratio across the whole spectrum, the counting time is usually weighted so that the data points at higher energies are counted for longer. Teo and Lee suggested\(^{54}\) weighting of 3 and 2 for atomic number \(Z < 36\) and \(36 < Z < 57\) respectively.

One of the limitations of EXAFS is that backscattering contributions from atoms of a similar atomic number (e.g. C, N and O) can rarely be distinguished. Thus for metal centres in proteins, EXAFS cannot distinguish between O donor ligands and N donor ligands or between S and Cl.

### 2.6.3 Detection Methods

The absorption of an X-ray photon leads to the creation of an electron vacancy. The excited atom subsequently relaxes to the ground state by a cascade of secondary processes. An electron from the outer shell can fill the vacancy, accompanied by the emission of a fluorescence photon or Auger electrons. Thus the EXAFS data can be acquired by detecting the transmission intensity or the emitted fluorescence or the ejected Auger electrons. Each of these detection methods has its advantages but yields the same structural information.

The most direct method of collecting the EXAFS data is in transmission mode in which two ion chambers detect the incident and the transmitted photon flux. The concentration of the element in the sample under probe must be high (5% in atomic weight or 50 mM) so that the absorption by the element of interest is appreciable compared to the background absorption caused by the bulk of the material. Some
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Dilute chemical systems are purposefully designed to function at low concentrations of certain types of metal and almost no metalloprotein has metal concentration high enough for the transmission method. The high background absorption in transmission mode makes it impossible to extract the EXAFS from the absorption spectrum. For these materials, detecting the emitted fluorescence following photo-absorption is the most appropriate option. The increased sensitivity of fluorescence over transmission for EXAFS measurements is because background absorption due to the matrix does not contribute to the X-ray fluorescence.

2.6.4 Experimental

The solution samples were placed into 50 μl transparent sample cells made of polycarbonate perspex. The cells were then immersed in liquid nitrogen and the samples frozen. The sample cell was then placed under vacuum, at liquid nitrogen temperatures, and in the line of the X-ray beam and the scans collected. The number of scans recorded per sample was dependent on the concentrations of the samples and their metal content.

Small quantities of solid samples were mixed with boron nitride, (purchased from Aldrich chemical company), which is used as a filler and is transparent to X-rays above 4-5 keV, and ground up together into a homogeneous powder mixture. The powder was then placed in an aluminium sample cell between adhesive tape, and the cells placed in vacuum, in the beam and scans collected.

The EXAFS data were collected and processed by Dr. Ian Harvey at the Synchrotron Radiation source, Daresbury Laboratories, Warrington, UK. The X-ray data for manganese protein samples were recorded on stations 8.1 and 9.3, and for manganese complexes on station 9.3, and the manganese K edge was measured. Data were collected at liquid nitrogen temperatures in vacuum in fluorescence mode for protein samples, and transmission mode for complexes, using a 13-element germanium detector and a Si(III) double-crystal monochromator applying 80% harmonic rejection.
2.7 Magnetic Susceptibility Measurements

The magnetic susceptibility, $\chi$, of a compound is related to the effective magnetic moment, $\mu_{\text{eff}}$, by:

$$\mu_{\text{eff}} = 2.828\sqrt{\chi_m \times T}$$

where $\mu_{\text{eff}}$ is the effective magnetic moment in units of Bohr magnetons ($\mu_B$), $\chi_m$ is the molar magnetic susceptibility and $T$ is the temperature.

In a free atom or ion, both the orbital and spin angular momenta give rise to a magnetic moment and contribute to the paramagnetism. When the atom or ion is part of a complex, any orbital angular momentum may be quenched as a result of the interactions of the electrons with their non-spherical environment. The electron spin angular momentum survives and gives rise to spin-only paramagnetism, which is characteristic of many d-metal complexes. For octahedral complexes involving first-row d-block metals the value of $\mu_{\text{eff}}$ can be estimated by assuming that the contribution made by the orbital angular momentum is negligible. The spin-only magnetic moment, $\mu$, of a complex with total spin quantum number $S$ is:

$$\mu = 2\sqrt{S(S+1)}$$

Since each unpaired electron has a spin quantum number of $\frac{1}{2}$; $S = \frac{1}{2} \times n$, where $n$ is the number of unpaired electrons, therefore:

$$\mu = \sqrt{n(n+1)}$$

A measurement of the magnetic moment of a d-block complex can usually be interpreted in terms of the number of unpaired electrons it contains, and hence it can be used to determine the oxidation state of the metal, and also distinguish between high-spin and low-spin complexes.
2.7.1 Experimental

Magnetic susceptibility data were collected by Prof. Andrew Harrison at The University of Edinburgh, Department of Chemistry, on a Quantum Design MPMS2 dc superconducting quantum interference device (SQUID) magnetometer that operates over the temperature range 1.7-350 K and fields of 0-10000 Oe.

2.8 X-ray Crystallography

X-ray crystal structure determinations were performed by Dr. Simon Parsons and Dr. Robert Coxall at The University of Edinburgh, Department of Chemistry, using single crystal X-ray diffraction techniques. X-ray crystallographic data were recorded on either a Bruker SMART APEX CCD diffractometer or a Stoe Stadi-4 diffractometer, equipped with an Oxford Cryosystems low-temperature device.

2.9 Elemental Analysis

CHN analyses were performed by the elemental analysis service at The University of Edinburgh, Department of Chemistry, using a Perkin Elmer 2400 CHN Elemental Analyser.

2.10 Mass Spectrometry

Mass spectra were recorded by the mass spectrometry service at The University of Edinburgh, Department of Chemistry, on a Kratos M.S.50TC instrument using fast atom bombardment. Xenon gas was used at 7 kV and the matrix was CH$_3$CN/3-NOBA.

2.11 pH Measurements

All pH measurements were made using either a Corning 240 pH meter, a Corning 145 pH meter, or an Orion 710A pH/ISE meter, equipped with an Aldrich glass/calomel combination pH electrode and calibrated with reference standard pH
buffer solutions of 4.00, 7.00 and 10.00, purchased from either the Sigma Chemical Company or the Aldrich Chemical Company.

pH meter readings for D$_2$O solutions were not corrected for deuterium isotope effects and are termed pH$. pH$ values can be converted to pD values using:

$$\text{pD} = \text{pH} + 0.4$$

2.12 **Centrifugation and Ultrafiltration**

Centrifugation was carried out using a Sanyo MSE Micro Centaur for small samples and a Beckman GS-15R centrifuge with a temperature control system for larger sample volumes.

Ultrafiltration was carried out using Amicon Centricon centrifugal filter tubes, with either a 10 or 30 kDa molecular weight cut off, in the Beckman GS-15R centrifuge. The low adsorption hydrophilic membranes allow solvents and low molecular weight solutes to go through the membrane while molecules above the specified molecular weight are retained above the membrane. All Centricon filter tubes were washed thoroughly before use. Ultrapure water was added to a clean, dry Centricon filter tube which was then centrifuged for 30 minutes to remove any impurities on the filter membrane.

2.13 **Lyophilisation**

All samples were frozen in liquid nitrogen prior to lyophilisation, which was carried out at -40°C using an Edwards Modulyo freeze drier and vacuum pump.
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2.14 References


9 B. G. Jenkins, R. B. Lauffer, 1988, 80, 328-336.


Chapter 2

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Chapter 3

Synthesis and Characterisation of Manganese Transferrins

3.1 Introduction

Transferrin is an 80 kDa iron-transport protein present in blood serum at a concentration of ca. 35 μM.\(^1\) It carries Fe(III) in blood at pH 7.4, delivers it to cells via receptor-mediated endocytosis, and releases it at pH 5.5 in endosomes.\(^2\) Transferrin is a bilobal glycoprotein with two (similar but not identical) high-affinity octahedral binding sites for Fe(III), one located in each lobe.\(^5\) Each Fe(III) ion is directly coordinated to the phenolate side-chains of two tyrosine residues, the imidazole ring of a histidine, and the carboxylate group of an aspartic acid residue. The remaining two coordination sites are occupied by a synergistic bidentate carbonate anion, which is also bonded to polar and charged groups of the protein.\(^5\)

The major receptor recognition sites on serum transferrin are thought to be localised on the C-lobe,\(^6\)-\(^8\) supported by the recent X-ray crystal structure of the human transferrin receptor.\(^8\) First Fe(III) binds to apo-transferrin and induces a major conformational change of the protein, from a lobe-opened to lobe-closed form. Then the iron-saturated holo-transferrin binds to the specific transferrin receptors on the cell surface and is internalised by clathrin-coated vesicles into endosomes. Here the pH is mildly acidic (pH ca. 5.0–5.5) and Fe(III) is released from the transferrin.

3.1.1 Manganese Binding to Transferrin

In human serum, only ca. 30% of the metal ion binding sites of transferrin are saturated with iron,\(^9\) and as a consequence there is capacity for the uptake and transport of other metal ions. A number of other transition metal ions, including those of therapeutic, diagnostic and toxic importance, have been reported to bind reversibly to human serum transferrin (hTf).\(^1\)\(^4\)\(^5\) The essential metal ion manganese is also likely to be transported in the blood by hTf. Manganese is thought to bind
strongly and to be transported as Mn(III) and not Mn(II),\textsuperscript{10} since the strength of binding of metal ions to human serum transferrin correlates with the acidity of metal ions (strength of hydroxide binding).\textsuperscript{11} This is illustrated in Figure 4.1, by a plot of stability constants (log $K^*$) for divalent and trivalent metal ions binding to the first lobe of apo-transferrin versus the stability constants for hydroxide binding [log $K_1$(OH)].\textsuperscript{11} Log $K_1$(OH) values are related to p$K_a$ values by log$K_1$(OH) = 14 - p$K_a$, and hence it can be seen from Figure 4.1 that the most readily hydrolysed (most acidic) metal ions (Fe(III), Bi(III)) bind the most strongly to transferrin. Mn(III) is a very acidic metal ion with reported p$K_a$ values of between −0.6 and 1.05 (at 298 K, $I > 0$) for the aqua ion,\textsuperscript{12} where $K_a$ is the hydrolysis constant.

![Figure 4.1 Correlation of the strength of transferrin and RO⁻ binding to metal ions (adapted from ref. 11).\textsuperscript{11} Correlation of the first metal-binding constants of human serum transferrin (hTf) for divalent and trivalent metal ions with that for hydroxide binding (or metal ion acidity: log $K_1$(OH) = 14 − p$K_a$, where $K_a$ is the hydrolysis constant). Black circles (•) are for experimental data and green circles (○) represent predicted values. The correlation coefficient is 0.977 and the intercept of this correlation is ca. −3, a value consistent with a flexible ligand that becomes more organised on metal binding.](image-url)
As can be seen from Figure 4.1 Mn(II) binds but only very weakly. The stability constant for the binding of the first equivalent of Mn(II) to human serum apotransferrin has been reported\textsuperscript{10} to be log $K_1 = 4.06 \pm 0.13$ (at pH 7.4 in 0.1 M $N$-(2-hydroxyethyl)piperazine-$N'$-2-ethanesulfonic acid (HEPES)). The second stepwise macroscopic equilibrium constant for the formation of Mn$_2$-hTf has been reported\textsuperscript{10} to have a value of log $K_2 = 2.96 \pm 0.13$. Such weak binding is unlikely to be involved in Mn transport in the body, since the concentration of free Mn in blood serum is low (ca. 20 nM).\textsuperscript{13} An equilibrium model for the speciation of Mn(II) in serum has been developed\textsuperscript{10} which estimates that almost 90\% of Mn(II) is bound to serum proteins, but only $\sim$1\% is bound to transferrin. The computer model confirms that albumin has a sufficient binding affinity to complex most of the Mn(II) in serum in competition with the common low molecular weight ligands in serum. The weak binding of Mn(II) to apo-transferrin and the inability of transferrin to compete with albumin indicates that the appearance of Mn-transferrin as a major serum species \textit{in vivo} must involve oxidation of the metal to form the much more stable Mn(III)-transferrin complex. Although Mn(III) has been reported to bind to transferrin,\textsuperscript{14} there has been no direct structural characterisation of the bound Mn ion. Since aquated Mn(III) ions, and many other small Mn(III) complexes are unstable in aqueous solution, Mn(III) transferrins are difficult to prepare by the routes normally employed for other metal ions.

In this study, routes for introduction of Mn(III) \textit{via} oxidation of weakly bound Mn(II) were employed. Procedures for the selective introduction of Mn(III) into either the C-lobe or N-lobe, or both, were investigated. Manganese transferrin was then characterised by UV-Vis absorption spectroscopy and by X-ray absorption spectroscopy (XAS). XAS has been extensively used in probing the environment of metals in biological and biochemical systems.\textsuperscript{15} There are also a few studies which have reported the application of EXAFS to the investigation of the metal ion binding sites of transferrin and some anion and metallosubstituted derivatives.\textsuperscript{16-21} EXAFS analysis can provide local structural and geometrical information to a resolution similar to that obtained in small molecule crystallography. This study was aided by the work on Mn(III)EHPG reported in Chapter 4.

An attempt to incorporate Mn(III) into the mono-lobe bacterial transferrin, ferric binding protein (FBP), is also reported. FBP is used by pathogenic bacteria, such as
*Neisseria gonorrhoeae*, to transport Fe(III). FBP binds Fe(III) with extremely high affinity, comparable to that of transferrin, and also shows remarkably similar overall 3D folds and Fe(III) binding site despite a lack of amino acid sequence similarity. In contrast to transferrin, FBP uses two tyrosine residues, a glutamate residue and a histidine residue from the protein, along with a molecule of H$_2$O and a monodentate phosphate (PO$_4^{3-}$) anion to bind Fe(III).

### 3.2 Experimental

#### 3.2.1 Purification and Characterisation of Apo-hTf and Apo-FBP

Apo-hTf (iron-free hTf, approx. 60 mg) was dissolved in 0.1 M KCl solution (approx. 1 – 2 ml). The colourless apo-hTf solution was placed in a washed Amicon Centricon 30 centrifuge tube and centrifuged for three 60 minute periods at 4°C, washing with 0.1 M KCl solution during each interval. This procedure allowed for the removal of any low molecular mass impurities of MWt ≤ 30,000, which can be present in the protein solution.

Apo-FBP was prepared by treatment of holo-FBP (iron-loaded FBP, pink) solutions (supplied by Dr. Weiqing Zhong) in Amicon Centricon 10 centrifuge tubes with a 250 mM sodium citrate solution at pH 4.5, at least 4 times for approx. 45 min periods at 14°C, until no pink colour was observed. The colourless apo-FBP was then sequentially washed 4 times with 2 ml of 0.1 M KCl for approx. 45 min periods at 14°C to remove any low molecular mass impurities, of MWt ≤ 10,000, present in the protein solution.

#### 3.2.2 Uptake of Manganese

*Preparation of Mn$_2$-hTf*

Mn$_2$-hTf was prepared by the addition of 2 mol. equiv. of an (ICP emission standard) Mn(II)acetate solution to a solution of the purified apo-hTf in HEPES buffer, pH 7.4, and in the presence of 20 mM bicarbonate. The protein solution was kept at 37°C for two weeks. Every day the vessel was oxygenated for ca. 30–60 s using gas from a cylinder.
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Preparation of $\text{Mn}_N\text{Fe}_C\text{-hTf}$

Reaction of apo-hTf with Fe(III)(NTA)$_2$ over the pH range 6 to 8.5 is known to lead to preferential binding of Fe(III) to the C-lobe.\textsuperscript{1,26} $\text{Mn}_N\text{Fe}_C\text{-hTf}$ was prepared by the addition of one mol. equiv. of a solution of Fe(III)(NTA)$_2$ to a solution of the purified apo-hTf in HEPES buffer, pH 7.4, containing a physiological concentration of bicarbonate (20 mM). One mol. equiv. of Mn(II)acetate was then added and the protein solution kept at 37°C for two weeks. Oxygen was again added on a daily basis.

Preparation of $\text{Fe}_N\text{Mn}_C\text{-hTf}$

Reaction of Fe(III)citrate with apo-hTf over the pH range 7.4 to 8.5 is known to lead to preferential loading of the N-lobe with Fe(III).\textsuperscript{1,26} $\text{Fe}_N\text{Mn}_C\text{-hTf}$ was prepared by the addition of one mol. equiv. of a solution of Fe(III)citrate to a solution of the purified apo-hTf in HEPES buffer, pH 7.4, and in the presence of 20 mM bicarbonate. One mol. equiv. of Mn(II)acetate was then added and the protein solution kept at 37°C for two weeks. Oxygen was added on a daily basis.

Preparation of $\text{Mn-hTf}$

To a solution of the purified apo-hTf in HEPES buffer, pH 7.4, containing 20 mM bicarbonate, one mol. equiv. of a solution of Mn(II)acetate was added. The protein solution kept at 37°C for two weeks. Oxygen was again added on a daily basis.

Attempted Preparation of $\text{Mn-FBP}$

Two separate attempts were made to load Mn into FBP. In the first attempt to prepare Mn-FBP, a solution of Mn(II)acetate was added to a solution of the purified apo-FBP in HEPES buffer, pH 7.4, containing a physiological bicarbonate concentration (20 mM), to give a 1:1 molar ratio. The protein solution was kept at 37°C for two weeks. Oxygen was added on a daily basis.

In the second attempt to load Mn into FBP, a freshly prepared aqueous solution of $[\text{Mn(III)}(\text{cyclam})(\text{Cl}_2)]\text{Cl}_2\text{H}_2\text{O}$ was added to a solution of the purified apo-FBP in
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0.1 M KCl, pH 7.4, containing 3 mM phosphate, to give a 1:1 molar ratio. The protein solution was kept at 37°C for at least 24 h.

3.2.3 Purification of Metal Loaded Proteins

Metal loaded hTf and FBP were purified in a similar manner to their corresponding apo-forms (described in section 3.2.1). The protein solutions were washed three times with 0.1 M KCl, using Amicon Centricon 30 centrifuge tubes for hTf samples and Amicon Centricon 10 centrifuge tube for FBP samples, to remove any unreacted metal that may be present.

3.2.4 Characterisation of Proteins

UV-Vis Spectroscopy

UV-Vis spectroscopy was used to determine the concentration of the protein samples, both for the apo- and lobe loaded forms. Acquisition details are given in section 2.4.6.

Protein concentrations were determined spectrophotometrically using the Beer-Lambert law on the basis of an $\varepsilon_{280}$ value of 93,000 M$^{-1}$ cm$^{-1}$ for hTf$^{27}$ and 44,200 M$^{-1}$ cm$^{-1}$ for FBP.

ICP-AES

The concentration of metal bound to the proteins was measured using ICP-AES. Samples were prepared as described in section 2.5.4.

NMR

$^1$H NMR spectra of Mn$_n$Fe$_C$-hTf and Fe$_n$Mn$_C$-hTf samples were recorded in 10% D$_2$O/90% H$_2$O solutions on a Bruker 500 MHz NMR spectrometer at 298 K, using large frequency widths (-100 to 100 ppm) due to the paramagnetic nature of the complexes. pH values were measured to be 7.4. Typical pulsing conditions were: acquisition time 0.164 s, 402 scans, 90° pulses 8 µs, 32 k data points, relaxation
delay 1.00 s. Prior to Fourier transformation, data were apodized with a Gaussian function using a $-1.0$ Hz line-broadening and a gb function of 0.0005. Water suppression was via presaturation during the relaxation delay.

**EXAFS**

Acquisition details are given in section 2.6.3. Data were collected only for the coordination spheres of the Mn centres (and not for iron) using the K edge. Data analysis was carried out by Dr. Ian Harvey (Daresbury).

EXAFS was also used to determine the order of lobe loading of hTf with Mn(III).

### 3.3 Results and Discussion

#### 3.3.1 Preparation of Mn Proteins

The Mn(III)-transferrin complexes were prepared from Mn(II) in the presence of bicarbonate and O$_2$. A characteristic brown colour developed slowly under these conditions, which indicates that O$_2$ appears to be the agent responsible for oxidising Mn(II) to Mn(III) during complex formation.

**Selective Lobe Loading**

For the preparation of transferrin with Mn in one lobe and iron in the other, firstly Fe(III) was selectively loaded into the required lobe using previously reported procedures and then Mn(II) was added to the Fe-hTf sample, to allow the uptake of Mn(III) into the empty lobe.

Fe(III), as [Fe(III)(NTA)$_2$] was added to apo-hTf at pH 7.4 to give an Fe:hTf molar ratio of 1:1. The addition of one mol. equiv. of Fe(III) as [Fe(III)(NTA)$_2$] leads to the uptake of Fe(III) preferentially into the C-lobe. The subsequent addition of Mn(II), as Mn(II)acetate therefore leads to the uptake of Mn(III) into the empty lobe, i.e. the N-lobe. Similarly Fe(III), as Fe(III)citrate was added to apo-hTf at pH 7.4 to give an Fe:hTf molar ratio of 1:1. The addition of one mol. equiv. of Fe(III) as Fe(III)citrate leads to the uptake of Fe(III) preferentially to the N-lobe.
subsequent addition of Mn(II), as Mn(II)acetate therefore leads to the uptake of Mn(III) into the empty lobe, i.e. the C-lobe.

All Mn-transferrin samples displayed characteristic absorption bands in the visible region.

The uptake of Mn by FBP was not successful by the methods of introduction that were used. Very little or virtually no binding was detected.

Although UV-Vis and resonance Raman data have shown that Mn(III) binds to transferrin, there have been no previous reports on the structural characterisation of the binding sites or the order of lobe loading with Mn(III).

X-ray spectroscopy (EXAFS and XANES) were used to investigate the oxidation state of Mn bound to human serum transferrin (hTf), and to compare the coordination spheres of Mn in the N-lobe and the C-lobe of transferrin. The protein possesses two similar metal binding sites in the N- and C-lobes which differ in their affinities. The factors which direct metal ions to specific lobes of hTf are poorly understood.

### 3.3.2 Oxidation State

The oxidation state of Mn in all protein samples was found to be +III, as can be seen from Figure 3.2, which is a plot of the energy of the X-ray absorption K edge relative to Mn metal versus oxidation state for all the Mn-transferrin samples and reference compounds. The energy of the X-ray absorption edge of the Mn-transferrin samples is characteristic of Mn(III).

