REDUCED METAL TRANSFERRIN BINDING IN NEUROLOGICAL DISEASES

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By employing G75 gel-filtration chromatography, it has been demonstrated that human plasma gallium speciation (and by implication, Al speciation) is bimodal. Normally, gallium was predominantly bound to a high molecular weight fraction which was presumably transferrin. Literature reviews and experimental work throughout this thesis provided evidence to support this idea. An aluminium-transferrin species was assumed to be relatively non-toxic and a protective function for this complex has been suggested. A second, low molecular weight species of gallium was observed and its identity has been suggested to be citrate. The results of this thesis support the concept that citrate was a gallium binding ligand present in the plasma, but there was another species (tentatively identified as phosphate) which bound gallium to a much greater degree than did citrate in the majority of samples studied. The consequence of a low molecular weight species of aluminium is the possibility that this leads to a more rapid, uncontrolled deposition of the metal in the brain compared to a transferrin mediated mechanism.

Plasma speciation studies in Alzheimer's disease, Parkinson's disease, Down's syndrome, and neonates has revealed an altered ratio of the two gallium species found in control subjects. In all groups there was an increase in the potentially more neurotoxic low molecular weight species. These observations have led to a suggested mechanism of accumulation of metals in the brain, which is known to occur in the first three groups. Possible pathogenic mechanisms are described. The results can also offer an explanation to the reported increased sensitivity to the toxic effects of aluminium in the neonate.

Speciation studies on normal plasma have shown the balance between high and low molecular weight species of gallium to be influenced by many physiological factors. There appears to be a fine equilibrium between both species which can be altered without any great difficulty. Therefore, in the diseased groups studied, it is possible that there are subtle biochemical changes within the circulatory system to affect the equilibrium which results in an increased low molecular weight species of aluminium. Furthermore, it has been demonstrated that there is a group of normal controls with no clinical signs of Alzheimer's or Parkinson's disease who have reduced transferrin binding. This indicates there is a population of healthy people who are at risk to the development of either disease.

Key Words - Aluminium, Transferrin, Oxidative damage, Alzheimer's disease, Parkinson's disease.

DEDICATION

I would like to dedicate this thesis to my mother. For without her determined encouragement throughout my educational years, I would not be in the position I am today.

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CHAPTER ONE

INTRODUCTION

1.1 ALZHEIMER'S DISEASE

1.1.1 INTRODUCTION

Alzheimer's disease (AD) leads to the progressive degeneration of the brain and results in the impaired ability to think, remember, and behave. A person with AD may experience confusion, undergo a personality change, and fail to complete daily tasks. Symptoms of the disease include a gradual loss of short term memory, the decline in the ability to perform simple routine tasks, and a loss of language skills. During the later stages of the disease, 24 hour care is required as patients are unable to look after themselves. Eating, grooming, and toileting all require assistance (Alzheimer's Association personal communication 1990).

AD is the most common form of dementia in the Western world, is the fourth most common cause of death in the U.S. (Glenner and Wong 1984) (behind heart disease, cancer, and strokes) and makes up one of the largest categories of chronic disease in the elderly. The prevalence rate of AD in the population increases from 2 - 3% of those aged 65 - 70 to 30% of the population aged over 90. The prevalence rate has also been shown to increase exponentially with age (Hafner 1990). Furthermore, the prevalence rate in women was 2.5 times greater than that found in men (Knoefel et al 1990). Mortality studies show people with AD have an age-specific death rate two to four times that of the general population (Katzman and Saitoh 1991), life expectancy upon the first recognition of any cognitive decline is approximately seven to eight years and about four years from the diagnosis of dementia (Hafner 1990).

The significance of AD lies in the fact that huge demographic changes are occurring within the population. The life expectancy of people in developing countries is somewhat less than that found in the Western world: fewer people reach an age where AD becomes more common, therefore AD does not pose such a threat to these countries. However, with improvements (primarily in medical care) the populations of developing countries (along with industrialised countries) are experiencing a marked aging (WHO 1982). In many industrialised countries, as many as 15% of the total population are now aged 65 years or over. There are increased numbers of the elderly (65+ years) with the greatest increase being in the very elderly (80+ years) for whom the risk of dementia is highest. It has been estimated that in the U.S., by the year 2050 there will be 67.5 million people 65 years old or over, compared to 25.5 million now, of those 12 - 14 million will become demented. A similar increase in the elderly is expected in the U.K. The aging population represents a financial burden to the younger, working section of the population. With the reduced birth rate experienced in many industrial countries, this socio-medical problem will become very critical. Not only in terms of the huge financial burden upon the health service, but also in the distress suffered by families who have a demented relative. In the U.S. the financial cost for the care of AD patients is estimated to be more than $80 billion each year.