Thus, the EXAFS measurements indicate that Mn is bound to transferrin as Mn(III). This is in agreement with the static magnetic susceptibility measurements reported previously by Aisen et al. Aisen et al determined a d⁴ electronic configuration and a spin state of S = 2 for the Mn₂-transferrin complex.
Figure 3.2 Oxidation state of Mn-transferrins. Variation of the energy of the Mn K absorption edge with oxidation state of Mn. (a) Mn(0) foil, (b) Mn(II)Cl₂, (c) Mn(III)acetate, (d) Mn(IV)O₂, (x) Mn-hTf (all samples). The edge shift is non-linear with oxidation state, and the Mn centres of all protein samples were found to be in the +III oxidation state.

Figure 3.3 Mn K edge structure (XANES) for Mn₅Fe₃-hTf (blue line) and Fe₅Mn₃-hTf (red line).
The XANES spectra of Fe\textsubscript{N}Mn\textsubscript{C}-hTf and Mn\textsubscript{N}Fe\textsubscript{C}-hTf are shown in Figure 3.3. There is a slight difference in the XANES spectra of the two samples. This is probably due to the difference in the Jahn-Teller splitting observed in the EXAFS data with Fe(III) in the N-lobe and Mn(III) in the C-lobe or Mn(III) in the N-lobe and Fe(III) in the C-lobe, respectively (Figure 3.4B and Figure 3.5B, respectively). The Jahn-Teller splitting for Mn\textsubscript{N}Fe\textsubscript{C}-hTf is bigger than for Fe\textsubscript{N}Mn\textsubscript{C}-hTf, i.e. the Jahn-Teller splitting upon Mn(III) coordination to the N-lobe is bigger than that for the C-lobe. Thus there is a difference between the two Mn binding sites.

The Jahn-Teller splitting can be seen clearly in the Fourier transform of the \(k^3\) weighted EXAFS spectrum of Mn\textsubscript{N}Fe\textsubscript{C}-hTf, Figure 3.4, in which there are two well separated first shell peaks.

### 3.3.3 Mn Coordination Sphere

The \(k^3\) weighted EXAFS spectrum of Mn\textsubscript{N}Fe\textsubscript{C}-hTf and its radial distribution function, with a first shell fit, are shown in Figure 3.4A and Figure 3.4B, respectively and the \(k^3\) weighted EXAFS spectrum of Fe\textsubscript{N}Mn\textsubscript{C}-hTf and its radial distribution function are shown in Figure 3.5A and Figure 3.5B, respectively.

The radial distribution function of Figure 3.4B shows two major peaks (peak I and peak II) due to backscattering from atoms in the first coordination shell. The EXAFS measurements indicate that the Mn(III) ion in Mn\textsubscript{N}Fe\textsubscript{C}-hTf is coordinated by six low Z ligands (oxygens/nitrogens) split into two shells (distances). The first shell distances for the N-lobe of Mn\textsubscript{N}Fe\textsubscript{C}-hTf are 2 oxygens/nitrogens at 1.94 Å (Debye-Waller factor 0.015 Å\(^2\)) and 4 oxygens/nitrogens at 2.20 Å (Debye-Waller factor 0.018 Å\(^2\)). The first shell distances are indicative of a Jahn-Teller distortion with two short axial ligands and four long equatorial ligands.

A comparison of the experimental EXAFS data with a theoretical EXAFS spectrum based upon the coordination of one imidazole nitrogen atom, two phenolate oxygen atoms and three additional low-Z ligands is given in Figure 3.4. The coordination numbers used to obtain the fit are 2.0 oxygens/nitrogens at 1.937 Å (Debye-Waller factor 0.015 Å\(^2\)) and 4 oxygens/nitrogens at 2.204 Å (Debye-Waller factor 0.018 Å\(^2\)).
Figure 3.4 (A) The $k^3$ weighted EXAFS spectrum, and (B) Fourier transforms of the $k^3$ weighted EXAFS spectrum of the Mn edge of Mn$_2$FeC-hTf (Mn in N-lobe and Fe in C-lobe, solid line) with a first shell fit (dotted line). The coordination numbers used to obtain the fit are 2.0 oxygens/nitrogens at 1.937 Å (Debye-Waller factor 0.015 Å$^2$) and 4 oxygens/nitrogens at 2.204 Å (Debye-Waller factor 0.018 Å$^2$).
Figure 3.5 (A) The $k^3$ weighted EXAFS spectrum, and (B) Fourier transforms of the $k^3$ weighted EXAFS spectrum of the Mn edge of $\text{Fe}_N\text{Mn}_C$-hTf (Fe in N-lobe and Mn in C-lobe).
The analysis of the EXAFS spectra of FeNMnc-hTf (Figure 3.5) gives essentially the same results but with less certainty. The spectra can be seen to be almost identical, indicating great similarity in the nature of the binding sites.

Due to the data quality, there is some uncertainty in the C-lobe coordination numbers of FeNMnc-hTf. Due to poor data quality as a result of noise, the EXAFS data range was truncated and the EXAFS data range is shorter than that of MnNFec-hTf. This results in line broadening and a broader first coordination shell peak is observed in the Fourier transform spectrum and it is therefore at a lower resolution. With the short EXAFS data range, splitting of the first shell is not justified. The average is 5.3 oxygens/nitrogens at 2.16 Å (Debye-Waller factor 0.048 Å²). Although the coordination number is closer to 5 than 6, the uncertainty with such a data range is close to 20%.

The first coordination shell in the Fourier transform spectrum in Figure 3.4B shows well resolved components (two peaks) due to the extended data range of Figure 3.4A compared with that of Figure 3.5A and Figure 3.5B.

The beat patterns observed in the EXAFS data in the ranges \( k = 5.5 \) to \( 7 \) Å\(^{-1}\) and \( 7 \) to \( 9 \) Å\(^{-1}\) are typical of imidazole coordination because of the strong multiple scattering from the outer atoms of imidazole.\(^{28}\) This multiple scattering contribution from the outer atoms of the imidazole group is also clearly evident in the Fourier transform of the Mn-transferrin EXAFS data, which show peaks at \(~3\) Å and \(~4\) Å.

It is difficult to assign the values obtained to any specific residues. A comparison of the coordination sphere of the N-lobe binding site for Mn and Fe is shown in Figure 3.6. The assignments of bond lengths to specific bonds for the Mn complex are arbitrary.

Both the Fe and Mn N-lobe binding sites are distorted from octahedral geometry. The distortion to the coordination sphere of Fe(III) is random,\(^{29}\) and is probably due to the geometric requirements of the binding site of the protein, whereas the distortion to the coordination sphere of Mn(III) is more regular in accordance with Jahn-Teller effects. The bond lengths of the Fe(III) complex are from the X-ray
structure of Fe-hTf/2N,\textsuperscript{29} and are summarised in Table 3.1, along with the bond lengths of the N-lobe of Mn\textsubscript{2}Fe\textsubscript{C}-hTf.

![Figure 3.6 Configurations of (A) Mn(III) and (B) Fe(III) metal ions in the N-lobe of hTf (taken from the X-ray structure of the N-lobe).\textsuperscript{29} The coordination spheres of both of the metal centres are distorted from that of a regular octahedron. The distortion of the Fe(III) centre is random whereas the distortion of the Mn(III) centre is more regular and in accordance with Jahn-Teller effects.]

Table 3.1 Metal-ligand bond lengths for the N-lobe of hTf with Mn(III) or Fe(III) bound.

<table>
<thead>
<tr>
<th>Mn N-lobe in Mn\textsubscript{2}Fe\textsubscript{C}-hTf</th>
<th>Bond length (Å)</th>
<th>Fe N-lobe in Fe-hTf/2N</th>
<th>Bond length\textsuperscript{29} (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Mn-O/N</td>
<td>1.937</td>
<td>5 Fe-O</td>
<td>1.99, 1.90 (Tyr);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.02 (Asp);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.06, 2.24 (CO\textsubscript{3}\textsuperscript{2-})</td>
</tr>
<tr>
<td>4 Mn-O/N</td>
<td>2.204</td>
<td>1 Fe-N</td>
<td>2.10 (His)</td>
</tr>
</tbody>
</table>

Distorted stereochemistries are to be expected for Mn(III) complexes as a consequence of the non-spherical symmetry of the d\textsuperscript{4} electronic configuration. The vast majority of six-coordinate Mn(III) compounds are static Jahn-Teller effects.
systems, with tetragonally elongated coordination octahedra. Examples include \([\text{Mn(III)(BBPEN)}][\text{PF}_6]\)\(^{30}\), where \(\text{H}_2\text{BBPEN}\) is \(N,N'\)-bis(2-hydroxybenzyl)-\(N,N'\)-bis(2-methylpyridyl)ethylenediamine, in which there are two long axial Mn-N bonds of 2.237 and 2.252 Å and four short equatorial Mn-O (1.846 and 1.888 Å) and Mn-N (2.078 and 2.124 Å) bonds, and \([\text{Mn(III)(Hvanpa)}]_2\text{N}_3\)\(^{31}\), where \(\text{H}_2\text{vanpa}\) is 1-(3-hydroxysalicylaldeneamino)-3-hydroxypropane, in which the two axial Mn-O bond lengths of 2.274 Å are ca. 0.3 Å longer than the four short equatorial Mn-O and Mn-N bonds of 1.8711 and 2.0124 Å, respectively.

There are also examples of Mn(III) complexes which have the inverse distortion of two short axial and four long equatorial coordination bonds (Jahn-Teller compression) as seen in the Mn(III)-transferrin complexes. These include \(K[\text{Mn(III)(EDTA)}].2\text{H}_2\text{O}\)\(^{32,33}\), which has two short axial Mn-O bonds in the range 1.889-1.909 Å and four long equatorial Mn-O and Mn-N bonds in the ranges 2.004-2.050 and 2.176-2.237 Å, respectively, and \([\text{Mn(III)L}_2]\)\(^34\), where \(\text{H}_2\text{L}\) is \(N-(3,5\)-dichloro-2-hydroxybenzyl)glycine, which has two short axial Mn-O bonds of 1.880 Å and four long, Mn-O and Mn-N bonds of 2.137 and 2.118 Å, respectively in the equatorial plane.

The analysis of the data of Mn\(_2\)-hTf reveals that the spectrum is simply the average of the data for the separate lobes.\(^{35}\) Averaging the two spectra gives the EXAFS spectrum for Mn\(_2\)-hTf (as expected), within the noise level of the three data sets.

These results indicate that the two bound Mn(III) ions do not exhibit major differences in their geometry, at least not in the limits of resolution of the EXAFS experiment. Thus both the N-lobe and the C-lobe bind Mn(III) in a similar but not identical manner. There are small differences in the XANES and EXAFS/Fourier transforms of the two lobes with Mn bound. The Jahn-Teller splitting for Mn\(_N\) is 0.267 Å and for Mn\(_C\) is 0.225 Å, and is clearly observable by EXAFS (Figure 3.4B and Figure 3.7B, respectively).

### 3.3.4 Selective Lobe Loading

The two binding sites of transferrin are very similar but not identical which often results in the preferential uptake of metal ions into one lobe first and then the other lobe.
Since there is a distinct difference in the EXAFS/XANES of N-lobe Mn and C-lobe Mn, the preferential uptake of one mol. equiv. of Mn(III) was investigated.

From the overlayed plots of Mn-hTf and Fe\textsubscript{N}\textsubscript{Mn}\textsubscript{C}-hTf (Figure 3.7), it can be seen that the Mn-hTf data set is very similar to that of the Fe\textsubscript{N}\textsubscript{Mn}\textsubscript{C}-hTf data set. Due to noise, the Fe\textsubscript{N}\textsubscript{Mn}\textsubscript{C}-hTf EXAFS data was truncated and the EXAFS data range is shorter. As a result a broader peak is observed in the Fourier transform spectra, and it is therefore at a lower resolution. The first peak in the Fourier transform spectra for these two data sets overlay each other very closely. The ‘peak’ in the EXAFS at 6 Å\textsuperscript{-1} in the Fe\textsubscript{N}\textsubscript{Mn}\textsubscript{C}-hTf data is a monochromator or sample alignment defect. A sample alignment defect is more likely as the ‘peak’ is absent from all other data sets.

From the overlayed plots of Mn-hTf and Mn\textsubscript{N}\textsubscript{Fe}\textsubscript{C}-hTf (Figure 3.8), it can be seen that the EXAFS goes out of phase between 7-9 Å\textsuperscript{-1}, indicating some differences in distances. There are also significant differences in EXAFS intensity at higher Å\textsuperscript{-1}. This is seen in the Fourier transform as a much larger split in the distances of the ligand atoms, with two peaks being clearly resolved for Mn\textsubscript{N}\textsubscript{Fe}\textsubscript{C}-hTf. Thus the Mn\textsubscript{N}\textsubscript{Fe}\textsubscript{C}-hTf data set is very different to that of Mn-hTf. These differences suggest that the sample of hTf loaded with one mol. equiv. of Mn(III), designated Mn-hTf, contains C-lobe bound Mn, (Mn\textsubscript{C}-hTf). Thus Mn(III) is preferentially taken up and bound by the C-lobe of transferrin from Mn(II)acetate at near physiological conditions and pH values, i.e. pH 7.4, HEPES buffer, 20 mM carbonate. Although the C-lobe binds more strongly to Fe(III) than the N-lobe, the binding site is slightly less accessible since there is an extra disulfide bond present compared to the N-lobe, which restricts the opening of the C-lobe.\textsuperscript{5}

Thus the order of lobe loading of transferrin with Mn(III) has been determined. EXAFS provides a method for determining the order of lobe loading of transferrin with Mn(III). The reaction of one mol. equiv. of Mn(II)acetate with apo-hTf in the presence of O\textsubscript{2}, at pH 7.4 led to the preferential occupation of the C-lobe with Mn(III). Thus the C-lobe selectively binds to Mn(III) first followed by the N-lobe.
Figure 3.7 Comparison of (A) the $k^3$ weighted EXAFS spectra, and (B) Fourier transforms of the $k^3$ weighted EXAFS spectra of the product from reaction of one mol. equiv. of Mn(II)acetate with human serum transferrin (solid line) and Fe$_N$Mn$_C$-hTf (iron in N-lobe and Mn in C-lobe, dotted line). The close correspondence suggests that there is preferential loading of the C-lobe.
Figure 3.8 Comparison of (A) the $k^3$ weighted EXAFS spectra, and (B) Fourier transforms of the $k^3$ weighted EXAFS spectra of the product from reaction of one mol. equiv. of Mn(II)acetate with human serum transferrin (solid line) and Mn$_N$Fe$_C$-hTf (Mn in N-lobe and iron in C-lobe, dotted line). The poor correspondence suggests that Mn preferentially loads the C-lobe (see Figure 3.6).
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The results obtained here for the order of lobe loading with Mn(III) is similar to that obtained for other metal ions such as Fe(III) and Bi(III) with preferential binding to the C-lobe.\(^1,36,37\)

Although previously a number of different techniques such as EPR, NMR, etc., have been used to investigate the order of lobe loading of hTf with a variety of different metal ions, no such applications have been made to Mn(III) on account of its paramagnetic properties.

The uncertainty in the C-lobe coordination numbers of Fe\(_{\text{NMnc}}\)-hTf (due to the data quality) can now be addressed. The Mn-hTf data, which has a longer range but is noisy, gives a more satisfactory total coordination number of 6 for the C-lobe, however it varies between 1+5 and 2+4. The distances are 1.97-1.98 and 2.19-2.20 Å for Mn-hTf. The first shell distances for the C-lobe of Fe\(_{\text{NMnc}}\)-hTf are 1-2 oxygens/nitrogens at 1.93-1.94 Å (Debye-Waller factor 0.018-0.042 Å\(^2\)) and 4-5 oxygens/nitrogens at 2.16 Å (Debye-Waller factor 0.018-0.023 Å\(^2\)). The presence of two short and four long bonds for 3d\(^4\) Mn(III) suggests the influence of a Jahn-Teller distortion.

The Debye-Waller factors are quite high for all the Mn samples and are similar to Ti\(_2\)-hTf.\(^35\) The values range from 0.02-0.04 Å\(^2\) for the first shell. This indicates a higher (than typical) degree of thermal/structural disorder. It can be envisaged that this might easily be related to the degree of difficulty in loading these metals into hTf.

Since the shift in energy of the absorption edge for all Mn-transferrins is very similar to that of Mn(III)acetate, it can be concluded that Mn(II) is oxidised as it is taken up, and this was confirmed by the appearance of the characteristic phenolate-to-Mn(III) charge-transfer bands in the absorption spectrum. The detailed mechanism of this process appears to be unknown, but most probably involves O\(_2\) as the oxidant.

Fe(III) binding was confirmed by the appearance of the characteristic tyrosinate-to-Fe(III) charge-transfer band at 465 nm.\(^1\)
3.3.5 $^1$H NMR Spectra

The $^1$H NMR spectrum of Fe$_n$Mn$_c$-hTf in 10%D$_2$O/90%H$_2$O is shown in Figure 3.9. The feature in the region of 6-13 ppm can be assigned to resonances due to NH and aromatic protons and the peaks between 0 and 3 ppm to resonances due to aliphatic protons. The peak at ~5 ppm can be assigned to water. There is also a pronounced hump in the spectrum centred at ca. 25 ppm. Although considerable efforts were made to determine its origin, it is still unclear. It seems likely that it arises from an instrumental artefact such as acoustic ringing of the probe since such a hump was observed in other spectra for which wide sweep widths were used. The peak centred at ~49 ppm is an artefact due to the probe.

![Figure 3.9 $^1$H NMR spectrum of Fe$_n$Mn$_c$-hTf.](image_url)

The $^1$H NMR spectrum of Mn$_n$Fe$_c$-hTf in 10%D$_2$O/90% H$_2$O was also recorded and was found to be virtually identical to that of Fe$_n$Mn$_c$-hTf.

Due to the presence of the paramagnetic ion, Mn(III), isotropic shifts were expected, as observed for the model complex Mn(III)EHPG (Chapter 4). It may be that the
protein complex yields peaks which are shifted outside of the detection range used (frequency width –100 to 100 ppm).

### 3.3.6 Uptake of Mn Transferrin by Receptor-Mediated Endocytosis

The uptake and storage of Mn by a clone of human neuroblastoma cells SHSY5Y has previously been studied. The results demonstrate that the transferrin receptor on SHSY5Y cells can bind and internalise Mn-transferrin as efficiently as an iron-transferrin. Since Mn-transferrin is able to bind to transferrin receptors and undergo receptor mediated endocytosis to enter cells, this implies that Mn(III) induces similar conformational changes as Fe(III) upon binding to transferrin and that both lobes are in the closed conformation. Thus the Jahn-Teller distortion is localised to the binding site and does not affect the overall protein structure of the closed conformation.

This is further evidence that the metal binding site is flexible, and adapts to the requirements of the metal ion and does not affect the overall protein structure.

### 3.4 Conclusions

These EXAFS studies have established directly for the first time that manganese binds to both the N-lobe and the C-lobe of human serum transferrin as Mn(III) with octahedral coordination. The coordination spheres of the Mn(III) centres in all protein samples are Jahn-Teller distorted.

It is not possible to distinguish readily between N and O ligands by EXAFS measurements but it seems likely that carbonate is bound to Mn(III) as a synergistic anion as it is to Fe(III).

The procedure used to introduce Mn(III) into the protein (binding of Mn(II) followed by oxidation with O$_2$) was successful although very slow, typically taking two weeks to reach completion.

In all four Mn(III)-hTf samples with Mn in one lobe or both lobes, or in one lobe with Fe(III) in the other lobe, (Mn-hTf, Mn$_2$-hTf, Mn$_n$Fe$_c$-hTf, and Fe$_n$Mn$_c$-hTf),
Mn(III) was six-coordinate with a Jahn-Teller splitting resulting in two short (1.9-1.97 Å) and four long (2.13-2.18 Å) ligand bonds.

It is demonstrated for the first time that the reaction of hTf with Mn(II) in the presence of O₂ leads to the preferential uptake of Mn(III) by the C-lobe. The EXAFS data allows a distinction between the N-lobe and the C-lobe to be made when Mn is bound. They have different Jahn-Teller distortions, which leads to different Jahn-Teller splittings in the EXAFS spectrum. This may be of biological significance.

Only when hTf is loaded with metal ions in both lobes does it bind strongly to the receptor and so the conformational changes that accompany metal binding are of direct relevance to its recognition by cells. Reaction of hTf with two mol. equiv. of Mn(II)acetate gave rise to both N- and C-lobe loading, with Mn(III).

Although the metal binding site of FBP resembles that of transferrin, little binding of Mn was observed. This may be because Mn(II) binds very weakly or that FBP is unable to oxidise Mn in the binding site, or perhaps because there is a more exposed site.
3.5 References


35 *Personal communication with Dr. Ian Harvey, Daresbury.*


Chapter 4

Manganese(III) and Titanium(IV) Complexes of Ethylenebis[(α-hydroxyphenyl)glycine]

4.1 Introduction

Low molecular mass compounds have successfully been used as models for the metal ion binding sites of metalloproteins. Ideally, these should closely resemble the metal ion binding site in the protein, in terms of their donor ligands and have similar physical and chemical properties. Studies of the stereochemistry and coordination geometry of the donor ligands and electronic and magnetic properties (e.g. absorption bands, magnetic moments, oxidation states) of the specific metal ion binding sites of human serum transferrin have long been aided by studies of metal complexes of the model ligand ethylenebis[(α-hydroxyphenyl)glycine] (EHPG or H4EHPG).1-10

EHPG is a hexadentate ligand which contains the same number and similar types of donor groups as are found in the two metal ion binding sites of transferrin.11 EHPG possesses three pairs of chemically equivalent donor atoms: two amine nitrogens, two carboxylate oxygens and two phenolate oxygens (Figure 4.1) and offers an octahedral coordination environment to metal ions.

Previous studies on the Fe(III) complex of EHPG have shown that it shares many similar physical properties with Fe(III)-transferrin.2,3 The EHPG ligand has also proved useful in providing insights into the structure and chemistry of a number of other iron-phenolate proteins.10,12,13

The Fe(III)EHPG complex, rac-Fe(III)EHPG, was recognised as a potential model for Fe(III)-transferrin originally on the basis of its optical and resonance Raman spectra and then later on the basis of its EPR g values and solution chemistry.2,3 The Fe(III)EHPG and Fe(III)-transferrin complexes also have similar isomer shifts and quadrupole splitting in the Mössbauer spectra.14
Figure 4.1 Schematic representation of the ligand, $N,N'$-ethylenbis[(o-hydroxyphenyl)glycine] (EHPG or H$_4$EHPG). The stars (*) indicate the chiral $\alpha$-carbons. The labelling scheme indicated is used for protons in NMR spectra.

The EHPG ligand represents an excellent model for methods of analysis that are most sensitive to metal-phenolate interactions. In UV-Vis spectroscopy, for example, phenolate coordination to a redox active metal ion is usually accompanied by the appearance of relatively intense ligand-to-metal charge-transfer bands, which often lie in the visible region of the spectrum. The EHPG ligand can provide two phenolate oxygens for coordination just as in transferrin. Moreover, the charge-transfer bands in the UV-Vis spectra of metal-EHPG complexes display all the characteristic features of the corresponding metallo-transferrin complexes for a wide variety of metal ions, although the charge-transfer bands are often shifted in energy or intensity.

The EHPG ligand itself possesses two asymmetric carbons atoms (Figure 4.1) and chelation to a metal introduces two further chiral centres at the nitrogen atoms. EHPG has been widely used as a ligand in studies of a range of metal ions other than Fe(III) and several X-ray crystal structures of metal-EHPG complexes have been reported.

In order to aid the characterisation of Mn(III) and Ti(IV) transferrin, the Mn(III) and Ti(IV) complexes of the model ligand EHPG have been synthesised in the work described here and characterised by NMR and UV-Vis spectroscopy and X-ray crystallography.
The solution structure of the Ti(IV)EHPG complex has been determined by 2D $^1$H NMR spectroscopy and compared with the solid state X-ray crystal structure. The stoichiometry of binding of Ti(IV) to EHPG and the kinetics of reaction of Ti(IV)citrate with EHPG have been studied by $^1$H NMR spectroscopy. 2D $^1$H NMR EXSY (exchange spectroscopy) was used at high pH (8.1) to study the solution dynamics of the Ti(IV)EHPG complex. The pH dependence of both the Ti(IV) and Mn(III) complexes of EHPG was studied using $^1$H NMR spectroscopy and UV-Vis spectroscopy, respectively.

4.2 Experimental

4.2.1 Materials and Methods

The commercially-available ligand consists of an unspecified mixture of mesomeric (R,S) and racemic (R,R/S,S) isomers, which were subsequently determined to be in a ca. 1:1 ratio in our samples. No attempt was made to separate them.

**UV-Vis Spectroscopy**

Electronic absorption spectra were recorded as described in section 2.4.6 at ambient temperature (between the ranges of 200 nm and 800 nm), unless stated otherwise. The measurements were made using typically $10^{-4}$-$10^{-5}$ M solutions in aqueous media, and baseline correction were made for the solvents/buffers used.

**$^1$H NMR Spectroscopy**

Acquisition details for NMR spectroscopy are given in section 2.2.12. The $^1$H NMR spectra of Mn(III) and Ti(IV) EHPG complexes were recorded in D$_2$O. Free ligand spectra were obtained in alkaline or acidic D$_2$O (pH adjusted with NaOD or DNO$_3$), respectively. $^1$H chemical shifts were internally referenced to either 1,4-dioxane (3.764 ppm at 298 K), TSP (0 ppm), or HOD (4.60 ppm). All $^1$H NMR spectra were recorded at 298 K unless stated otherwise. Solutions were typically 5 mM. $^1$H NMR spectra of Mn(III)EHPG were recorded over a large sweep width (100 to -100 ppm) due to the paramagnetic nature of the complex.
**pH Measurements**

The pH values of samples were measured and adjusted as described in section 2.11. The pH values for D₂O solutions are recorded as pH⁺ values, and are uncorrected for deuterium isotope effects.

### 4.2.2 Preparation of Mn(III) and Ti(IV) EHPG Complexes

**Preparation of Na[Mn(III)(EHPG)]**

Mn(III)EHPG complexes were prepared following a reported procedure.⁴ To a suspension of H₄EHPG (1.442 g, 4 mmol) in ca. 25 ml water, two mol. equiv. of NaOH (0.320 g, 8 mmol) were added under an atmosphere of N₂. Manganese(III) acetate dihydrate (1.178 g, 4 mmol) was added and the resultant dark brown solution stirred overnight at ambient temperature. The solution was then filtered and evaporated to dryness in vacuo. The product was recrystallised from ethanol/water (70%, v/v) at 277 K to yield brown crystals of rac-Na[Mn(EHPG)].3H₂O (1) suitable for X-ray crystallographic analysis. The crystals were filtered off, washed with ice-cold water and dried in vacuo at ambient temperature over P₂O₅ for a period of 24 h. Yield: 0.12 g, 5.9%. Anal. Calc. for Na[Mn(C₁₈H₁₆N₂O₆)].4H₂O, C₁₈H₁₆N₂O₆MnNa.4H₂O: C, 42.69; H, 4.74; N, 5.53. Found: C, 42.70; H, 3.85; N, 6.75%. UV-Vis (H₂O): \( \lambda_{\text{max}} \) 423 nm (\( \epsilon = 810 \text{ M}^{-1}\text{cm}^{-1} \)), \( \lambda \) 480 nm (shoulder, \( \epsilon = 390 \text{ M}^{-1}\text{cm}^{-1} \)), \( \lambda \) 345 nm (\( \epsilon = 1930 \text{ M}^{-1}\text{cm}^{-1} \)).

This preparation was repeated but recrystallisation was carried out from a more concentrated solution in ethanol/water (70%, v/v). This also yielded brown crystals, which in the subsequent X-ray crystallographic analysis were found to be rac,meso-Na[Mn(EHPG)].H₂O (2). The crystals were filtered off, washed with ice-cold water and dried in vacuo at ambient temperature over P₂O₅ for a period of 24 h. Yield: 0.412 g, 20.4%. Anal. Calc. for Na[Mn(C₁₈H₁₆N₂O₆)].4H₂O, C₁₈H₁₆N₂O₆Mn.4H₂O: C, 42.69; H, 4.74; N, 5.53. Found: C, 43.37; H, 4.97; N, 6.32%. UV-Vis (H₂O): \( \lambda \) 437 nm (\( \epsilon = 840 \text{ M}^{-1}\text{cm}^{-1} \)), \( \lambda \) 351 nm (\( \epsilon = 2450 \text{ M}^{-1}\text{cm}^{-1} \)).
**Preparation of Rac-[Ti(IV)(EHPG)(H₂O)]₁₁/₃H₂O**

To a suspension of H₄EHPG (18.1 mg, 0.05 mmol) in water (2 ml), a few drops of NH₄OH were added until a clear pale peach solution was obtained (i.e. all the ligand had dissolved). The pH of the solution was adjusted to pH 5.4 by dropwise addition of HCl to mimic endosomal conditions.

The above 25 mM EHPG solution (2 ml, 0.05 mmol) was added to an aqueous solution of Ti(IV)citrate (0.5 ml, 100 mM, 0.05 mmol), pH 7.1, which had been prepared as reported previously,¹⁷ in a 1:1 molar ratio. The resultant orange solution was stored at 277 K and yielded orange crystals of rac-[Ti(IV)(EHPG)(H₂O)]₁₁/₃H₂O (3) after 3-4 days, suitable for X-ray crystallographic analysis. The pH of the final solution was found to be pH 6.1. The crystals were filtered off, washed with ice-cold water and dried in air at ambient temperature. Yield: 8.4 mg, (65.6% based on rac-EHPG). Anal. Calc. for Ti[(CtsHtsN₂O₆)(H₂O)]₅H₂O, CtsHtsN₂O₇Ti.₅H₂O: C, 42.20; H, 5.47; N, 5.47. Found: C, 42.00; H, 5.32; N, 5.28 %. UV-Vis (H₂O): λₘₐₓ 386 (ɛ = 9600 M⁻¹cm⁻¹), λ 260 (ɛ = 11200 M⁻¹cm⁻¹).

### 4.2.3 Solution Studies of Mn(III) and Ti(IV) EHPG Complexes

**pH Dependence**

In order to investigate the pH stability of the rac-Mn(III)EHPG complex (1), UV-Vis spectra at different pH values were recorded in aqueous solutions. Solutions were typically 10⁻⁴-10⁻⁵ M in 0.1 M NaCl. The pH values of the solutions were adjusted by the addition of µl aliquots of standard aqueous 0.1 M HCl or NaOH solutions. Dilution was negligible.

Similarly, ¹H NMR spectra were also recorded at different pH* values in D₂O for Ti(IV)EHPG (complex 3). Solutions were typically 5 mM. The pH of the solutions was adjusted by the addition of µl aliquots of standard solutions of 0.1 M NaOD or DNO₃ in D₂O. Again, dilution was negligible.
Temperature Dependent $^1$H NMR Spectra

The effect of temperature on the signals in the $^1$H NMR spectra of the paramagnetic rac-Mn(III)EHPG complex (1) was studied to investigate paramagnetic behaviour and aid interpretation of the spectrum. The measurements were made in D$_2$O between 20°C and 60°C, in 10°C increments.

Determination of Metal-to-Ligand Stoichiometry

Titrations were carried out to determine the metal-to-ligand stoichiometry of the reaction between Ti(IV)citrate and EHPG in solution. Complex formation was monitored by the decrease in intensity of the resonance peaks due to the ligand and increase in intensity of the peaks due to the formation of the complexes, in the $^1$H NMR spectra, upon addition of aliquots of Ti(IV)citrate to solutions of EHPG, in D$_2$O solutions. Metal titrations were carried out at ca. pH* 7.4, close to cellular physiological pH values. Final concentrations of both reactants were ca. 5 mM.

Kinetic Studies

The kinetics of reaction between Ti(IV)citrate and EHPG was studied by $^1$H NMR spectroscopy in D$_2$O solutions. Complex formation was monitored by the decrease in intensity of the resonance peaks due to the ligand and increase in intensity of the peaks due to the formation of the complex in the $^1$H NMR spectrum.

Reactions were carried out at pH* 7.1 in D$_2$O at 310 K. An aliquot of a Ti(IV)citrate solution (pH* 7.4) was added to a solution of EHPG (pH* 7.1) to give a final concentration of 5 mM of each reactant. $^1$H NMR spectra were recorded immediately, and then subsequently at various time intervals thereafter. There was no measurable change in pH* during the reaction. For kinetic analysis of NMR spectra, relative concentrations of each species were calculated at each time point from peak integrals.

For comparison, reactions of Ti(III)citrate (prepared by mixing Ti(III)Cl$_3$ with one mol. equiv. of citrate) with EHPG were also carried out. Ti(III)citrate on its own was only slowly oxidised to Ti(IV)citrate (several hours).
4.3 Results and Discussion

Commercially-available $H_4EHPG$, which consists of a ca. 1:1 mixture of mesomeric $(R,S)$ and racemic $(R,R/S,S)$ isomers,$^{4,6,7}$ was used in all metal-EHPG preparations.

Both rac and meso forms of Mn(III)EHPG and the rac form of Ti(IV)EHPG were crystallised and their X-ray structures were determined (by Dr. Simon Parsons). The ligand forms stable 1:1 complexes with Mn(III) and Ti(IV) in which both phenolate groups are coordinated.

**Stereochemistry**

The EHPG ligand itself contains two chiral carbon atoms, and so therefore has two diastereomeric forms: a pair of racemic enantiomers $(R,R$ and $S,S)$ and a meso isomer $(R,S$ or $S,R)$. However, there are also two prochiral nitrogen atoms, and so the two amine nitrogens become chiral upon coordination with a metal ion. Thus there are four chiral atoms in metal-EHPG complexes, which can, in theory, give rise to a total of 16 possible configurations. Since, only a gauche conformation for the NCH$_2$CH$_2$N group in the metal complexes is possible,$^{6,18}$ and the meso isomers are related by $C_2$ symmetry, and are therefore equivalent, the original 16 possible configurations are reduced to only 6.

In the three isomers with nitrogen atoms in the N(S,S) configuration, the chirality at the asymmetric carbon atoms can be either C(R,R), C(S,S), or C(R,S). If the nitrogen atoms are in the N(R,R) configuration, a similar argument holds, since they are mirror images of the three N(S,S) isomers. The complex with the absolute configuration N(S,S)C(R,R), which has been designated as $(R,R)$ rac$^{18}$ or AR$^6$ in some previous reports, and its enantiomer N(R,R)C(S,S), ΔSS$^6$ both have trans carboxylate ligands in five-membered chelate rings in axial sites and cis phenolate ligands in six-membered chelate rings in the equatorial plane defined by the five-membered en chelate ring. The enantiomer with the N(R,R)C(S,S) configuration is shown in Figure 4.2A. The complex with the absolute configuration N(S,S)C(S,S), which has been previously designated as (S,S) rac$^{18}$ or ΔRR$^6$ in the literature, and its enantiomer N(R,R)C(R,R), both have trans phenolate ligands in six-membered chelate rings in axial sites and cis carboxylate ligands in five-membered chelate rings.
in the equatorial plane defined by the five-membered en chelate ring. The enantiomer with the N(S,S)C(S,S) configuration is shown in Figure 4.2B. The complex with the absolute configuration N(S,S)C(R,S), which has been previously designated as (R,S) meso in the literature,\textsuperscript{18} and its enantiomer N(R,R)C(S,R), have cis configurations of the ligands, i.e. both phenolate and carboxylate ligands are mutually cis, as shown in Figure 4.2C.

![Diagram](image)

(A) N(R,R)C(S,S)  
(B) N(S,S)C(S,S)  
(C) N(R,R)C(S,R)

Figure 4.2 Schematic representations of the rac (A, B) and meso (C) isomers of octahedral metal-EHPG complexes.
4.3.1 Preparation of Mn(III) and Ti(IV) EHPG Complexes

Preparation of Mn(III)EHPG Complexes

Mn(III) complexes of EHPG were prepared via reaction of Mn(III)acetate with EHPG in water, although the yields were relatively low. From the first preparation, only the racemic isomer, compound 1 crystallised, whereas the second preparation gave rise to crystals of compound 2 containing both the meso and rac isomers in complexes 2a and 2b, respectively.

Patch et al have reported the preparation of Mn(III) complexes of EHPG in low yield, starting from either Mn(II) followed by air oxidation, or directly from Mn(III). They attributed the low yields to ligand oxidation by Mn(III), although Mn(III) complexes of EHPG are stable to internal redox reactions. Frost et al have noted that it is impossible to titrate Mn(II) solutions of EHPG at high pH, due to irreversible oxidation of Mn(II) in the presence of EHPG, even in an inert atmosphere, and have concluded that oxidation of Mn(II) occurs at the expense of decomposition of the solvent or reduction of the ligand. They noted that oxidation of the Mn(II) ion did not appear to occur at low pH.

The magnetic moment of (rac,meso) complex (2), $\mu_{\text{eff}}$, was determined to be 5.1 $\mu_B$ by SQUID magnetic susceptibility measurements, a value close to the spin-only value of 4.9 $\mu_B$ for the high-spin $d^4$ ground-state electronic configuration expected for octahedral Mn(III) ion, and the same as that reported for rac-Na[Mn(EHPG)].1.5H2O. The EHPG ligand has an overall charge of -4 when in its fully deprotonated form, which is balanced by the Mn(III) ion and Na$^+$ counter cation.

Preparation of Ti(IV)EHPG Complexes

Ti(IV) complexes of EHPG were prepared from aqueous Ti(IV)citrate solutions, at near physiological pH values. NH4OH was added to the suspension of EHPG in water to aid solubility, by deprotonating the ligand upon raising the pH of the solution. All preparations gave rise to crystals of compound 3 containing, only the racemic isomer, in complexes 3a and 3b, respectively. Ti(IV) complexes of EHPG
can also be prepared from the antitumour agent titanocene dichloride, \( \text{Cp}_2\text{TiCl}_2 \).\(^7\) The \(-4\) charge of the EHPG ligand in its fully deprotonated form is balanced by the titanium ion, which is in its +4 oxidation state.

The Mn(III) and Ti(IV) EHPG complexes were characterised using NMR and UV-Vis spectroscopy and X-ray analysis. The solution studies and X-ray crystal structure of Mn(III) and Ti(IV) EHPG complexes are reported.

### 4.3.2 X-ray Analysis

Crystallographic data for complexes 1, 2, and 3 are summarised in Table 4.1.

**X-ray Crystal Structure of Complex 1**

Compound 1 crystallised from an aqueous ethanol solution in the space group \( P2_1/n \) as \( \text{rac-} \text{Na[Mn(EHPG)]}.3\text{H}_2\text{O} \). The structure and atom labelling are shown in Figure 4.3. Mn(III) complexes of the racemic \((R,R)\) isomer of EHPG with the absolute configuration \( \text{N}(\text{S}, \text{S})\text{C}(\text{R}, \text{R}) \) at the N and C stereogenic centres, as well as complexes of the enantiomer \( \text{N}(\text{R}, \text{R})\text{C}(\text{S}, \text{S}) \), are present in the unit cell.

![Figure 4.3](image)

**Figure 4.3** Thermal ellipsoid plot of the \( \text{N}(\text{S}, \text{S})\text{C}(\text{R}, \text{R}) \) enantiomer of the anion in complex 1, \( \text{rac-} \text{Na[Mn(EHPG)]}.3\text{H}_2\text{O} \). The axial trans carboxylate Mn-O bonds in this isomer are long (2.202 and 2.162 Å) due to the Jahn-Teller distortion. The axial O-Mn-O bond angle (168.3°) deviates significantly from linearity. Thermal ellipsoids enclose 30% probability surfaces.
Table 4.1 Crystal data, data collection and refinement for complexes 1, 2 and 3.

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<td>0 ≤ k ≤ 16,</td>
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<td>-12 ≤ l ≤ 26</td>
<td>0 ≤ l ≤ 23</td>
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<td>4709</td>
<td>9463</td>
</tr>
<tr>
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<td>4690 (R_{int} = 0.0588)</td>
<td>5662 (R_{int} = 0.0270)</td>
</tr>
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<td>Max. and min. transmission</td>
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<td>0.467 and 0.190</td>
<td>-</td>
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<td>4690 / 0 / 367</td>
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<td>R1 = 0.0499</td>
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<td>(2076 data)</td>
<td>(4428 data)</td>
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<td>wR2 = 0.2732</td>
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<td>Largest difference peak and hole (eÅ⁻³)</td>
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<td>1.066 and −0.575</td>
<td>0.752 and −0.379</td>
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Chapter 4
Mn(III) and Ti(IV) Complexes of EHPG

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Mn(III) in complex 1 is coordinated to four oxygen atoms and two nitrogen atoms and has a distorted octahedral geometry. The two axial carboxylate Mn-O bonds are long (Mn(1)-O(71), 2.202(7); Mn(1)-O(121), 2.162(7) Å) and the O-Mn-O axis deviates significantly from linearity (O(71)-Mn(1)-O(121) angle, 168.3(3)°). In the equatorial plane, the two amine nitrogen atoms (Mn(1)-N(8), 2.058(8); Mn(1)-N(11), 2.063(8) Å) are trans to phenolate oxygen atoms (Mn(1)-O(1), 1.865(7), Mn(1)-O(18), 1.901(6) Å). The in-plane bond angles are close to 90° for the six-membered chelate rings (N(8)-Mn(1)-O(1) angle, 91.9; N(11)-Mn(1)-O(18) angle, 88.8(3)°) but are much smaller, only 83.7(3)°, for the five-membered en chelate ring. Selected bond lengths and bond angles for complex 1 are listed in Table 4.2. The water molecules and sodium ions in the lattice are disordered. Complex 1 has virtual C2 symmetry, with the pseudo 2-fold symmetry axis passing through the midpoint of the C-C bond of the five-membered en chelate ring backbone, bisecting the Mn(III) ion.

X-ray Crystal Structure of Complex 2

Compound 2 was crystallised using the same procedure as for compound 1, but from a more concentrated solution. Compound 2 crystallised as rac,meso-Na[Mn(EHPG)].H2O in the space group Pbcn with two independent molecules, 2a and 2b, in the unit cell. Their structures, together with the atom labelling schemes, are shown in Figure 4.4. Molecule 2a contains the meso (S,R) isomer of the EHPG ligand, whereas molecule 2b contains the racemic (S,S) isomer. The absolute configurations at the stereogenic centres of the diastereomeric complexes are N(R,R)C(S,R) and N(R,R)C(S,S) for molecules 2a and 2b, respectively. The space group is centrosymmetric and the N(S,S)C(R,S) and N(S,S)C(R,R) enantiomers of 2a and 2b, respectively, are also present in the unit cell. The water molecules and sodium ions in the lattice are disordered.

The rac isomer 2b has crystallographic .2. symmetry and adopts a similar structure to the rac isomer, molecule 1, but is slightly less distorted. The two trans axial Mn-O carboxylate bond lengths are now equal (but still long, Mn-O, 2.165(7) Å), as are the two equatorial Mn-O phenolate bonds (Mn-O, 1.890(7) Å), and the two Mn-N amine bonds (Mn-N, 2.012(8) Å, ca. 0.05 Å shorter than for 1). The bond angles are very similar to those of complex 1. Selected bond lengths and angles are listed in Table 4.2. Molecules 1 and 2b are enantiomers.
Figure 4.4 Thermal ellipsoid plots for one of the enantiomers of (A) the meso, and (B) the rac anions in complex 2, Na[Mn(EHPG)].H₂O. The axial trans carboxylate Mn-O bonds in the rac isomer based on Mn(1') are long (2.165 Å) and the trans amine Mn-N (2.194 Å) and carboxylate Mn-O (2.152 Å) bonds for the meso isomer based on Mn(1) are long due to Jahn-Teller distortions. The axial N-Mn-O bond angle (154.3°) in the meso isomer shows an even greater deviation from linearity than for the rac isomer (169.0°). Thermal ellipsoids enclose 30% probability surfaces.
In the *meso* isomer 2a, Mn(III) also has a distorted octahedral geometry. In contrast to the *rac* isomer (molecules 1 and 2b), there is only one long axial carboxylate Mn-O bond (Mn(1)-O(121), 2.152(8) Å). The other is replaced by a long axial amine Mn-N bond (Mn(1)-N(8), 2.194(8) Å), and the O-Mn-N axis deviates even more from linearity (N(8)-Mn(1)-O(121) angle, 154.3(3)°) than the corresponding O-Mn-O axis in the *rac* isomer, bond angles of 168.3(3) and 169.0°, in molecules 1 and 2b, respectively. In the equatorial plane the other amine nitrogen (Mn(1)-N(11), 2.005(8) Å) is trans to a phenolate oxygen (Mn(1)-O(1), 1.897(7) Å), and the second phenolate oxygen (Mn(1)-O(18), 1.901(7) Å) is trans to a carboxylate oxygen (Mn(1)-O(71), 1.988(7) Å). All three pairs of chemically equivalent donor atoms are mutually cis. Selected bond lengths and bond angles for 2a are given in Table 4.2.

The asymmetric unit of compound 2 contains a [meso-Mn(EHPG)]⁺ ion, 2a, in a general position, with the all-cis configuration of the ligand, and also a [rac-Mn(EHPG)]⁺ ion, 2b, with trans carboxylate groups, on a crystallographic 2-fold axis. In total there are 12 molecules in the unit cell, eight of which are *meso* and four of which are *rac*. Thus, there are twice as many *meso* isomers as there are *rac* isomers in the unit cell.

For all three Mn(III)EHPG complexes, the (N-Mn-O) bond angles of the six membered phenolate chelate rings are close to 90° (range 88.8-91.9°) whereas the N-Mn-O and N-Mn-N bond angles of the five-membered carboxylate and en chelate rings are smaller (range 75.1-78.3° and 82.4-85.2(3)°, respectively).

The range of values of Mn-N and both Mn-O carboxylate and Mn-O phenolate bond lengths found in both the Mn(III)EHPG complexes, 1 and 2, is comparable to those reported previously for other Mn(III) complexes.20-23

As in the crystal lattice of the Mn(III)EHPG complexes, the Na⁺ ions in the crystal lattice of Na₂[Cu(EHPG)] and Na[Fe(EHPG)] complexes are also disordered.6,24 Such disorder has been attributed6 to a manifestation of the multitude of satisfactory Na⁺ coordination environments offered by the oxygen atoms of the solvent water molecules and the coordinated EHPG ligands in these crystal structures. This is also probably the reason for disorder in the Mn(III)EHPG complexes.
Table 4.2 Selected bond lengths (Å) and angles (°) for complexes 1, 2a and 2b.

<table>
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<th></th>
<th>1</th>
<th>2a</th>
<th>2b</th>
</tr>
</thead>
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<td>1.897(7)</td>
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<td>Mn(1)-O(18)</td>
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<td>1.901(7)</td>
<td>1.890(7)</td>
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<td>Mn(1)-N(8)</td>
<td>2.058(8)</td>
<td>2.194(8)</td>
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<td>Mn(1)-N(11)</td>
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<td>2.005(8)</td>
<td>2.012(8)</td>
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<tr>
<td>Mn(1)-O(71)</td>
<td>2.202(7)</td>
<td>1.988(7)</td>
<td>2.165(7)</td>
</tr>
<tr>
<td>Mn(1)-O(121)</td>
<td>2.162(7)</td>
<td>2.152(8)</td>
<td>2.165(7)</td>
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<tr>
<td><strong>Angles(°)</strong></td>
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<tr>
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<td>88.9(3)</td>
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<td>N(8)-Mn(1)-O(121)</td>
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<td>154.3(3)</td>
<td>95.6(3)</td>
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<td>75.1(3)</td>
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<td>76.2(3)</td>
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<td>89.3(3)</td>
<td>95.6(3)</td>
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<tr>
<td>O(121)-Mn(1)-O(71)</td>
<td>168.3(3)</td>
<td>89.1(3)</td>
<td>169.0(5)</td>
</tr>
</tbody>
</table>

The Mn(III)EHPG complexes exhibit interesting structural features. In both complexes 1 and 2, two of the bonds to Mn(III) are significantly longer than the other four. In the rac isomer (1 and 2b), there are two long axial Mn-O (carboxylate) bonds, and four short equatorial bonds, two Mn-O (phenolate) and two Mn-N (amine) bonds. The meso isomer, 2a, also has two long axial bonds and four short equatorial bonds. However, in contrast to the racemic isomers, 1 and 2b, the meso isomer, 2a, has only one long axial Mn-O (carboxylate) bond. The other is replaced...
by a long axial Mn-N (amine) bond. The four short equatorial bonds, now involve only one Mn-N (amine) bond together with three Mn-O bonds (two Mn-O phenolate bonds and one Mn-O carboxylate bond). The two amine nitrogens in both isomers 1 and 2 are cis due to the geometric requirements of the five-membered en chelate ring.

In the case of the rac isomer in complexes 1 and 2b, the two axial Mn-O carboxylate bonds are ca. 0.2 Å longer than the equatorial Mn-O carboxylate bonds in the meso isomer 2a. For 2a (meso isomer) as well as a long axial Mn-O carboxylate bond, one axial Mn-N bond is ca. 0.2 Å longer than the equatorial Mn-N bond. For both rac and meso isomers, the Mn-O phenolate bonds are always equatorial and short, ca. 0.1 Å shorter than the equatorial Mn-O carboxylate bonds and ca. 0.25 Å shorter than the axial Mn-O carboxylate bonds.

There is substantial distortion from octahedral symmetry for Mn(III) in both the isomers. The lengthening of the axial Mn-O and Mn-N bonds compared to the equivalent equatorial bonds can be attributed to a Jahn-Teller distortion. Tetragonal distortions are expected for six-coordinate complexes of high spin d^4, 5E_g (t^2_g e_g), Mn(III).

Crystal structures of other Mn(III) complexes with Jahn-Teller distorted octahedral geometries have been reported. Distortion so as to give two long axial and four short equatorial bonds (Jahn-Teller elongation), as observed in the present case, is relatively common. Examples include [Mn(III)(BBPEN)][PF_6],^22 where H_2BBPEN is N,N'-bis(2-hydroxybenzyl)-N,N'-bis(2-methylpyridyl)ethylenediamine, in which there are two long axial Mn-N bonds of 2.237 and 2.252 Å and four short equatorial Mn-O (1.846 and 1.888 Å) and Mn-N (2.078 and 2.124 Å) bonds, and [Mn(III)(Hvanpa)_2]N_3,^25 where H_2vanpa is 1-(3-hydroxyalicycaldeneamino)-3-hydroxypropane, in which the two axial Mn-O bond lengths of 2.274 Å are ca. 0.3 Å longer than the four short equatorial Mn-O and Mn-N bonds of 1.8711 and 2.0124 Å, respectively.

In the octahedral d^9 Cu(II) complex with rac-EHPG, rac-Na_2[Cu(EHPG)],^6 there is a similar Jahn-Teller distortion with elongation of the two trans axial Cu-O (carboxylate) bonds (2.404, 2.462 Å). These are substantially longer (by ~0.45 Å) than the equatorial Cu-O (phenolate) bonds (1.944, 1.937 Å).
It has been pointed out\(^5\) by Patch and Carrano that such a Jahn-Teller distortion could be significant in Cu(II) complexes of transferrin. Since the Cu(II)EHPG complex is severely distorted\(^6\) it is not unreasonable to assume that the Cu(II)-transferrin complex would be similarly distorted. This is indeed what has been observed in the X-ray crystal structure of the Cu(II) complex of lactoferrin and by EXAFS studies of Cu(II)-transferrin.\(^{26-28}\) Thus, since the Mn(III)EHPG complexes are Jahn-Teller distorted, the Mn(III)-transferrin complex can also be expected to be distorted.

There are examples of Mn(III) complexes that have the inverse distortion of two short axial and four long equatorial coordination bonds (Jahn-Teller compression). These include K[Mn(III)(EDTA)].\(2\)H\(_2\)O,\(^{29,30}\) which has two short axial Mn-O bonds in the range 1.889-1.909 Å and four long equatorial Mn-O and Mn-N bonds in the ranges 2.004-2.050 and 2.176-2.237 Å, respectively, and [Mn(III)L\(_2\)]\(^{31}\) where H\(_2\)L is \(N-(3,5\)-dichloro-2-hydroxybenzyl)glycine, which has two short axial Mn-O bonds of 1.880 Å and four long, Mn-O and Mn-N bonds of 2.137 and 2.118 Å, respectively in the equatorial plane.

### 4.3.3 X-ray Crystal Structure of Rac-[Ti(EHPG)(H\(_2\)O)].11/3H\(_2\)O (3)

Compound 3 crystallised from an aqueous solution as rac-[Ti(EHPG)(H\(_2\)O)].11/3H\(_2\)O in the space group \(C2/c\) with two independent molecules, 3\(a\) and 3\(b\), in the unit cell. Their structures, together with the atom labelling schemes, are shown in Figure 4.5. Both molecules, 3\(a\) and 3\(b\), contain the racemic (S,S) isomer of the EHPG ligand. The absolute configuration of the Ti(IV) complexes at the N and C stereogenic centres is N(S,S)C(S,S). The space group is centrosymmetric and the N(R,R)C(R,R) enantiomers of molecules 3\(a\) and 3\(b\) are also present in the unit cell.

Molecules 3\(a\) and 3\(b\) have similar structures but molecule 3\(b\) is slightly more distorted. They differ slightly in their bond lengths and bond angles (Figure 4.5 and Table 4.3). Ti(IV) in both molecules is seven-coordinate with a distorted pentagonal bipyramidal geometry. All six chelating atoms of the EHPG ligand, the four oxygen atoms from the two carboxylate and two phenolate groups as well as the two nitrogen atoms from the two amine groups are coordinated to the Ti(IV) ion together with an additional oxygen atom from a water molecule.
Figure 4.5 Thermal ellipsoid plots of the molecules in complex 3, rac-[Ti(EHPG)(H₂O)].11/3H₂O, with axial trans phenolate Ti-O bonds. X-ray crystal structure and atom labelling scheme for one of the enantiomers of each of the molecules 3a (A) and 3b (B) in rac-[Ti(EHPG)(H₂O)].11/3H₂O. The axial O-Ti-O bond angle (171.22 and 168.24°, for molecules 3a and 3b respectively) deviates significantly from linearity.

In molecule 3a, the two phenolate oxygen atoms of the ligand are trans to each other and are located in the axial positions (Ti-O, 1.869(2) Å) and the O-Ti-O axis deviates significantly from linearity (O-Ti-O angle, 171.22(14)°). The other five coordinated
atoms, the two amine nitrogen atoms (Ti-N, 2.210(2) Å), and the two carboxylate oxygen atoms (Ti-O, 2.061(2) Å) of the ligand, along with the additional oxygen atom from a water molecule (Ti-OH₂, 2.091(3) Å), are nearly equally distributed in the equatorial pentagonal plane of the Ti(IV) ion.

The axial Ti-O phenolate bond lengths are equal, as are the equatorial Ti-O carboxylate and Ti-N amine bonds. The in-plane bond angles are in the range 71.16 to 73.12°. Selected bond lengths and bond angles for molecule 3a are listed in Table 4.3. The coordinated water molecule forms H-bonds with each of the nearby coordinated oxygen atoms of the carboxylate groups (OH···O distance, 2.35 Å). The molecule has virtual C₂ symmetry, with a pseudo 2-fold symmetry axis passing through the midpoint of the C-C bond of the five-membered en chelate ring backbone, bisecting the Ti(IV) metal ion and the oxygen atom (the Ti(11)-O(1W1) bond) of the coordinated water molecule.

The asymmetric unit contains enantiomers of rac-[Ti(IV)(EHPG)(H₂O)] molecules, with trans phenolate groups. Molecule 3a, lies on a crystallographic 2-fold axis while molecule 3b occupies a general position in the asymmetric unit. There is extensive hydrogen bonding between the rac-[Ti(IV)(EHPG)(H₂O)] molecules, 3a and 3b, and solvent water molecules in the unit cell of complex 3.

**Hydrogen Bonding**

The water molecules of crystallisation in the crystal lattice form hydrogen bonds with both the carboxylate (bound and unbound) and phenolate oxygen atoms of molecule 3a. H-bonds are also formed between the NH protons of molecule 3b and the oxygen atoms of the water molecules of crystallisation in the crystal lattice. There is also intermolecular hydrogen bonding between the OH₂ group of one molecule and the uncoordinated carboxylate oxygen atoms of another molecule (OH₂···O; 1.85 Å from molecule 3a to 3b and 1.99 Å from molecule 3b to 3a). The water molecules of crystallisation also form intermolecular hydrogen bonds.
Table 4.3 Selected bond lengths (Å) and angles (°) for complexes 3a and 3b.

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<td>Ti(11)-O(1W1)</td>
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<td>Ti(22)-O(2W2)</td>
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<table>
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<th><strong>Angles (°)</strong></th>
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</tr>
</thead>
<tbody>
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</tr>
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<td>O(11)-Ti(11)-O(821#)</td>
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<td>O(11)-Ti(11)-O(1W1)</td>
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<td>N(92)-Ti(22)-N(122)</td>
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<td>144.05(6)</td>
<td>N(92)-Ti(22)-O(2W2)</td>
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</tbody>
</table>
In both molecules, 3a and 3b, the equatorial Ti-N amine bond lengths are the longest (average Ti-N, ca. 2.212 Å) followed by the equatorial Ti-OH₂ bond distance (average Ti-OH₂, ca. 2.084 Å). The equatorial Ti-O carboxylate bond lengths are slightly shorter than the Ti-OH₂ bond distance (average Ti-O, ca. 2.059 Å) and the axial Ti-O phenolate bond lengths are the shortest (average Ti-O, ca. 1.871 Å). The axial Ti-O phenolate bonds are ca. 0.2 Å shorter than the equatorial Ti-O carboxylate and coordinated water bond distances (average Ti-O, ca. 2.068 Å). The angle O-Ti-O between the two axial bonds deviates significantly from linearity (O-Ti-O angle, 171.22(14) and 168.24(10)° for 3a and 3b respectively). The two amine nitrogen atoms (Ti-N bonds) are cis due to the geometric requirements of the five membered en chelate ring.

The average bond angle in the pentagonal plane is 72.09° for both molecules, 3a and 3b, which is very close to the ideal angle of 72.0°. The six membered N-Ti-O phenolate chelate rings are in axial positions and have larger bond angles, as expected (average N-Ti-O angle, 82.33°).

The Ti-N amine and Ti-O carboxylate bonds, as well as the short axial phenolate Ti-O bonds are comparable in length to those reported previously for Ti(IV)-phenoxo complexes. The coordinated water molecule is slightly further away, with a bond length similar to those in the seven-coordinate aqua metal-EDTA complexes, such as [Ti(IV)(EDTA)(H₂O)] (Ti-OH₂, 2.084(2) Å), [Co(III)(EDTAH)(H₂O)] (Co-OH₂, 2.073(2) Å) and M[Fe(III)(EDTA)(H₂O)] (where M = Rb or Li and Fe-OH₂ is 2.106(11) and 2.107(3) Å, respectively).

The structure of the rac-Ti(IV)EHPG complexes, 3a and 3b, reveals a unique geometry and stereochemistry previously unobserved for crystalline metal-EHPG complexes. An interesting feature is the seven-coordinate geometry of rac-Ti(IV)EHPG as all the other structurally characterised metal-EHPG complexes reported (containing either rac or meso isomers of the EHPG ligand) are only six-coordinate. In the rac-Ti(IV)EHPG complexes the seven-coordinate pentagonal bipyramidal Ti(IV) ion is coordinated to two trans phenolate oxygen atoms in axial positions, whereas, all other metal-EHPG complexes have an octahedral or pseudo-octahedral geometry in which the carboxylate ligands are trans to each other and are located in the axial positions.
Rac-Ti(IV)EHPG is seven-coordinate with a water molecule as a co-ligand (Figure 4.5). Ti(IV) has empty 3d orbitals and an ionic radius of ca 0.68 Å. Similar to its 4d$^0$ and 5d$^0$ analogues Zr(IV) and Hf(IV), respectively, Ti(IV) is readily isolated in solvated form with the metal achieving seven-coordination, and several X-ray structures of seven-coordinate complexes have been reported. Examples include [Ti(IV)(EDTA)(H$_2$O)], and the Ti(IV)(THPED) dimer complex, ([Ti(IV)(THPED)$_2$]), where H$_4$THPED is N,N,N',N'-tetrakis-(2-hydroxypropyl)ethylenediamine). Fackler et al have suggested that a spherically symmetric charge distribution (e.g. d$^0$ and high spin d$^5$ electronic configurations) favours the pentagonal-bipyramidal geometry if the central metal ion is of sufficient size to tolerate a coordination number higher than 6, or if the ligand-field splitting does not greatly favour an octahedral arrangement. With its relatively large size, and a spherically symmetric charge distribution, Ti(IV) (d$^0$) can adopt coordination numbers from 2 to 11.

Hoard et al. have pointed out that for metal-EDTA complexes, as the size of the central metal ion increases and the M-N bond lengths increase, seven-coordination becomes favourable via the binding of a water molecule to fill the “hole” created by the expansion of the O-M-O bond angles trans to the M-N bonds of the five-membered en chelate ring. This also seems to be the case for metal-EHPG complexes. Table 4.4 compares data for available X-ray crystal structures of metal-EHPG complexes. It can be seen that Ti(IV) has a relatively large ionic radius (0.68 Å, only Cu(II), 0.72 Å is bigger), and that Ti(IV)EHPG complexes have the longest M-N bonds (> 2.2 Å), and therefore the smallest N-M-N five-membered chelate ring bond angles. Modelling studies reveal that increases in the M-N bond lengths of the five-membered en chelate ring lead to movement of the trans oxygen atoms connected to the nitrogens by chelate rings and to concomitant opening of the corresponding O-M-O bond angles trans to the M-N bonds. In the case of Ti(IV)EHPG complexes, the M-N bond lengths are long and the N-M-N bond angles of the five-membered en chelate ring are small (71-73°). Concomitant opening of the O-M-O bond angles trans to the M-N bonds is large enough (>143.5°) to encourage the coordination of a water molecule in the large hole thus formed. Therefore, it is understandable that Ti(IV) becomes seven-coordinate in these circumstances.
Table 4.4 Comparison of data for metal-EHPG complexes.

<table>
<thead>
<tr>
<th>Metal</th>
<th>d</th>
<th>Radius</th>
<th>C.N.</th>
<th>Geom</th>
<th>M-O</th>
<th>M-N</th>
<th>(\angle)NMN</th>
<th>(\angle)OMO</th>
<th>Ligand</th>
<th>Complex Chirality</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti(IV)</td>
<td>0</td>
<td>0.68</td>
<td>7</td>
<td>pent</td>
<td>1.976</td>
<td>2.229</td>
<td>72.94</td>
<td>143.50</td>
<td>meso</td>
<td>N(R,R)C(R,S) or N(S,S)C(S,R)</td>
<td>This work</td>
</tr>
<tr>
<td>Ti(IV)</td>
<td>0</td>
<td>0.68</td>
<td>7</td>
<td>pent</td>
<td>1.987</td>
<td>2.214</td>
<td>72.41</td>
<td>145.92</td>
<td>rac</td>
<td>N(R,R)C(R,R) or N(S,S)C(S,S)</td>
<td>7</td>
</tr>
<tr>
<td>Ti(IV)</td>
<td>0</td>
<td>0.68</td>
<td>7</td>
<td>pent</td>
<td>1.990</td>
<td>2.210</td>
<td>71.91</td>
<td>146.24</td>
<td>rac</td>
<td>N(R,R)C(R,R) or N(S,S)C(S,S)</td>
<td>7</td>
</tr>
<tr>
<td>Mn(III) (1)</td>
<td>4(H.S.)</td>
<td>0.65</td>
<td>6</td>
<td>oct</td>
<td>2.033</td>
<td>2.061</td>
<td>83.73</td>
<td>97.03</td>
<td>rac</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
<td>This work</td>
</tr>
<tr>
<td>Mn(III) (2a)</td>
<td>4(H.S.)</td>
<td>0.65</td>
<td>6</td>
<td>oct</td>
<td>1.985</td>
<td>2.100</td>
<td>82.43</td>
<td>111.53</td>
<td>meso</td>
<td>N(R,R)C(R,S) or N(S,S)C(S,R)</td>
<td>This work</td>
</tr>
<tr>
<td>Mn(III) (2b)</td>
<td>4(H.S.)</td>
<td>0.65</td>
<td>6</td>
<td>oct</td>
<td>2.028</td>
<td>2.012</td>
<td>85.23</td>
<td>97.04</td>
<td>rac</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
<td>This work</td>
</tr>
<tr>
<td>V(IV)</td>
<td>1</td>
<td>0.63</td>
<td>6</td>
<td>oct</td>
<td>1.894</td>
<td>2.208</td>
<td>78.20</td>
<td>103.24</td>
<td>rac</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
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</tr>
<tr>
<td>Fe(III)</td>
<td>5(H.S.)</td>
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<td>6</td>
<td>oct</td>
<td>1.977</td>
<td>2.166</td>
<td>79.86</td>
<td>105.51</td>
<td>rac</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
<td>24</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>5(H.S.)</td>
<td>0.64</td>
<td>6</td>
<td>oct</td>
<td>1.967</td>
<td>2.151</td>
<td>80.61</td>
<td>107.17</td>
<td>rac</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
<td>24</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>5(H.S.)</td>
<td>0.64</td>
<td>6</td>
<td>oct</td>
<td>1.967</td>
<td>2.157</td>
<td>79.17</td>
<td>112.66</td>
<td>meso</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
<td>24</td>
</tr>
<tr>
<td>Ga(III)</td>
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<td>0.62</td>
<td>6</td>
<td>oct</td>
<td>1.949</td>
<td>2.090</td>
<td>82.96</td>
<td>96.53</td>
<td>rac</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
<td>6</td>
</tr>
<tr>
<td>Cu(II)</td>
<td>9</td>
<td>0.72</td>
<td>6</td>
<td>oct</td>
<td>2.187</td>
<td>2.005</td>
<td>85.94</td>
<td>91.55</td>
<td>rac</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
<td>6</td>
</tr>
<tr>
<td>Co(III)</td>
<td>6(L.S.)</td>
<td>0.63</td>
<td>6</td>
<td>oct</td>
<td>1.907</td>
<td>1.938</td>
<td>86.59</td>
<td>87.14</td>
<td>rac</td>
<td>N(R,R)C(S,S) or N(S,S)C(R,R)</td>
<td>6</td>
</tr>
</tbody>
</table>

\(^a\) ionic radius (Å), \(^b\) Key: pent, pentagonal bipyramid; oct, octahedral, \(^c\) Average bond distance (Å), \(^d\) NMN angle (°), \(^e\) OMO angle trans to NMN angle (°).
The V(IV)EHPG complex \(^{16}\) also has long M-N bonds, but V(IV) has a smaller ionic radius than Ti(IV) and one of the oxygen ligands (V=O) in the \(\text{trans} \ O-V-O\) unit is not connected by a chelate ring, and thus the O-V-O angle is only \(\text{ca.} \ 103^\circ\)\(^{16}\), not large enough to accommodate a water molecule. An interesting feature of the vanadium complex is that although it is six-coordinate, only five of the six chelating atoms of the hexadentate EHPG ligand form bonds with the V(IV) ion. One of the phenolate oxygen atoms of the EHPG ligand is protonated in the complex and thus is not coordinated to the metal ion. An oxygen is coordinated instead in its place.

A similar analysis may also apply to the spherically symmetrical high-spin \(d^5\) Fe(III)EHPG complexes.\(^{15}\) Fe(III) has a medium ionic radius and the Fe(III)EHPG complexes have relatively long Fe-N bond lengths (\(\text{ca.} \ 2.16 \, \text{Å}\)) and as a result the \(\text{trans} \ O-Fe-O\) bond angles are in the range 105-112\(^\circ\). Although they are six-coordinate in the crystal lattice, optical spectroscopic properties suggest\(^3,^{13}\) that Fe(III)EHPG may be seven-coordinate in aqueous solution with an additional bound water ligand.

The binding of a water molecule in the equatorial plane \(\text{trans}\) to the Ti-N bonds can also be understood from the \(\text{trans}\) effect. Ti(IV), \(d^0\), is a “hard” metal ion and its affinity for nitrogen donors is weak, as evidenced from the long Ti-N bond lengths (>2.2 \, Å) in the Ti(IV)EHPG complex as well as other Ti(IV) complexes with N-donors.\(^{32,33,38}\) This causes a weak \(\text{trans}\) effects, i.e. the position \(\text{trans}\) to the Ti-N bonds has greater affinity for electron donors. If there is enough space, as in complexes 3a and 3b, it could accept an additional oxygen from a solvent molecule at the \(\text{trans}\) position, to become seven-coordinate. The preference for seven coordination exhibited by both the spherically symmetrical \(d^0\) Mg(II) and high spin \(d^5\) Fe(III) chelate complexes of EDTA,\(^{35,39}\) by the binding of a solvent (water) molecule \(\text{trans}\) to the M-N bonds, in the equatorial plane, have also been largely attributed, albeit indirectly, to the disproportionately low affinities of these cations for amine nitrogen relative to their affinities for carboxylate oxygen.\(^{39}\)

### 4.3.4 Comparison with other EHPG Complexes

The most striking difference between the meso and racemic isomers of the metal-EHPG complexes is the coordination geometry about the metal ion. Complexation of the metal ion by the racemic isomer occurs via \(\text{trans}\) carboxylate or \(\text{trans}\)
phenolate coordination, whereas in the meso species all chelating groups are mutually cis. This difference in coordination has a significant impact on the stability of the metal complexes of the two isomers.

Molecular mechanics calculations\textsuperscript{40} predict that optimal stability will be achieved when the EHPG ligands adopt an equatorial coordination plane defined by the five-membered en chelate ring, containing a 6,5,6 combination of chelate ring sizes, with two five-membered axial chelate rings. This corresponds to the (R,R) rac isomer. The 5,5,5 combination of chelate ring sizes in the equatorial coordination plane defined by the en chelate ring, with two six-membered axial chelate rings corresponds to the (S,S) rac isomer and is the least stable configuration. The (R,S) meso isomer has been assigned to the 5,5,6 combination of chelate ring sizes in the coordination plane defined by the en chelate ring, with a stability in between the two rac isomers ((R,R) rac and (S,S) rac isomer).

Table 4.4 compares the stereochemistry for reported X-ray crystal structures of metal-EHPG complexes. N(S,S)C(R,R) and N(R,R)C(S,S) ((R,R) rac, ARR and ASS) isomers, as in complexes 1 and 2b, have previously been found in X-ray crystal structures of Fe(III), Co(III), Ga(III), Cu(II) and V(IV) EHPG complexes. Molecular mechanics studies\textsuperscript{18} of Fe(III)EHPG complexes have shown that these are the most stable isomers, both in vacuo and in aqueous solution, while the least stable is the N(S,S)C(S,S) or N(R,R)C(R,R) ((S,S) rac, ASS or ARR) isomer. Stability constants for the (R,R) rac and (R,S) meso complexes of EHPG with Fe(III), Ni(II), Zn(II), Cu(II), Ga(III) and In(III) have been determined,\textsuperscript{9} and the results show that all the (R,R) rac isomers are more stable than the (R,S) meso isomer by 2-3 kcal/mol (8.4-12.6 kJ/mol).

For octahedral metal-EHPG complexes, greater stability has been proposed\textsuperscript{18,41} for (R,R) rac isomers due to the geometric selectivity in the placement of the two six-membered chelate rings (with their greater bite angle) in the equatorial plane defined by the five-membered ethylenediamine chelate ring and with two five-membered chelate rings in axial positions. In the (S,S) rac isomer, both of the six-membered chelate rings are in axial positions, which is a very strained arrangement. The structure of the (R,S) meso isomer, for which the only possible arrangement is the one with all coordinating groups cis to one another, is intermediate between the highly strained (S,S) rac arrangement and the most favourable (R,R) rac
arrangement. In the plane defined by the five-membered en chelate ring there is now only one six-membered phenolate chelate ring, the other is replaced by a five-membered carboxylate chelate ring. The only other reported X-ray crystal structure of a six-coordinate meso-EHPG metal complex is that of Fe(III),\textsuperscript{15,24} although a seven-coordinate aqua meso-Ti(IV)EHPG complex is known\textsuperscript{7}.

One of the most interesting features of the Ti(IV)EHPG complexes is the stereochemistry. Although X-ray crystal structures of EHPG complexes containing the N(S,S)C(R,R) and N(R,R)C(S,S) ((R,R) rac, ΔRR and ΔSS) isomers of Fe(III), Co(III), Ga(III), Cu(II), V(IV) and the (R,S) meso isomer of Fe(III) have previously been reported, there have been no previous reports of the crystal structure of an EHPG complex containing the N(S,S)C(S,S) or N(R,R)C(R,R) ((S,S) rac, ΔSS or ΔRR) isomer, as far as we are aware. The absence of the N(S,S)C(S,S) or N(R,R)C(R,R) ((S,S) rac, ΔSS or ΔRR) isomers in crystals has previously been attributed to unfavourable crystal packing,\textsuperscript{15,24} or to the inherent instability of the (S,S) rac isomer.\textsuperscript{6,18} Thus the presence of the (S,S) rac isomers (N(S,S)C(S,S) and its enantiomer N(R,R)C(R,R) as pairs in the unit cell) in crystals of 3 was unexpected. None of the preparations gave rise to N(S,S)C(R,R) or N(R,R)C(S,S) ((R,R) rac, ARR and ΔSS) isomers of Ti(IV)EHPG, which have always been observed for other metals studied so far and is the most stable isomer. The major factor in the stability of the metal-EHPG complex isomers, has been attributed to the concept of internal ring strain.\textsuperscript{18}

The unusual N(S,S)C(S,S) or N(R,R)C(R,R) ((S,S) rac, ΔSS or ΔRR) chirality of rac-Ti(IV)EHPG in 3 can be understood by considering the factors leading to metal complex stability. The two major factors are ligand basicity (i.e. the sum of the log protonation constants) and coordination geometry.\textsuperscript{9} Experimental results reported previously have suggested that the rac and meso isomers of the EHPG ligand have the same basicities (rac 38.0 and meso 37.9).\textsuperscript{9} Therefore it is the coordination geometry that is likely to play a major role in the stability of the metal-EHPG complexes. Since the bite angle of a six-membered chelate ring\textsuperscript{6,40} is about 90-95° and substantially greater than that of a five-membered chelate ring (ca 70-80°),\textsuperscript{6,40} more satisfactory octahedral coordination is achieved when both six-membered chelate rings are bound to the metal in the equatorial plane that contains the five-membered ethylenediamine chelate ring. This is the case for the (R,R) rac isomer in most of the reported metal-EHPG complexes. For the rac-Ti-EHPG complexes,
Chapter 4

Mn(III) and Ti(IV) Complexes of EHPG

Ti(IV) is seven coordinate with a pentagonal bipyrimidal geometry, which will be very strained if any of the six-membered chelate rings bound to the metal ion are in the pentagonal plane. More satisfactory pentagonal bipyrimidal coordination is achieved with the six-membered chelate rings in the axial positions, \textit{i.e.} the (S,S) \textit{rac} configuration, which results in [Ti(EHPG)(H$_2$O)$_3$]$\cdot$1113H$_2$O crystallising as the N(S,S)C(S,S) and N(R,R)C(R,R) ((S,S) \textit{rac}, ASS or ΔRR) isomers.

4.3.5 UV-Vis Spectra

The electronic absorption spectra of aqueous solutions of both Mn(III)EHPG complexes 1 and 2 and the \textit{rac}-Ti(IV)EHPG complex 3 are dominated by moderately intense charge-transfer bands in the visible region.\(^5\)

Complex 1 (\textit{rac}-Mn(III)EHPG) exhibits bands at 345 nm and 423 nm with a shoulder at 480 nm, whereas the bands for complex 2 (\textit{rac},meso-Mn(III)EHPG) at 351 nm and 437 nm are slightly shifted to higher wavelength. The band at 480 nm in complex 1 (\textit{rac}) has been assigned\(^4\) as a \textit{d}$\rightarrow$\textit{d}, $^5$E$_g$ $\rightarrow$ $^5$T$_{2g}$ transition, although the complex is not strictly octahedral on account of the Jahn-Teller distortion. High-spin d$^4$ Mn(III) complexes usually display a visible absorption band due to a spin allowed and Laporte forbidden, \textit{d}$\rightarrow$\textit{d} transition. The intensity of the \textit{d}$\rightarrow$\textit{d} transition is weak compared to the intensity of a charge-transfer band. Hence the weak \textit{d}$\rightarrow$\textit{d} band at 480 nm observed for complex 1 (\textit{rac}), is probably also present for complex 2 (\textit{rac},meso) but is most probably hidden by the broad intense charge-transfer absorption band at 437 nm.

The \textit{rac}-Ti(IV)EHPG complex (3) exhibits two characteristic UV-Vis absorption bands, one below and one above 300 nm (at 290 nm and 386 nm, respectively). The higher energy band of \textit{rac}-Ti(IV)EHPG at $\lambda$ = 290 nm is due to internal ligand transitions, whereas the visible absorption band at 386 nm can be assigned as a ligand-to-metal charge-transfer (LMCT) band\(^5\) and is the origin of the orange colour of the d$^0$ complex. The absorption of light in the visible region of the electromagnetic spectrum by phenolate complexed to a metal ion is well established. The two higher energy absorption bands observed for the Mn(III)EHPG complexes can also be assigned as LMCT transitions.\(^5\) Similar LMCT bands in the visible region have been observed for the spherically symmetrical Fe(III)EHPG (high-spin d$^5$) and VO-EHPG (d$^0$) complexes and assigned to transitions from a πt orbital on
the bound phenolate oxygen to half-filled d-orbitals on Fe(III) or empty d-orbitals on V(V), respectively.\textsuperscript{2,3,5,10} The LMCT bands for complexes 1 and 2 can therefore be attributed to a LMCT transition from a \( p\pi \) orbital of the phenolate oxygen atoms to partially-filled Mn(III) d-orbitals. Similar absorption bands have also been observed for other Mn(III) complexes with phenolate ligands, and assigned to LMCT transitions.\textsuperscript{20,22}

Such transitions are characteristic of phenolate coordination to all easily reducible metal ions. Similar bands have also been observed for other phenoxyo Ti(IV) complexes, and assigned to LMCT transitions.\textsuperscript{42} The visible bands for complex 3 can therefore be attributed to LMCT transition from a \( p\pi \) orbital of the phenolate oxygen atoms to empty Ti(IV) d-orbitals. A similar broad band at 300 to 400 nm has been observed for Ti(IV)-transferrin.\textsuperscript{17} Similar UV-Vis absorption bands have also been reported for the \textit{meso}-Ti(IV)EHPG dimer complex.\textsuperscript{7}

The higher energy LMCT band observed for other Mn(III) complexes with phenolate ligands, has been assigned to \( p\pi \rightarrow d\sigma^* \) transitions and the lower energy LMCT bands to \( p\pi \rightarrow d\pi^* \) transitions from a \( p\pi \) orbital of the phenolate oxygen atoms to a partially-filled Mn(III) d-orbital.\textsuperscript{22} Hence the lower energy relatively intense LMCT visible absorption bands at ca. 423 nm for 1 and 437 nm for 2 can be assigned as \( p\pi \rightarrow d\pi^* \) charge transfer transitions and the higher energy intense LMCT absorption bands at 345 nm and 351 nm, for 1 and 2 respectively, to \( p\pi \rightarrow d\sigma^* \) transitions from a \( p\pi \) orbital of the phenolate oxygen atoms to a partially-filled Mn(III) d-orbital. Such \( p\pi \rightarrow d\pi^* \) and \( p\pi \rightarrow d\sigma^* \) transitions, of similar energies and intensities have been previously reported for other Mn(III) complexes with phenolate ligands.\textsuperscript{20,22}

The LMCT band at 437 nm for the Mn(III) \textit{meso},\textit{rac} complex 2, is slightly red-shifted compared with that for the \textit{rac} complex 1 (423 nm). A similar shift is observed for the \textit{meso} and \textit{rac}-Fe(III)EHPG complexes. \textit{Rac}-Fe(III)EHPG exhibits a band at 475 nm (\( \varepsilon = 4450 \text{ M}^{-1} \text{ cm}^{-1} \))\textsuperscript{2} whereas \textit{meso},\textit{rac}-Fe(III)EHPG has a band at the slightly higher wavelength of 480 nm (\( \varepsilon = 4300 \text{ M}^{-1} \text{ cm}^{-1} \)).\textsuperscript{3} For Fe(III) the pure \textit{meso} complex has also been isolated and exhibits a band at 486 nm (\( \varepsilon = 4105 \text{ M}^{-1} \text{ cm}^{-1} \)).\textsuperscript{3} Therefore the spectrum of the \textit{meso},\textit{rac} complex is the sum of bands for the individual diastereomers. On this basis, \textit{meso}-Mn(III)EHPG would be expected to exhibit an absorbance maximum at ca. 450 nm, \textit{i.e.} shifted to higher wavelength compared to the \textit{meso},\textit{rac} complex 2. Patch \textit{et al.} have reported\textsuperscript{4} an absorbance
maximum of 427 nm for rac-MnEHPG, but 432 nm for meso-MnEHPG, although they did not confirm the stereochemistry of their complexes by X-ray crystallography.

Mn(III) human serum transferrin exhibits two (broad) bands at 330 and 430 nm which have been assigned to phenolate-to-metal charge-transfer transitions. Although the X-ray structure of Mn(III)-transferrin has not been determined, by analogy with Fe(III), the Tyr ligands are likely to be trans to oxygen ligands from carbonate and Asp. This situation is not mimicked in either complex 1 or 2 in which one or both of the phenolate O atoms are trans to N. The apparent difference in the distortion probably also contributes to the positions of the LMCT bands. The LMCT band of rac-Mn(III)EHPG is more similar to that of Mn(III)-transferrin than that of the meso complex.

4.3.6 Solution Studies of the Rac-Mn(III)EHPG Complex (1)

**pH Dependence**

UV-Vis spectra of the rac-Mn(III)EHPG complex (1) at different pH values were recorded in aqueous 0.1 M NaCl solutions. The pH was either lowered or raised using microlitre aliquots of standard aqueous solutions of 0.1 M HCl or HNO₃, respectively. The binding and release of Mn(III) was monitored by the increase or decrease in absorbance at 345 and 423 nm.

Electronic absorption spectra of complex 1 (rac) over the pH range 1.9 to 11.5 in (0.1 M NaCl) aqueous solutions are shown in Figure 4.6. There are three bands at pH 7.5, one relatively weak d→d band at 480 nm and two relatively intense visible phenolate-to-Mn(III) LMCT bands at 351 and 423 nm.

As can be seen from the LMCT bands in Figure 4.6, complex 1 is more stable at low pH than at high pH. Complex 1 is stable between pH 3.4 and 7.5. Thus Mn(III) is stabilised over biologically relevant pH values, 3-8. At pH 2.5 most of the absorbance at 351 and 423 nm is lost and by pH 1.9 the LMCT bands have almost completely disappeared, corresponding to the loss of Mn(III)-phenolate bonding (and protonation of the ligand at low pH). This is consistent with the complete loss of
colour observed, with the appearance of a new absorption band, as a result of the decomposition of the \textit{rac}-Mn(III)EHPG complex.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4_6.png}
\caption{UV-Vis spectra of an aqueous solution of compound 1, \textit{rac}-Mn(III)EHPG, showing the LMCT bands at various pH values: (A) 7.5-1.4; (B) 7.5-11.5. Concentration, $5.2 \times 10^{-4} \text{ M}$, $\varepsilon_{423} = 810 \text{ M}^{-1} \text{ cm}^{-1}$, $\varepsilon_{345} = 1930 \text{ M}^{-1} \text{ cm}^{-1}$, pH 7.5 (298 K, $\mu = 0.1 \text{ M NaCl}$).}
\end{figure}
Upon raising the pH value of the solution the LMCT bands appear to increase in intensity. The LMCT absorption band at 345 nm at pH 7.4 appears to shift to lower energy whilst also concomitantly increasing in intensity upon raising the pH value of the solution. At pH 11.5 the absorption band has shifted (17 nm) to 362 nm. Formation of a brown precipitate occurred in the solution at pH 11.5. No loss of colour was observed. At high pH there is hydroxide attack followed by the formation of a mixed valence complex, although this has not been conclusively proved. In oxidation states III or IV, Mn, like Fe(III) and Cr(III), readily forms clusters related to simple hydroxide or oxide chemistry. The three elements can also form clusters in mixed oxidation states. The exact formulation of many such complexes is now known from X-ray diffraction studies; it is found that oxo groups can bridge two or three Mn atoms in many types of clusters, including cubane and adamantane forms. The Mn(III) in these clusters may then disproportionate resulting in Mn(II) species and oxo-species of MnO(IV).

$^1H$ NMR Spectra

The paramagnetic $^1H$ NMR spectrum of rac-Mn(III)EHPG, complex 1, in D$_2$O is shown in Figure 4.7. Three broadened and isotropically upfield or downfield shifted proton resonances are observed.

![Figure 4.7. $^1H$ NMR spectrum of a 5.65 mM solution of complex 1 (rac-Mn(III)EHPG) in D$_2$O. The proton labelling scheme is shown in Figure 4.1.](image)
Two relatively sharp peaks are observed, one upfield at −33.1 ppm (2H), and the other downfield at +26.2 ppm (2H). The third isotropically-shifted resonance is broader and is observed upfield at −18.0 ppm (2H). All three isotropically-shifted resonances can be assigned to the phenyl ring protons.

The chemical shifts of the resonances of the phenyl protons of complex 1 (rac-Mn(III)EHPG) in D₂O are very similar to those observed by Li and Pecoraro for their rac-Mn(III)EHPG preparation in MeOH and DMF.⁴⁶ Thus the isotropically-shifted phenyl proton resonances can be assigned by analogy. The two upfield shifts at −33.1 and −18.0 ppm can be assigned to H₄ and H₆, respectively, and the downfield shifted resonances at +26.2 ppm to H₅. The resonance due to the H₃ proton which is ortho to the Mn-O phenolate bond is not observed. This is also the case for the Mn(III) complexes with Schiff base-type ligands.⁴⁷ This may be due to its close proximity to the Mn(III) ion, which results in broadening and/or shifting of the H₃ proton resonance beyond the frequency width used. Additionally in the ¹H NMR spectrum of the rac-Mn(III)EHPG complex (1), two further resonances are observed in the diamagnetic region at +1.6 and +3.2 ppm, which have previously not been reported. The sharp peak at +3.2 ppm (2H) is most probably due to the α-C proton. Li and Pecoraro have reported⁴⁶ a ¹H NMR peak at −2.4 ppm for meso-Mn(III)EHPG, due to the α-C proton. The chemical shifts and assignments are summarised in Table 4.5.

### Table 4.5 ¹H NMR chemical shifts of rac-Mn(III)EHPG, complex 1.

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical shift (ppm)ᵃ</th>
<th>Line width, Δν½ (Hz)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₄</td>
<td>−33.1</td>
<td>ca. 650</td>
</tr>
<tr>
<td>H₅</td>
<td>+26.2</td>
<td>ca. 340</td>
</tr>
<tr>
<td>H₆</td>
<td>−18.0</td>
<td>ca. 2000</td>
</tr>
<tr>
<td>α-CH</td>
<td>+3.2</td>
<td>ca. 20</td>
</tr>
<tr>
<td>unassigned</td>
<td>+1.6</td>
<td>ca. 250</td>
</tr>
</tbody>
</table>

ᵃ All shifts are relative to TSP, with downfield shifts as negative. ᵇ Errors are large (± 10%) since peaks are broad.
The rac-Mn(III)EHPG complex (1), gives rise to isotropically-shifted ligand proton NMR resonances due to the presence of the paramagnetic high-spin d⁴ Mn(III), S = ⁵⁄₂ centre. Although the lines are broad, the chemical shifts are quite large, so that a relatively well-resolved spectrum is observed. Broad resonances that are shifted both upfield and downfield were observed. These features are the result of the mechanism of spin delocalisation, which gives rise to large chemical shifts for the phenolate protons. Relaxation was too fast to allow observation of 2D spectra.

For metals of the appropriate electronic spin relaxation time, the chemical shift pattern of |δ(o-H)| > |δ(m-H)| > |δ(p-H)| with alternating upfield and downfield shifts is observed when spin delocalisation into a π molecular orbital system occurs primarily via a scalar mechanism. Such a pattern is observed for Fe(III)(salen)X. A similar pattern of alternating upfield and downfield resonances is observed for complex 1 (rac-Mn(III)EHPG) and the Mn(III)EHPG complexes previously reported. Li and Pecoraro have previously reported the ¹H NMR shifts of the isotropically-shifted phenyl ring protons of Mn(III)EHPG complexes of both meso and rac isomers (in both MeOH and DMF).

In general, both dipolar (through space) and contact (through bond) mechanisms are expected to contribute to the isotropic shifts observed in the ¹H NMR spectra of transition metal complexes, and neither mechanisms can be neglected a priori in the case of high-spin d⁴ Mn(III).

In the case of the Fe(III)(salen)X complexes, the dipolar contribution is negligible because of the near-spherical electronic symmetry of the high spin d⁵ Fe(III) ion and the paramagnetic shifts observed are predominantly, if not solely contact in origin; i.e. they arise from the delocalisation of unpaired spin density onto the salen phenolate ligands. This interaction can transfer only down-spin from the ligand to Fe(III), since each metal orbital is singly occupied. It is reasonable to assume that the shifts observed in the rac-Mn(III)EHPG complex are dominated by the contact component, given the large distances between the H4 and H5 atoms and the Mn(III) centre, and the 1/r³ distance dependence of the dipolar mechanism. This expectation is supported by a lack of significant curvature in the Curie temperature dependence of the spectra over 20-60°C. However, analysis of the spectra of the Mn(III) complex in terms of a contact mechanism is complicated by the possibility for transfer of up or down-spin density from the ligand to Mn(III), by the possible...
existence of $\pi$ and $\sigma$ delocalisation mechanisms of similar absolute magnitude for the H3 and H6 atoms. Delocalisation involving transfer of up-spin from the phenolate to Mn(III) would be expected to give alternating shifts that are opposite in sign from those found in the Fe(III)Schiff base complexes.

The $^1$H NMR spectra of Mn(III)(salen)X complexes also do not show isotropically shifted resonances due to the ethane backbone. The considerable line broadening of the H6 resonance at -18 ppm is consistent with increased dipolar line broadening due to closer proximity to the Mn(III) centre. The chemically equivalent protons are also magnetically equivalent and therefore give rise to a single set of peaks.

The observation of three sets of two approximately equal intensity resonances observed in the $^1$H NMR spectrum of meso-Mn(III)EHPG by Li is consistent with the X-ray crystal structure of the meso-Mn(III)EHPG complex 2a. The chemically equivalent protons are no longer magnetically equivalent (as in rac-Mn(III)EHPG, complex 1) and thus interact with the magnetic field differently and experience different shifts.

In the temperature dependent NMR experiments on complex 1 (rac-Mn(III)EHPG) all signals shift upon changing the temperature (Figure 4.8). The observed isotropic shifts of the rac-Mn(III)EHPG resonances are plotted verses $T^{-1}$ in Figure 4.9. The Curie law applies to such systems if the contact effects are of major importance and therefore straight-line plots should be obtained for the isotropic shifts versus $1/T$ plots. As shown in Figure 4.9, the Curie law does apply to compound 1 between 20° and 60°C. From these measurements it can be inferred that the contact shift mechanism dominates for this system and not dipolar contributions. These findings are in agreement with the conclusions drawn for other Mn(III) complexes.
Figure 4.8 Temperature dependence of the signals in the $^1$H NMR spectra of a 5.65 mM solution of compound 1 (rac-Mn(III)EHPG) in D$_2$O. The pH* of the dark yellow solution was 7.4. $^1$H NMR spectra were recorded over a large sweep width (100 to -100 ppm) due to the paramagnetic nature of the complex. The measurements were made between 20°C and 60°C, in 10°C increments.
Figure 4.9 Plot of isotropic $^1$H NMR shift verses $T^{-1}$ for rac-Mn(III)EHPG resonances. Linear relationships are found indicating that the Curie law is obeyed and that the shifts are essentially contact in origin.

### 4.3.7 Solution Studies of the Rac-Ti(IV)EHPG Complex (3)

**NMR of Complex 3**

Solution state $^1$H NMR studies of rac-Ti(IV)EHPG, complex 3, allowed comparison of some of the structural features of the solid-state with those in solution. Peak assignments and connectivities were established by 2D NMR techniques. 1D and 2D $^1$H NMR studies indicate that the structure of the rac-Ti(IV)EHPG complex (3) in solution is similar to that in the solid state. The 2D COSY and 2D NOESY NMR spectra of complex 3 in D$_2$O solution, pH 6.5, are shown in Figure 4.10A and Figure 4.10B, respectively.
Figure 4.10 (A) 2D COSY and (B) 2D NOESY (800 ms mixing time) spectra of complex 3, rac-Ti(IV)EHPG, in D$_2$O, pH $^*$ 6.5, at 308 K, which establish the NMR resonance assignments. Strong NOE peaks are observed for the phenyl protons, NCH$_2$CH$_2$N protons and for $\alpha$-CH to NCH$_2$CH$_2$N and for $\alpha$-CH to H$_6$. For labelling system, see Figure 4.1; a, axial protons; e, equatorial protons.

The chemical shifts and assignments are summarised in Table 4.6, together with the data for the rac isomer of the EHPG ligand. Both upfield and downfield shifted signals were observed for the rac-Ti(IV)EHPG complex compared with the corresponding ligand. The $^1$H NMR spectrum of complex 3, consists of four signals for the phenyl protons centred at 6.73(d)(2H), 7.08(t)(2H), 7.40(t)(2H) and 7.40(d)(2H) which can be assigned to H$_3$, H$_5$, H$_4$, and H$_6$, respectively. The signals due to the phenyl protons H$_6$(2H) (doublet) and H$_4$(2H) (triplet) are superimposed on top of each other and therefore the signal appears as a multiplet in the spectrum at 7.40 ppm (4H). There are also two quasi-quartets due to the ethylenediamine protons at 3.01 and 2.66 ppm (4H) and a singlet at 4.86 ppm (2H) which can be assigned to the $\alpha$-H's on the chiral carbons. The two deceptively simple 4-line patterns centred at 3.01 and 2.66 ppm (4H) represents an A$_2$B$_2$ system, indicating a gauche conformation of the en portion of the EHPG ligand.
Table 4.6 Comparison of the $^1$H NMR chemical shifts ($\delta$) of complex 3, rac-Ti(IV)EHPG, and the rac isomer of the EHPG ligand, in D$_2$O at ca. pH$^*$ 1.4, at 298K. For atom labels see Figure 4.1. $\Delta\delta$ = coordination shift (ppm).

<table>
<thead>
<tr>
<th></th>
<th>Rac-EHPG</th>
<th>Complex 3</th>
<th>$\Delta\delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH$^*$</td>
<td>1.38</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-H</td>
<td>5.08(s)</td>
<td>4.86(s)</td>
<td>-0.22</td>
</tr>
<tr>
<td>methylene</td>
<td>3.43(m),</td>
<td>3.01(d),</td>
<td>-0.42</td>
</tr>
<tr>
<td></td>
<td>3.21(m)</td>
<td>2.66(d)</td>
<td>-0.55</td>
</tr>
<tr>
<td>phenol H3</td>
<td>6.96(d)</td>
<td>6.73(d)</td>
<td>-0.23</td>
</tr>
<tr>
<td>H4</td>
<td>7.41(t)</td>
<td>7.40(t)</td>
<td>-0.01</td>
</tr>
<tr>
<td>H5</td>
<td>7.00(t)</td>
<td>7.08(t)</td>
<td>+0.08</td>
</tr>
<tr>
<td>H6</td>
<td>7.28(d)</td>
<td>7.40(d)</td>
<td>+0.12</td>
</tr>
</tbody>
</table>

* Negative values are for resonances that have shifted upfield upon Ti(IV) coordination.

The phenolate-Ti(IV) coordination of the rac isomer causes a relatively large upfield shift of the H3 resonance (ca. 0.2 ppm), but downfield shifts for H6 (ca. 0.12 ppm), whereas H5 and H4 peaks are only slightly shifted (< 0.08 ppm).

As expected, only one single peak was observed for the two $\alpha$-Hs in complex 3 (rac-Ti(IV)EHPG), indicating that the two $\alpha$-Hs are magnetically equivalent. The $\alpha$-Hs of complex 3 gave rise to a peak at 4.86 ppm, which was not affected by pH over the range 1.0-7.0, in contrast to the large chemical shift changes of the $\alpha$-Hs of the free ligands due to the protonated and deprotonated forms.

**pH Stability of Complex 3**

The effect of pH on the stability of rac-Ti(IV)EHPG (complex 3) in D$_2$O and aqueous solutions, was studied using $^1$H NMR and UV-Vis spectroscopy, respectively. The pH was either lowered or raised using µl aliquots of a standard aqueous solution of 0.1 M HCl and NaOH, respectively and the pH$^*$ was adjusted using either DNO$_3$ or NaOD (0.1 M) solutions. All spectra were recorded at 298 K.
Figure 4.11 The UV-Vis spectra of aqueous solution of rac-Ti(IV)EHPG, complex 3, showing the LMCT bands at various pH values. (A) pH 0.73-4.12; (B) pH 4.12-8.06. Concentration, $3.08 \times 10^{-5}$ M, $\varepsilon_{386} = 9,600$ M$^{-1}$cm$^{-1}$, pH 4.1 (298 K, $\mu = 0.1$ M, 0.1 M NaCl).

The electronic absorption spectra of complex 3 (rac-Ti(IV)EHPG) over the pH range 0.7 to 8.0 in aqueous (0.1 M NaCl) solutions are shown in Figure 4.11. The pH was lowered using $\mu$l aliquots of a standard solution of 0.1 M HCl to pH 0.7 and raised using $\mu$l aliquots of a standard solution of 0.1 M NaOH to pH 8.06. UV-Vis spectra were recorded at various intermediate pH values as well as at both pH limits. There are two bands at low pH, one below and one above 300 nm. Complex 3 exhibits strong visible phenolate-to-Ti(IV) charge-transfer absorption bands near 387 nm.

As can be seen from the LMCT bands in Figure 4.11, complex 3 (rac-Ti(IV)EHPG) is more stable at low pH than at high pH. Complex 3 is stable between pH 1.0 and 6.5. At pH 7.0, ca. 40% of the absorbance at 386 nm is lost, and by pH > 8.0, the LMCT band has nearly disappeared. The loss of colour and the decrease in intensity of the LMCT band is indicative of the loss of the Ti(IV)-phenolate bond.
Figure 4.12 $^1$H NMR spectra of a 6.6 mM solution of rac-Ti(IV)EHPG, complex 3, in $D_2O$ at different pH$^*$ values. When crystals of 3 were dissolved in $D_2O$ the pH$^*$ was 6.8. This was then subsequently adjusted using either 0.1 M NaOD or DNO$_3$. For labelling scheme see Figure 4.1.
The effect of pH on the stability of the rac-Ti(IV)EHPG complex (3), at different pH* values was further investigated by ¹H NMR (Figure 4.12). The pH* of the solution in D₂O was lowered using 0.1 M DNO₃ solutions to pH 1 and then raised to pH 8.23 using 0.1 M NaOD solutions. NMR spectra were recorded at various intermediate pH* values as well as at both pH* limits. Complex 3 was stable over the range pH* 1 to 7, but below pH* 1.0, dissociation occurred slowly to give a colourless solution, the ¹H NMR spectrum being consistent with the appearance of signals due to the free ligand. However, at pH* ≥ 7.5, many new peaks appeared in both the aromatic and aliphatic regions of the ¹H NMR spectrum, which is indicative of the formation of new species, which are probably hydrolysis products of the rac-Ti(IV)EHPG complex, resulting from the high pH* of the solution. Thus rac-Ti(IV)EHPG decomposes at alkaline pH*.

These results are in agreement with the UV data. No attempt was made to identify all the decomposition products.

Kinetic Studies of Reactions in Solution

The reaction solutions were composed of a mixture of racemic and mesomeric ligands.

The interaction between Ti(IV)citrate and EHPG was followed by ¹H NMR spectroscopy in D₂O at pH* 7.1. The appearance of new peaks in the ¹H NMR spectrum of the solution (Figure 4.13) is indicative of the formation of new species. At pH* 7.1, the citrate ligands were readily replaced by EHPG. At this pH* only the racemic isomer of the ligand binds Ti(IV), as can been seen from the decrease in intensity of the α-H NMR peak due to the racemic isomer of the ligand. Peaks due to the rac-Ti(IV)EHPG complex (3) can be identified clearly in the ¹H NMR spectrum of the solution. No change is seen in the peak due to the α-H of the meso isomer of the ligand, which indicates that it does not bind. The formation of the rac-Ti(IV)EHPG complex is accompanied by an increase in colour intensity. The increase in intensity of the two deceptive quasi-quartets at 2.99 and 2.64 ppm (NCH₂CH₂N), the doublet at 6.72 ppm (H3), the triplet at 7.06 ppm (H5) and the multiplet at 7.39 ppm (H4 and H6) is indicative of the gradual formation of the rac complex 3 with t₁/₂ of ca. 30 min at 310 K.
Figure 4.13 Kinetics of the reaction between Ti(IV)citrate and EHPG in D₂O at pH 7.1, 310 K followed by ¹H NMR spectroscopy, showing the gradual formation of complex 3, rac-Ti(IV)EHPG. Peak labels: m and r, meso and rac isomers of the free ligand H₄EHPG. For peak labelling see Figure 4.1.
Reactions of EHPG with Ti(III)citrate were also studied. This gave rise to exactly the same spectra as that from the reaction of Ti(IV)citrate with EHPG, and it can be concluded that oxidation of Ti(III) to Ti(IV) (presumably due to O₂ from air) occurred readily.

**Stoichiometry**

The interaction between Ti(IV)citrate and EHPG was followed by \(^1\)H NMR spectroscopy in D₂O by the sequential addition of 0.2 molar equivalents of Ti(IV)citrate to solutions of EHPG. Since, titanium transfer from citrate to EHPG displayed slow reaction kinetics on the NMR time-scale, the spectra were recorded at 30 min intervals. The appearance of new peaks in the \(^1\)H NMR spectrum of the solution is indicative of the formation of new species. The NMR spectra produced by the sequential addition of aliquots of Ti(IV)citrate to EHPG shows that binding occurs in a 1:1 ratio (Figure 4.14).

The peaks due to the α-H of both *meso* and *rac* isomers of the ligand are easily identified. Decreases in intensity of the α-H NMR peaks due to both the *meso* and *rac* isomers of the EHPG ligand are indicative of the formation of Ti(IV)EHPG complexes of both *meso* and *rac* isomers at this pH*. At pH* 7.4, the citrate ligands were readily replaced by EHPG. Although peaks due to both isomers are seen to decrease in intensity simultaneously, the peaks due to the *rac* isomer decreases more and faster than those for the *meso* isomer which indicates preference of binding of Ti(IV) to the *rac* isomer of EHPG. Thus there is more favourable formation of the *rac* complex.

Peaks due to the formation of the *rac*-Ti(IV)EHPG complex (3) can be identified clearly in the spectra. The NCH₂CH₂N peaks of the *rac*-Ti(IV)EHPG complex that forms are hidden under the citrate peaks. The formation of the *rac*-Ti(IV)EHPG complex is accompanied by an increase in colour intensity. The increase in intensity of the two deceptive quasi-quartets at 2.99 and 2.64 ppm (NCH₂CH₂N), the doublet at 6.72 ppm (H3), the triplet at 7.06 ppm (H5) and the multiplet at 7.39 ppm (H4 and H6) is indicative of the gradual formation of the *rac* complex 3, upon addition of aliquots of Ti(IV)citrate to the reaction mixture.
Figure 4.14 $^1$H NMR spectra generated by titrating aliquots of a solution of Ti(IV)citrate into a solution of EHPG in different ratios, at pH* 7.4. Peak labels: cit, citrate; m and r, meso and rac isomers of the free ligand EHPG. For peak labelling see Figure 4.1. Free citrate appears as an AB quartet centred at ca. 2.6 ppm.
A number of other new peaks are also seen to appear in the $^1$H NMR spectra upon addition of aliquots of Ti(IV)citrate, which have not been previously observed and cannot be attributed to the rac-Ti(IV)EHPG complex (3), and therefore are most probably due to other diastereomeric complexes of Ti(IV)EHPG.

Although peaks characteristic of the rac-Ti(IV)EHPG complex (3) were observed, no NMR peaks characteristic of the meso dinuclear oxygen-bridged complex were observed during the reaction, suggesting that the meso ligand forms other types of complexes under these conditions. No further attempts were made to identify them.

No further increase in the intensities of the product peaks was observed upon increasing the molar ratio of EHPG to Ti(IV)citrate from 1:1 to 1:1.2. The binding ratio is close to 1:1 for both isomers.

**EXSY**

The 2D $^1$H NMR EXSY spectrum generated for a solution of rac-Ti(IV)EHPG, complex 3, at pH* 8.1 is shown in *Figure 4.15*.

Three sets of exchange cross-peaks were clearly present in this spectrum. One cross-peak in the EXSY spectrum is found between protons with chemical shifts of 5.88 ppm and 4.21 ppm. The system exhibits two site exchange and so the rate of exchange can be calculated. Two mixing times were used to obtain such spectra, 300 ms and 500 ms, and from these $k_{ex}$ was calculated to be $7 \pm 1$ s$^{-1}$. The data therefore show that at this pH complex 3 is partly converted into a second species which can undergo slow exchange with complex 3.

No further attempt was made to identify this product.
Figure 4.15 2D EXSY $^1\text{H}$ NMR spectrum of a solution of rac-Ti(IV)EHPG, complex 3, in D$_2$O at pH$^*$ 8.1.
Chapter 4

4.4 Conclusions

In this chapter I have studied some new chemistry of Mn(III) and Ti(IV) complexes of the ligand EHPG as models for metal-transferrin complexes.

I crystallised for the first time rac-Na[Mn(EHPG)].3H₂O (complex 1) which contains N(S,S)C(R,R) stereogenic centres. It has a distorted octahedral geometry and two long axial Mn-O bonds due to Jahn-Teller distortion as expected for a 3d⁴ ion. A rac,meso isomer Na[Mn(EHPG)].H₂O was also crystallised. This was also Jahn-Teller distorted but now with one long Mn-O (carboxylate) bond and one long axial Mn-N (amine) bond.

The Ti(IV) complex rac-[Ti(EHPG(H₂O)].11/3H₂O was also crystallised. This contains N(S,S)C(S,S) stereogenic centres. Ti(IV) in this complex is seven-coordinate with a distorted pentagonal bipyramidal geometry, a geometry and stereochemistry previously unrecognised for metal-EHPG complexes.

The electronic absorption spectra of Mn(III)EHPG and Ti(IV)EHPG showed moderately intense ligand-to-metal charge-transfer bands in the visible region at similar wavelengths to those observed for Mn(III) and Ti(IV) complexes of human serum transferrin.

Titration of rac-Mn(III)EHPG with pH showed that the complex was stable over the range pH 3-8. Therefore if the stability of Mn(III)-transferrin is similar, then if transferrin delivers Mn(III) to cells, release of Mn(III) inside the cell may require the presence of a chelating agents such as ATP. Rac-Ti(IV)EHPG was stable in aqueous solution in the range pH 1-6.5, and again release of Ti(IV) from transferrin inside cells may require the assistance of a chelating agent.

Due to the paramagnetic nature of the high-spin d⁴ Mn(III) ion and relatively short electron spin–lattice relaxation times, paramagnetically shifted ¹H NMR peaks with relatively large isotropic shifts of up to ca. ± 35 ppm were observed for rac-Mn(EHPG), complex 1, and such shifts may be useful for observing binding of Mn(III) by transferrin.
The transfer of Ti(IV) from citrate to EHPG in aqueous solution was studied by $^1$H NMR spectroscopy. The reactions were complicated and pH dependent. The half-life of the reaction between Ti(IV)citrate and EHPG was determined to be approximately 30 min. There appeared to be preferential complexation of Ti(IV) to the rac isomer of EHPG at pH* 7.1. 2D NMR exchange studies showed that two species existed in aqueous solution at pH* 8.1 in slow exchange.

Since Ti(IV) binds strongly to EHPG, and to transferrin, transferrin may play a role in the delivery of Ti(IV) from the anticancer drug titanocene dichloride or budotitane to cancer cells which have a high density of transferrin receptors. Ti(IV) is very "hard" and readily hydrolyses to form insoluble polymeric species at neutral pH. Both titanocene dichloride and budotitane undergo rapid and complete hydrolysis to form insoluble polymers at physiological pH. However, biological studies reveal that titanium is accumulated in the cellular nucleic acid-rich regions (mainly the nucleus) after in vivo application of titanium drugs. Therefore biomolecules must be involved in the stabilisation of Ti(IV) and its transport.

It is clear that metal binding to the model ligand EHPG and to the protein transferrin can be finely controlled both by pH and by kinetic factors. Further biological investigations of titanium transferrin or other phenolate proteins are therefore warranted.
4.5 References


Chapter 5

Manganese Cyclam and Bicyclam Complexes

5.1 Introduction

Macrocyclic ligands often form stable complexes with transition metal ions in a less common oxidation state. Macrocyclic ligands with nitrogen donors have been reported to form stable complexes with virtually all kinds of transition metal ions and also some other metal ions.¹

Cyclam

![Figure 5.1 Schematic representation of cyclam, (1,4,8,11-tetra-azacyclotetradecane).](image)

Cyclam (1,4,8,11-tetra-azacyclotetradecane, [14]aneN₄), Figure 5.1, is a 14-membered heterocycle which has been studied extensively.² It was first synthesised in 1937,³ and has since been found to have a wide range of applications in a variety of areas, including catalysis, coordination, bioinorganic and biomimetic chemistry. Depending on the spatial alignment of the NH protons there are five possible configurational isomers of metal cyclam with both folded (cis) and planar (trans) geometries. The trans geometries are RSRS(++++), RSRR(+++), RRSS(+-+), RSSR(++−) and RRRR(+-+).⁴ The “+” sign indicates that the hydrogen atom
attached to the nitrogen atom is above the plane of the molecule, while "-" sign indicates that it is below the plane. The isomers are designated trans-I to trans-V, respectively, as shown in Figure 5.2. Semi-quantitative analysis of the relative strain energies of the five isomers has indicated that the trans-III form is the most stable in octahedral coordination.\textsuperscript{5,6}

![Figure 5.2 The five trans and one cis configurational isomers of cyclam. Colour code: dark blue, N; light blue, metal. Only the H atoms on N are shown.](image)

Mn(III) complexes of cyclam have been shown to be effective contrast agents for magnetic resonance imaging,\textsuperscript{7} and have also been shown to have superoxide dismutase activity.\textsuperscript{8}

**Bicyclams**

Bicyclams are a new class of highly potent HIV inhibitors.\textsuperscript{9} They are highly selective agents that act by specifically blocking the CXCR4 coreceptor used by T-cell strains of HIV.\textsuperscript{10} They originated from the serendipitous discovery of anti-HIV activity in a monocyclam preparation that contained bicyclam as a contaminant.\textsuperscript{11} The monocyclam was not as active as the bicyclam, so further work was carried out on
the bicyclam by-product. The most potent of the bicyclams is [1,4-phenylenebis-(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane, also known as AMD3100. AMD3100 is a bicyclam where two cyclam units are linked together by a \( p \)-phenylene bridge, Figure 5.3. It was derived from JM2763, which has an aliphatic linker instead of the phenyl ring. The hydrochloride salt is in clinical trials as an anti-HIV drug.

Figure 5.3 Schematic representation of [1,4-phenylenebis-(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane, (AMD3100).

The anti-HIV activity exhibited by bicyclams is particularly intriguing due to the possibility that binding to the molecular target at the HIV-inhibitory step is transition metal mediated.

A number of transition metal complexes of AMD3100 and several other bicyclams have been prepared and have been found to exhibit anti-HIV activity. The Zn(II) complex (AMD3479) was slightly more active (10-fold) than AMD3100 and the Ni(II) complex (AMD3462) was as active as AMD3100 in its capacity to inhibit binding. The Cu(II) (AMD3469) and Co(II) (AMD3461) complexes were found to be 5-fold and 2220-fold less active respectively, than AMD3100. The Pd(II) complex (AMD3158) was virtually inactive.

Azamacrocycles such as cyclam can form many compounds with high thermodynamic stability with a range of transition metals. The aim of this work was to synthesise stable Mn(III) complexes, which amongst other things could be used to deliver Mn(III) to transferrin.
5.2 Experimental

5.2.1 Preparation of Trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O (4)

Trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O (4) was prepared according to a previously reported procedure.²¹

A solution of MnCl₂.4H₂O (0.5 g, 2.5 mmol) in methanol (50 ml) was added to a solution of cyclam (0.5 g, 2.5 mmol) in methanol (25 ml). Air was bubbled through the resultant dark green solution for 6 h, the colour darkening almost immediately. After approx. 1 hour, an apple-green precipitate formed at the bottom of the flask. Conc. HCl was added to the resultant dark brown solution, which resulted in a green solution with apple-green precipitate. The green precipitate was filtered off, dried in air, and recrystallised from warm HCl (1 M). The solution was kept at 277 K which yielded green needle-shaped crystals of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O (4) within 48 h suitable for X-ray crystallographic analysis. The crystals were filtered off, washed with acetone and diethyl ether, and dried in vacuo at ambient temperature for a period of approx. 3-4 h. Yield: 0.398 g, 42%. Anal. Calc. for [Mn(C₁₀H₂₄N₄)Cl₂]Cl.H₂O, C₁₀H₂₄N₄Cl₃Mn.H₂O: C, 31.62; H, 6.32; N, 14.76. Found: C, 31.47; H, 6.71; N, 14.52%.

Crystals of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O (4) were also obtained from solutions left for 12 h at ambient temperature, although in slightly lower yields.

5.2.2 Attempted Preparation of Trans-[Mn(III)(cyclam)(acetate)₂]Cl (5)

A solution of Mn(II)acetate.4H₂O (0.61 g, 2.5 mmol) in ethanol (25 ml) was added to a solution of cyclam (0.5 g, 2.5 mmol) in ethanol (25 ml). Air was bubbled through the resultant dark green solution for 4 h, the colour gradually darkening. Glacial acetic acid was added to 10 ml of the resultant dark green solution, which resulted in a lime green solution after 24 h. The volume was reduced and the solution placed at 277 K overnight. No crystals were obtained.
5.2.3 Preparation of $p$-Phenylene Bridged Bicyclam

The general reaction scheme for the synthesis of monosubstituted cyclam derivatives is shown in Figure 5.4.\textsuperscript{22}

Figure 5.4 The general reaction scheme for the synthesis of monosubstituted cyclam derivatives. For the preparation of the bridged bicyclam, 7, RBr = dibromo-$p$-xylene.

Some of the chemicals used in the preparation of $p$-phenylene bridged bicyclam (7) are carcinogenic or potentially carcinogenic and were handled with care; carbon tetrachloride is a carcinogen and although P(NMe\(_2\))\(_3\) is not, the oxidation product O=P(NMe\(_2\))\(_3\) is very carcinogenic. Hydrolytic oxidation of P(NMe\(_2\))\(_3\) occurs very easily in the presence of water and oxygen, \textit{i.e.} if exposed to air, so dry conditions were employed.
**Preparation of P(NMe₂)₃ Protected Cyclam (6)**

To a suspension of cyclam (501 mg, 2.5 mmol) in dry toluene (100 ml), one mol. equiv. of P(NMe₂)₃ (454 µl, 2.5 mmol) was added under an atmosphere of N₂. The reaction mixture was stirred for a few hours at room temperature and then heated under reflux overnight. The resultant clear, almost colourless solution was cooled to 273 K (which resulted in the formation of a white precipitate) and treated with a slight excess of dry carbon tetrachloride (375 µl, 3.9 mmol), which caused further precipitation. The reaction mixture was stirred overnight. All volatile components were removed under high vacuum, 10% sodium hydroxide solution (25 ml) was added to the white powder, and the mixture was stirred for several hours. The reaction mixture was extracted with chloroform (5 x 100 ml) and the combined organic phases were dried over anhydrous MgSO₄. The solution was evaporated to dryness in vacuo. The product, a yellow oil, was dried under a high vacuum at ambient temperature overnight, which yielded a white powder of the protected cyclam. Yield: 520 mg (0.520 g), 85%.

**Preparation of p-Phenylene Bridged Bicyclam (7)**

To a suspension of the protected cyclam (6) (249 mg, 1.02 mmol) in dry DMF (30 ml), α,α'-dibromo-p-xylene (134 mg, 0.51 mmol) and potassium carbonate (253 mg, 1.83 mmol) were added and the reaction mixture stirred at between ca. 90-120°C for 24 h. The mixture was then evaporated to dryness in vacuo and 10% aqueous HCl solution (80 ml) was added to the slightly yellow residue. The resultant pale yellow clear solution was heated under reflux for 1.5 days. The pH of the solution was raised to > pH 13 by the addition of sodium hydroxide pellets, which caused a white precipitate to form. This alkaline aqueous solution was extracted with chloroform (5 x 90 ml) and the combined organic phases dried over anhydrous sodium sulfate (Na₂SO₄). The solution was filtered and evaporated to dryness in vacuo, which yielded the sufficiently pure slightly yellow residue of the p-phenylene bridged bicyclam (7). Yield: 0.530 g.
5.2.4 Attempted Preparation of Mn(III)bicyclam (8)

A Mn(III) complex of the bicyclam (7) was prepared following a procedure similar to that used for the synthesis of the Mn(III)cyclam complex.

A solution of MnCl₂.4H₂O (80.1 mg, 0.4 mmol) in methanol (10 ml) was added to a suspension of bicyclam (100 mg, 0.2 mmol) in methanol (10 ml). Air was bubbled through the resultant cloudy pale green/yellow solution for 4 h, the colour gradually darkening. The resultant dark brown solution was filtered to remove any impurities. Conc. HCl (3 ml) was added dropwise to the dark brown solution, which resulted in the immediate formation of a very fine red precipitate. The mixture was placed at 277 K overnight. The red precipitate was filtered off but proved difficult to dry. It appeared to be very hygroscopic and so was redissolved in conc. HCl, which gave a yellow solution. The yellow solution was kept at 277 K which over a period of a few days went colourless. No solid product was obtained.

5.2.5 Solution Studies of Trans-[Mn(III)(cyclam)Cl₂]Cl.2H₂O (4)

The stability of trans-[Mn(III)(cyclam)Cl₂]Cl.2H₂O (4) in HEPES buffer, pH 7.4, was studied by UV-Vis spectroscopy at 298 K.

The effects of variation of pH and oxygen were studied on the stability of trans-[Mn(III)(cyclam)Cl₂]Cl.2H₂O (4) in aqueous solution with time. The pH was controlled with a non-coordinating buffer (HEPES). Reactions of the different halides; F⁻, Cl⁻, Br⁻, and I at pH 7.4 in HEPES buffer were also studied.

5.2.6 NMR Spectroscopy

The ¹H NMR spectrum of a freshly prepared 20 mM solution of trans-[Mn(III)(cyclam)Cl₂]Cl.2H₂O, complex 4, in D₂O was recorded. TSP was added as an internal reference.
5.2.7 EXAFS

EXAFS data on trans-[Mn(III)(cyclam)Cl]Cl.2H2O, complex 4, both as a solid and in aqueous solution (saturated solution of complex 4 in 20 mM HEPES buffer, pH 7.4) were collected. The solution spectrum was recorded immediately after dissolution. Data analysis was carried out by Dr. Ian Harvey (Daresbury). The solution spectra shown were averaged over a period of about 0.5 h after dissolution, during which time little change was observed.

5.3 Results and Discussion

5.3.1 Synthesis of Ligands and Mn Complexes

Preparation of Mn(III)cyclam Complexes

Complex 4, trans-[Mn(III)(cyclam)Cl]Cl.2H2O, was prepared following the procedure reported by Chan et al.\textsuperscript{21} The Mn(III) complex was prepared by air oxidation of Mn(II).

Trans-[Mn(III)(cyclam)Cl]Cl.2H2O (4) appears to stable indefinitely in the solid state which is in agreement with previous reports for other Mn(III)cyclam complexes.\textsuperscript{21}

The complex trans-[Mn(III)(cyclam)(acetate)2]Cl (5) was prepared in solution, as evidenced from the colour change during the reaction and the very dark solution obtained, but was not readily isolated. Although attempts were made to isolate this complex, these were unsuccessful and thus no further analysis was carried out.

Synthesis of Bicyclam

The bicyclam was synthesised using a general reported procedure for the preparation of monosubstituted cyclams.\textsuperscript{22} All reaction steps for the synthesis of bicyclam are best performed overnight although shorter times might also be possible. Due to the water sensitivity of P(NMe\textsubscript{2})\textsubscript{3} care was taken to maintain absolutely water-free
conditions. After stirring with carbon tetrachloride (which was used in a slight excess) and evaporation the flask was kept for some additional time under high vacuum to ensure that all the carbon tetrachloride was removed. The purity of the first step was checked by \(^{31}\text{P NMR}\) (1 singlet).

For the second step the solvent again had to be dry; water causes side reactions of this \(S_{N2}\) reaction. The amount of potassium carbonate used was in a 1.5 to 2-fold excess. The evaporation of the DMF was done to absolute dryness. Traces of this solvent (which is miscible with water) can dissolve some of the product in water and can lead to a lower yield.

The crude product was pure enough for further reactions (ca. 95%). Unfortunately this compound showed a very low solubility in all solvents both as the free amine and as the hydrochloride. The hydrochloride can be obtained by dissolving the free bicyclam in ethanol followed by the addition of a saturated solution of HCl gas in ethylacetate.

**Attempted Preparation of Mn-bicyclam**

There are no known reports of Mn-bicyclam complexes. Although attempts were made to prepare a Mn(III) complex of the bicyclam, attempts to isolate and characterise the complex were unsuccessful. The reaction of bicyclam with Mn(II)Cl\(_2\) resulted in the formation of a dark green solution, which is characteristic of Mn(III) binding to cyclam. However on the addition of HCl a red coloured precipitate was obtained. The colour change is indicative of the formation of a new complex. Upon redissolving the red precipitate in HCl a yellow solution was obtained which is indicative of further reactions taking place. The yellow colour of the solution then disappeared over time yielding a colourless solution which indicated that the species in solution undergoes further reactions, perhaps hydrolysis.

**5.3.2 X-ray Crystal Structure of Trans-[Mn(III)(cyclam)Cl\(_2\)]Cl\(_2\)H\(_2\)O (4)**

Crystallographic data for complex 4 are summarised in Table 5.1. A thermal ellipsoid diagram and atom labelling scheme of [Mn(III)(cyclam)Cl\(_2\)]Cl\(_2\)H\(_2\)O is shown in Figure 5.5. Selected bond lengths and bond angles are listed in Table 5.2.
Table 5.1 Crystal data, data collection and refinement for complex 4.

<table>
<thead>
<tr>
<th>Crystal data</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emperical formula</td>
<td>C_{10}H_{32}MnN_{4}O_{4}Cl_{3}</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>[Mn(cyclam)Cl_{2}]Cl.2H_{2}O</td>
</tr>
<tr>
<td>Formula weight</td>
<td>433.69</td>
</tr>
<tr>
<td>Crystal habit, colour</td>
<td>Block, green</td>
</tr>
<tr>
<td>Crystal size (mm)</td>
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</tr>
<tr>
<td>Crystal system</td>
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<tr>
<td>Space group</td>
<td>P2_1/n</td>
</tr>
<tr>
<td>a (Å)</td>
<td>9.795(3)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>6.473(2)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>16.529(7)</td>
</tr>
<tr>
<td>β (°)</td>
<td>106.96(3)</td>
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<tr>
<td>Volume (Å³)</td>
<td>1002.5(6)</td>
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<td>Z</td>
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</tr>
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<td>Density (calc.) (Mg m⁻³)</td>
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<tr>
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<td>F (000)</td>
<td>456</td>
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<td>Data collection and refinement</td>
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<td>Wavelength (Å)</td>
<td>0.71073</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>150(2)</td>
</tr>
<tr>
<td>θ Range for data collection (°)</td>
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</tr>
<tr>
<td>Index ranges</td>
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</tr>
<tr>
<td>No. reflections collected</td>
<td>1840</td>
</tr>
<tr>
<td>No. independent reflections</td>
<td>1761 (R_{int} = 0.0081)</td>
</tr>
<tr>
<td>Max. and min. transmission</td>
<td>0.903 and 0.746</td>
</tr>
<tr>
<td>Data / restraints / parameters</td>
<td>1761 / 0 / 110</td>
</tr>
<tr>
<td>R indices [F &gt; 4σ(F)]</td>
<td>R1 = 0.00602 (1542 data)</td>
</tr>
<tr>
<td>R indices (F² and all data)</td>
<td>wR2 = 0.1580</td>
</tr>
<tr>
<td>Goodness-of-fit on F²</td>
<td>1.071</td>
</tr>
<tr>
<td>Largest difference peak and hole (eÅ⁻³)</td>
<td>1.268 and -1.125</td>
</tr>
</tbody>
</table>

The geometry at the Mn(III) ion is trans-pseudo-octahedral as found for other related Mn(III)cyclam complexes. The cyclam macrocycle sits in the equatorial plane with all four nitrogens coordinated to the Mn(III) ion. The two chloride ligands occupy the axial positions with a Cl-Mn-Cl angle of 180°.
The crystal was twinned and no H atoms could be located on the two water molecules of solvation and the chloride anion was found to be present in the structure in two positions, having occupancies of 75% and 25%.

![Diagram of Mn(III)(cyclam)Cl2Cl2H2O (4)](image)

**Figure 5.5 Thermal ellipsoid diagram and atom labelling scheme for [Mn(III)(cyclam)Cl2]Cl2H2O (4).** The chloride anion and oxygen atoms of the two waters of recrystallisation are also shown.

The two independent Mn-N bond lengths are very similar and have values of 2.032(4) and 2.036(4) Å. These values for the Mn-N bond lengths are very similar to those reported for the corresponding bonds in other similar Mn(III)cyclam complexes.23 The axial Mn-Cl bond length of 2.5249(13) Å is very long, and is indicative of the axial elongation expected for this high-spin d^4 complex and has a similar value to that of the corresponding axial bonds in other similar Mn(III) cyclam complexes with axial Cl^- ligands.23 The *cis* N-Mn-N bond angles are 85.61(15)° and 94.39(15)°, with the smaller value being associated with the five-membered chelate rings and the larger value with the six-membered chelate rings, as expected.
The trans-[Mn(III)(cyclam)Cl_2]^+ cation adopts the most commonly-observed trans-III configuration, of N(R)N(R)N(S)N(S), with the hydrogens of two of the NH groups pointing 'up' and the hydrogens of the other two NH groups pointing 'down'. The six-membered rings are in a chair conformation.

### 5.3.3 EXAFS of Trans-[Mn(III)(cyclam)Cl_2]Cl_2H_2O

The Fourier transform spectrum, with a theoretical fit, of a powder sample of trans-[Mn(III)(cyclam)Cl_2]Cl_2H_2O is shown in Figure 5.6. The fit was obtained using the full molecule, full multiple scattering with bond length restraints (from the crystal structure). Mn-ligand bonds were not restrained, but bonds within the cyclam were. Just fitting the ligand atoms (all four nitrogens at the same distance and the two chlorides at the same distance) gave an R-factor of 29.4%. The full fit gives an R-factor of 25.6% (EXAFS 24.6%, deviation from 'ideal' bond lengths 1%). The difference is due to the amount of destructive interference by the carbon atoms at 3 Å taken into account for the two fits.
Figure 5.6 Fourier Transform spectrum (solid line) and theoretical fit (dotted line) for trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O (4) in the solid state. The theoretical fit is for the full molecule, using full multiple scattering with bond length restraints (from the crystal structure). Mn-ligand bond lengths were not restrained, but bonds within cyclam were.

From Figure 5.6, the Fourier transform spectrum and the theoretical fit for trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O in the solid state, it can be seen that the intensity of the peak at 3 Å is slightly less in the theoretical fit compared to that obtained from the experiment. The peak at 3 Å is due to the carbon atoms of the cyclam ligand. In the theoretical fit all the carbon atoms of cyclam have the same Debye-Waller factor and thus the Debye-Waller factor for these atoms is an average of three distances (2.8, 3.0 and 3.4 Å). The final Mn-Cl distance is 2.55 Å.

Thus, a good fit to the powder data has been obtained by using the full crystal structure. The EXAFS data only contains information regarding the first shell fit, however the R-factor was lower. The EXAFS data for the Mn(III)cyclam powder is in excellent agreement with the crystal structure. The EXAFS data suggest that there are 4 nitrogens at 2.05 Å and 2 chlorides at 2.52-2.55 Å, which is similar to that obtained for the crystals from the X-ray structure (4 N at 2.04 Å and 2 Cl at
2.525 Å). The R-factor from the EXAFS is lower than that obtained for the crystal structure from X-ray crystallography. As determined in the crystal structure there is internal symmetry.

The Fourier transform of trans-[Mn(III)(cyclam)Cl]Cl.2H2O in solution (HEPES buffer, pH 7.4) is compared with that of the powder in Figure 5.7. A saturated solution of trans-[Mn(III)(cyclam)Cl]Cl.2H2O in HEPES buffer was used. The data collection was complete in ca. 0.5 h and no change in the spectrum was observed over this time. After a week the data was recollected and no observable change occurred either in the spectra or in the colour of the solution (brown). The intensity of the peak at 2.5 Å for trans-[Mn(III)(cyclam)Cl]Cl.2H2O in solution is half that obtained for the powder. Analysis shows that there is only one chloride at 2.55 Å. Since this analysis is by single scattering from ligand atoms only, a comparison can only be made with a powder fit of just the ligand atoms which gives two chlorides at 2.52 Å. Thus the Cl distance obtained in solution is just slightly longer than that obtained from the powder spectrum with a first shell fit.

Although the broadening of the peak at 2 Å suggests that there might be an additional ligand at a shorter bond distance, to replace the lost Cl', analysis does not find it. This could easily be due to the short data range, and does not mean that the complex is five-coordinate, just simply that EXAFS can see only five ligands.

The distances of the ligand atoms obtained are four nitrogens at 2.07 Å and one chloride at 2.56 Å. These distances are very similar to those obtained for the powder sample.

The metal-ligand bond lengths obtained by EXAFS for trans-[Mn(III)(cyclam)Cl]Cl.2H2O (4) as a powder and in solution, along with those obtained by X-ray crystallography are listed in Table 5.3.
Figure 5.7 Fourier transform of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O in the solid state (powder form, solid line) and in solution (dotted line).

Table 5.3 Metal-ligand bond lengths of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O (4).

<table>
<thead>
<tr>
<th>Bond</th>
<th>Powder EXAFS</th>
<th>Solution EXAFS</th>
<th>Crystal Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Bond length Å</td>
<td>No.</td>
</tr>
<tr>
<td>Mn-N</td>
<td>4</td>
<td>2.05</td>
<td>4</td>
</tr>
<tr>
<td>Mn-Cl</td>
<td>2</td>
<td>2.52-2.55</td>
<td>1</td>
</tr>
</tbody>
</table>

* Average bond length (Å)

The X-ray absorption edge of powdered trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O is shifted only 8 ev from Mn metal, which suggests an oxidation state of 2+. The edge shift (10 ev) of the solution of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O suggests an increase in oxidation state to 2.5+. Further analysis is required to determine the oxidation state accurately.
5.3.4 Solution Studies of Trans-[Mn(III)(cyclam)Cl2]Cl.2H2O (4)

Although the complex trans-[Mn(III)(cyclam)Cl2]Cl.2H2O is stable indefinitely in the solid state, in aqueous solutions it undergoes a multistep reaction. It is postulated that initially hydrolysis occurs. In H2O, the absorption band in the visible region at 650 nm shifts to slightly higher energy and increases in intensity very slowly with time (days) at 298 K. A new band at 550 nm also appeared and increased in intensity with time (days). This is accompanied by a change in the colour of the solution from green to brown.

The stability of trans-[Mn(III)(cyclam)Cl2]Cl.2H2O (4) in HEPES buffer (20 mM) at pH 7.4 was investigated by UV-Vis spectroscopy. The reaction appeared to progress in four steps. The reaction proceeded at a much faster rate in HEPES buffer (20 mM, pH 7.4) compared to H2O, at 298 K, and the UV-Vis spectra for the initial stage of the reaction are shown in Figure 5.8. The UV-Vis spectra for later stages of the reaction (2 h 45 min – 26h 5 min) are shown in the Appendix (Figure A5-1).

Similar to the sample in H2O, two new visible absorption bands appeared at 640 nm and 550 nm for the sample in HEPES buffer, pH 7.4. Additionally another band also appeared at 525 nm. The intensity of all three absorption bands gradually increased for about 60 min, with an isosbestic point at 500 nm (Figure 5.8). After this time the intensities of all three bands continued to increase, although there was no longer an isosbestic point and the whole spectrum increased in intensity, until about 2 h 30 min. In the next phase of the reaction the absorption band at 640 nm decreased in intensity, although the absorption band at 525 nm continued to increase, and the band at 550 nm stayed almost unchanged. An isosbestic point was observed at ca. 606 nm, which shifted to 593 nm after about 2 h. This phase of the reaction continued for approx. the next 4 h, at which point the total reaction time was almost 7 h. After this time the absorption band at 525 nm continued to increase for a further approx. 5 h, while the absorption bands at 550 nm and 640 nm decreased in intensity. A new isosbestic point at 540 nm was observed. All three bands then decreased in intensity, although the lower energy region of the penultimate and final spectrum started to increase in intensity after a total reaction time of ca. 25 h. The greatest change took place in the first 3-4 h.
Figure 5.8 UV-Vis spectra of trans-[Mn(III)(cyclam)Cl]Cl.2H$_2$O in HEPES buffer (20 mM, pH 7.4) at 298 K, with times as indicated. The appearance of the new bands at 525, 550 and 640 nm in the initial stage of the reaction can be seen. This first stage takes approx. 2.5 h to reach completion. Initial concentration of trans-[Mn(III)(cyclam)Cl]Cl.2H$_2$O, 4.2 mM.

When the apple green trans-[Mn(III)(cyclam)Cl]Cl.2H$_2$O complex (4) was dissolved in HEPES buffer an immediate colour change from green to brown was observed. The intensity of the brown colour was found to increase over time.

**Effect of Oxygen on the Reaction**

Since the reaction is multistep and appears to be quite complex, the effect of oxygen on the reaction was monitored by bubbling N$_2$ through the HEPES buffer before dissolving the complex and maintaining the reaction under an atmosphere of N$_2$, thus excluding air from the reaction mixture. In the absence of oxygen, the reaction proceeded in the same manner as in the presence of oxygen for the first approx. 18 h, with the appearance of three new bands at exactly the same wavelength as previously
observed in the presence of oxygen. The reaction proceeded at approx. the same rate and isosbestic points occurred at similar positions, although slightly shifted in energy (Figure A5.2). After ca. 18 h, the intensity of the whole spectrum increased for 2 h, after which time the absorption band at 525 nm decreased and the band at 640 nm increased in intensity for the next 3 h, with an isosbestic point near 580 nm. After 23 h the intensity of the whole spectrum decreases again for the next 1.5 h. The absorbance at 640 nm then increased in intensity for the next 1.5 h, whilst no change is observed in the absorption band at 525 nm. The whole spectrum then starts to decrease in intensity.

Oxygen does not appear to play a role in the initial stages of the reaction, although it may well be involved in the later stages due to the differences in absorption bands observed with and without the presence of oxygen.

**Effect of Halides on the Reaction**

In the presence of 0.1 M NaBr, the reaction proceeded with similar rates as for the chloride complex, but with slightly shifted isosbestic points. Phase 2 took slightly longer (Figure A5-4 in the appendix). A plot of the absorbance intensity at 640 and 550 nm versus time in the presence of 0.1 M NaBr is shown in Figure 5.9. In the presence of 0.1 M NaI the reaction again proceeded in a similar manner but at a much faster rate (Figure 5.10). The first stage of the reaction, i.e. the appearance of the new bands, was complete in ca. 1 h, after which the bands decreased in intensity. A plot of the absorbance intensity at 643 and 549 nm versus time for the reaction in 0.1 M NaI is shown in Figure 5.11. In the presence of 0.1 M NaCl, the reaction again proceeded in a similar manner to before, but at a much slower rate. The first stage of the reaction took ca. 8 h for completion, and the intensity of the absorption bands was less than before (Figure 5.12). The second phase of the reaction also took 8 h and was much slower than before, although the third phase was of a similar duration. The final stage of the reaction began after almost 22 h. These are shown in Figure A5-3 in the appendix. A plot of the absorbance intensity at 643 and 549 nm versus time for the reaction in 0.1 M NaCl is shown in Figure 5.13.
Figure 5.9 Plot of absorbance intensity at 640 and 550 nm versus time over a period of 30 h for a ca. 4 mM solution of trans-[Mn(III)(cyclam)Cl₂]Cl.2H₂O in 0.1 M NaBr in HEPES buffer (20 mM, pH 7.4) at 298 K.

Figure 5.10 UV-Vis spectra of trans-[Mn(III)(cyclam)Cl₂]Cl.2H₂O in 0.1 M NaI in HEPES buffer (20 mM, pH 7.4) at 298 K, with times as indicated. Initial concentration of trans-[Mn(III)(cyclam)Cl₂]Cl.2H₂O, 3.4 mM.
Figure 5.11 Plot of absorbance intensity at 643 and 549 nm versus time over a period of 30 h for a ca. 4 mM solution of trans-[Mn(III)(cyclam)Cl]Cl.2H₂O in 0.1 M NaI in HEPES buffer (20 mM, pH 7.4) at 298 K.

Figure 5.12 UV-Vis spectra of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O in 0.1 M NaCl in HEPES buffer (20 mM pH 7.4) at 298 K, with times as indicated. This first stage took approx. 3.5 h to reach completion. Initial concentration of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O, 2.9 mM.
Figure 5.13 Plot of absorbance intensity at 640 and 550 nm versus time over a period of 30 h for a ca. 4 mM solution of trans-[Mn(III)(cyclam)Cl]Cl.2H₂O in 0.1 M NaCl in HEPES buffer (20 mM, pH 7.4) at 298 K.

In the presence of 0.1 M NaF no new absorption bands in the region between 450 and 800 nm were observed and correspondingly no colour change took place. Upon dissolution of complex 4, trans-[Mn(III)(cyclam)Cl]Cl.2H₂O, in 0.1 M NaF an apple green solution was obtained. Since this sample showed no new bands in the region λ > 400 nm, the bands at lower wavelengths were investigated by dilution of the sample (Figure A5-5). The absorption maxima at 268 nm and 362 nm had ε values of 10,690 and 865 M⁻¹ cm⁻¹, suggesting that these are ligand-to-metal charge-transfer bands. The formation constant of MnF²⁺ (Mn³⁺ + F⁻ = MnF²⁺) has been reported to be ≈ 10⁶ at ~23°C,²⁴ which implies that fluoride forms a strong bond with Mn(III). Since the fluoride complex probably forms in NaF and the bands are of similar energy as for the chloride complex (ε₂₆₆₅ = 6363 M⁻¹ cm⁻¹, ε₃₄₀ = 1000 M⁻¹ cm⁻¹), they may involve the nitrogen ligands rather than the axial ligands, (which are more weakly bound in view of the Jahn-Teller distortion).
Effect of pH on the Reaction

When the pH of the reaction was increased from 7.4 to 8.2 in HEPES buffer, the first phase of the reaction proceeded at a similar rate as at pH 7.4. However after the initial phase the reaction proceeded differently (Figure 5.14). After ca. 2 h, the absorption bands at 525 and 549 nm continued to increase in intensity and there was an isosbestic point at 644 nm. The lower energy region of the spectrum decreased in intensity. After 5.5 h the isosbestic point moved to 674 nm and all three absorption bands increased in intensity, with a slight decrease in the tail end of the spectrum.

Figure 5.14 UV-Vis spectra of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O in HEPES buffer (20 mM, pH 8.2) at 298 K, with times as indicated. Initial concentration of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O, 3.9 mM.
Figure 5.15 Plot of absorbance intensity at 637 and 549 nm versus time over a period of 30 h for a ca. 4 mM solution of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O in HEPES buffer (20 mM, pH 8.2) at 298 K.

The values of extinction coefficient for the bands in the visible region are ca. $10^2$ M$^{-1}$ cm$^{-1}$, which implies that they are relatively weak in comparison to the observed LMCT bands and therefore can be assigned to d-d transitions.

### 5.3.5 Reaction Course

Except for the NaF in HEPES buffer, in all the aqueous solvent systems studied, a multistep reaction occurs over the period monitored (approx. 24 h). The most significant change occurred over the initial period of the reaction (~3 h), which was accompanied by an immediate colour change from green to brown followed by further darkening of the brown colour. Three new weak absorption bands appear in the spectrum in the visible region at ca. 525, 550 and 640 nm in all the aqueous solvent systems studied (H$_2$O, HEPES buffer, and HEPES buffer containing either 0.1 M NaCl, NaBr, or NaI) except in the presence of 0.1 M NaF in HEPES buffer. The absorption bands at 550 and 640 nm reached an absorption maximum after a reaction time of approx. 2.5 h, with an isosbestic point at 500 nm for the first hour.
The reaction then proceeded at a much slower rate, with a slight increase in absorption at 525 nm and a decrease in absorption at 640 nm. Isosbestic points at ca. 600 nm were observed in all the aqueous solvent systems studied except for HEPES buffer at pH 8.2 and 0.1 M NaI in HEPES buffer, pH 7.4. In 0.1 M NaI in HEPES buffer, pH 7.4, no isosbestic point was seen and in HEPES buffer at pH 8.2 an isosbestic point was observed but it was shifted to lower energy ($\lambda = 675$ nm). In the solvent systems with isosbestic points, this suggests the presence of only two absorbing species (the Cl$\text{}_2$ complex and OH$_2$/OH product, Figure 5.16) during this time. In the next phase of the reaction the absorption band at 550 nm also decreased slightly, along with the band at 640 nm, whilst the band at 525 nm continued to increase. In the final phase of the reaction the whole spectrum decreased in intensity.

In some systems the long wavelength tail end of the spectrum increased in intensity, which may be attributed to light scattering due to the formation of a brown precipitate. The precipitate was observed in these systems and was also found to form in the other systems over a longer time period. The precipitation may be caused by the formation of a polymeric species.

The absorption profile was not affected significantly by the presence of excess NaBr or if carried out under an atmosphere of N$_2$. Thus oxygen does not appear to play a significant role in the reaction in the initial phases. The presence of excess NaCl slowed down the reaction significantly, implying that a back reaction is occurring involving the Cl$^-$ anion. In the presence of NaI in the system the reaction proceeded at a faster rate, with the first phase being over in only an hour. The intermediate stages of the reaction were not observed, with the final stage, i.e. decrease in intensity of the entire spectrum, occurring immediately after the first phase. Increasing the pH of the reaction from 7.4 to 8.2 did not appear to affect the initial stage of the reaction although a decrease in intensity is not observed in any of the absorption bands in the later stages. Thus the pH appears to affect the final stage of the reaction and thus the product formed. This can be related to the ionisation of axial OH$_2$/OH ligands and the formation of bridged species which could undergo redox reactions.

Therefore the same product is formed in the initial stage of the reaction under all of the above discussed reaction conditions, with the appearance of the same absorption bands in each of the systems and only a slight change in the rate of the reaction. In the system in which NaF is present no new absorption bands are observed in the
region between 400 and 800 nm, and the absorption profile does not change. Thus in
the presence of NaF a relatively stable complex is formed. We can thus speculate
that in all the other systems studied one or more of the axial Cl⁻ ligands are displaced
by water, and in the NaF systems F⁻ is bound in the axial positions. The F⁻ must be
bound fairly tightly as no change in the absorption profile is observed, which is
indicative of the formation of a stable complex in aqueous solutions.

It can be speculated, based on the results obtained that the reaction may proceed via
aqua and hydroxo products in the reaction mechanism shown in Figure 5.16.

![Figure 5.16. Hydrolysis of trans-[Mn(III)(cyclam)Cl]Cl₂H₂O (4) in aqueous
solution.](image)

Hydroxide and oxide are good bridging ligands and dimers and oligomers may
eventually form. Such a course of reaction is common for Fe(III) complexes of
tetraaza ligands (e.g. porphyrins). Deprotonation of the macrocycle NH groups
cannot be ruled out and redox reactions of bridged species may occur
(e.g. 2Mn(III) → Mn(II) and Mn(IV)). I attempted to synthesise a Mn(III) complex
of the bicyclam AMD3100 to investigate this further but did not obtain any
crystalline product. Colour changes from green to red and yellow were observed in
the presence of HCl and this would provide interesting further study.

Dinuclear mixed valence Mn(III)-Mn(IV) complexes with an oxygen bridging ligand
are fairly common. Crystal structures reported recently include that of the dinuclear
bis(µ-oxo)Mn(III)/Mn(IV) complex, [(pmap)Mn-(µ-O)₂-Mn(pmap)](ClO₄)₃, where
pmap is bis[2-(2-pyridyl)ethyl]-2-pyridylmethylamine,²⁵ and [Mn₂OL₂]³⁺, where
L is the monoanionic $N,N$-bis(2-pyridylmethyl)-$N'$-salicyliden-1,2-diaminoethane ligand.$^{26}$

After this work was completed the crystal structure of a dinuclear mixed-valence Mn(III)-Mn(IV) cyclam complex, $[(\text{cyclam})\text{Mn}^{III}(\text{ClO}_4)^-\text{Mn}^{IV}(\text{NO}_3)]$, was reported, containing two oxide bridges between Mn cyclams in the cis conformation.$^{27}$ The complex was self-assembled by the reaction of Mn(II) with cyclam in aqueous media. Such species may be formed during the later stages of the reaction of trans-$[\text{Mn}(\text{III})(\text{cyclam})\text{Cl}_2]\text{Cl}.2\text{H}_2\text{O}$ (4) in aqueous solutions.

The values of the extinction coefficients of the bands in the visible region are low ($10^2 \text{ M}^{-1} \text{cm}^{-1}$), which implies that the bands in the visible region are due to d-d transitions in origin. For a regular octahedral high-spin 3d$^4$ ion, a $^5T_2g \leftrightarrow ^5E_g$ spin-allowed transition would be expected, but in the present case the symmetry is likely to be much slower than this. In the spectrochemical series, the ligands of interest follow the order (increasing $\Delta_o$) $I^- < Br^- < Cl^- < F^- < OH^- < H_2O.$

5.3.6 NMR Spectrum of Trans-[Mn(III)(cyclam)Cl$_2$]Cl.2H$_2$O (4)

The $^1$H NMR spectrum of complex 4 in 10%D$_2$O/90%H$_2$O at 298 K is shown in Figure 5.17.

Peaks were observed over the wide spectral region of $-60$ to $+50$ ppm with broad peaks centred at $+37$, $+19$, $-9$, and $-47$ ppm with linewidths of ca. $1200$, $3650$, $7500$, and $9500$ Hz, respectively. These are consistent with the presence of a paramagnetic high-spin Mn(III) (d$^4$) centre in the complex with the shifts arising from through-space (pseudocontact) and through-bond (contact) effects. It seems likely that the largest shifts are observed for the NH protons which are close to the Mn(III) ion. This could be verified by recording a spectrum for a D$_2$O solution which would allow H-D exchange. The intense peak at $4.7$ ppm is from H$_2$O and the relatively small peak at $-50$ ppm is an artefact due to the probe. No further attempts were made to assign the peaks. Comparison of this spectrum with that of substituted cyclam derivatives would allow further assignments to be made. Such paramagnetically-shifted resonances should be useful in studies of the interaction of Mn(III) cyclams with biomolecules and in studies of Mn transfer reactions. Also it
may be possible to study the various configurations of cyclam which are present in solution. Since the fluoride complex is stable this would be a good starting point.

![NMR spectrum](image)

Figure 5.17 (A) $^1$H NMR spectrum of $\text{trans-}[\text{Mn(III)(cyclam)Cl}_2]\text{Cl}.2\text{H}_2\text{O}$, complex 4 (20 mM) after standing in 10%$\text{D}_2\text{O}/90\%\text{H}_2\text{O}$ for 10-15 min, (B) expansion of the region 1.0-8.5 ppm. The intense peak at 4.7 ppm is from $\text{H}_2\text{O}$. 
5.4 Conclusions

Water-soluble Mn(III) complexes may be useful for the delivery of Mn(III) to proteins such as transferrin and could find application as metallodrugs. In this Chapter, I have studied a Mn(III) complex of the tetraazamacrocycle cyclam.

The complex trans-[Mn(III)(cyclam)Cl]Cl.2H2O, complex 4, was crystallised and the X-ray structure showed that it is octahedral with two long axial Mn-Cl bonds (Jahn-Teller distortion) of 2.525 Å. The cyclam adopts the trans-III configuration with stable chair conformations for the two six-membered rings.

K-edge EXAFS measurements verified the Mn-N and Mn-Cl bond lengths, but in aqueous solution only a single Mn-Cl bond was detected, consistent with hydrolysis occurring. Hydrolysis was also detected by UV-Vis spectroscopy, a new d-d band appearing at ca. 640 nm which reached a maximum intensity after ca. 2.5 h at 298 K. However there were further subsequent reactions attributable to deprotonation of coordinated water, and possible oligomerisation and redox reactions. This made further analysis of the data difficult.

Hydrolysis was inhibited by the presence of excess chloride, but bromide had little effect and iodide increased the rate of reaction. In accordance with the 'hard' nature of Mn(III), fluoride strongly inhibited the hydrolysis. Thus fluoride complexes of such Mn(III) macrocycles could be useful in biological and pharmacological studies. Paramagnetically shifted 1H NMR peaks were observed for Mn(III)cyclam in water and this may be a promising method for further studies of the configurational isomers and hydrolysis reactions. The application of electrospray mass spectrometry may also assist with identification of the products of the hydrolysis reactions.
5.5 References


Figure A5-1 UV-Vis spectra of trans-[Mn(III)(cyclam)Cl2]Cl.2H2O in HEPES buffer (20 mM, pH 7.4) at 298 K, with times as indicated. Initial concentration of trans-[Mn(III)(cyclam)Cl2]Cl.2H2O, 4.0 mM.
Figure A5-2 UV-Vis spectra of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O in HEPES buffer (20 mM, pH 7.4) at 298 K, under an atmosphere of N₂, with times as indicated. Initial concentration of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O, 3.9 mM.
Figure A5-2 cont. UV-Vis spectra of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O in HEPES buffer (20 mM, pH 7.4) at 298 K, under an atmosphere of N₂, with times as indicated. Initial concentration of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O, 3.9 mM.
Figure A5-3 UV-Vis spectra of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O in 0.1 M NaCl in HEPES buffer (20 mM, pH 7.4) at 298 K, with times as indicated. Initial concentration of trans-[Mn(III)(cyclam)Cl₂]Cl₂H₂O, 2.9 mM.
Figure A5-4 UV-Vis spectra of trans-[Mn(III)(cyclam)Cl2]Cl.2H2O in 0.1 M NaBr in HEPES buffer (20 mM, pH 7.4) at 298 K, with times as indicated. Initial concentration of trans-[Mn(III)(cyclam)Cl2]Cl.2H2O, 3.7 mM.
Figure A5-5 UV-Vis spectrum of \( \text{trans-}[\text{Mn(III)}(\text{cyclam})\text{Cl}_2]\text{Cl}_2\text{H}_2\text{O} \) in 0.1 M NaF in HEPES buffer (20 mM, pH 7.4) after 5 hours at different concentrations of the complex. The spectrum did not change with time. In contrast to solutions in 0.1 M NaCl, NaBr or NaI there are no bands in the region \( \lambda > 450 \text{ nm} \).
Publications

(1) Stereo-selective formation of seven-coordinate titanium(IV) monomer and dimer complexes of ethylenebis(o-hydroxyphenyl)glycine

Maolin Guo, Hongzhe Sun, Shailja Bihari, John A. Parkinson, Robert O. Gould, Simon Parsons and Peter J. Sadler


(2) Manganese transferrin

Kerry E. Bunyan, Ben J. Tura, Ian Harvey, Shailja Bihari, David J. Harrison, Peter J. Sadler


(3) Stereoisomers of Mn(III) complexes of ethylenebis[(o-hydroxyphenyl)glycine]

Shailja Bihari, Pamela Smith, Simon Parsons, Peter J. Sadler