1.1.2 DIAGNOSIS OF ALZHEIMER'S DISEASE

As there is no specific marker for AD, clinical diagnosis is usually based upon the exclusion of other diseases, known causes of dementias (e.g. multi infarct dementia, depression, nutritional deficiencies, and head injuries), and by evaluation. Diagnostic methods currently employed include: computerised tomography, positron emission tomography, and nuclear resonance imaging. Clinicians assess the results of psychiatric, psychological, physical, and neurological reports and apply accepted criteria for the diagnosis of AD. Three sets of diagnostic criteria are commonly used: the diagnostic and statistical manual of the American Psychiatry Association (DSM III), the clinical diagnosis of AD of the National Institute of Neurological and Communicative Disorders and Stroke with the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA), and the Eisdorfer and Cohen research diagnostic criteria (ECRDC) for the diagnosis of primary neuronal degeneration of the Alzheimer's type (Kukull et al 1990).

There are many problems associated with correctly diagnosing AD which is reflected in the diagnostic rate confirmed at post mortem. Rarely is the accuracy rate for even the most experienced clinician above 80% and it can be as low as 46% (Homer et al 1988).

The presence of senile plaques and neurofibrillary tangles in specific brain sections is taken as confirmation of AD post mortem. Pathological examination of brains of non demented elderly subjects has shown the presence of similar lesions in very small numbers (Tomlinson et al 1968). As a result of these findings, it is the density of the lesions which determines the classification of AD.

1.1.3 TREATMENT OF ALZHEIMER'S DISEASE

Treatment of AD is only palliative. It is derived from medication for the patient and counselling for the patients family. Counselling and social management can provide general support, a safe and appropriate environment for the patient (Cooper 1991), and ease the burden of both patient and carer. Medication for AD patients is usually in the form of drugs to improve or preserve cognitive function and those to improve behaviour. Recently, McLachlan et al (1991) have used desferrioxamine (DFO) as a treatment for AD. They demonstrated a reduction in the rate of clinical deterioration in patients given DFO compared to controls. Generally, drug treatment offers little help to AD patients in terms of improving their quality or quantity of life.

1.1.4 NEUROPATHOLOGY

Alzheimer used the Bielschowsky method of staining (developed to specifically stain neurofibrils) and was able to illustrate that the brain of a demented subject was severely affected by plaques which had been previously described by Blocq and Marinesco (1882). Alzheimer also noted a form of neuronal degeneration which had not been previously described. These histological features were later described as neuritic or

senile plaques (SP) and neurofibrillary tangles (NFT) respectively. Other neuropathological features of AD include: Hirano bodies, granulovacuolar degeneration (of Simchowitz), cell loss in the basal forebrain, and congophilic angiopathy (amyloid deposits in the cerebral cortical and leptomeningeal blood vessels) (Perl et al 1991). Despite these other histological parameters, it is SP and NFT which distinguish AD patients from others (Perry and Perry 1988).

The greatest density of plaques are found in the frontal and temporal cortex and hippocampus (Mann 1985). Mature plaques are not seen in the cerebellum or thalamus (Terry et al 1981) although diffuse plaques are (Ogomori et al 1989). NFT are primarily found in the temporal cortex, hippocampus, and amygdala (Mann 1985 and references therein). Hyman et al (1984) have shown the localisation of NFT in neurons of the hippocampus are the neurons which connect the hippocampus to other areas of the cortex, basal forebrain, thalamus, and hypothalamus. Because this area of the brain is essential for learning and memory, it could account for much of the clinical symptoms which are apparent in AD. The density of SP and NFT in the cerebral cortex is correlated with the appearance and degree of dementia (Roth et al 1966), similar correlations have been reported by others.

1.1.4.1 NEUROFIBRILLARY TANGLES

NFT are almost entirely composed of paired helical filaments (PHF) (although tangles of straight filaments or straight filaments combined with PHF have been observed in a few cases of AD (Shibayama and Kitoch 1978, Yagishita et al 1981 cited by Iqbal and Grundke-Iqbal 1991)) and deposited in the cytoplasm of mainly pyramidal dying neurons. The structure of PHF has been elucidated using the electron microscope. They are 28 - 36 nm wide, consist of pairs of protein fibres (approximately 12 nm in diameter), and are anti clockwise, helically twisted about each other at regular intervals of 70 - 90 nm (Katzman and Saitoh 1991). PHF are derived from the cytoskeleton. The cytoskeleton contains three fibrous proteins which are responsible for structural and transport processes in the nerve cells: microtubules, microfilaments, and neurofilaments. Therefore, Anderton (1991) has suggested that in neurons which develop NFT, the normal cytoskeleton disappears and is replaced by an accumulation of PHF.

The protein composition of PHF is not yet fully known because they have been shown (electrophoretically) to be complex structures. A major component of the PHF is believed to be the microtubule associated protein tau (Kosik 1989), although it is not the same as normal brain tau. Ubiquitin (a biochemical protease tag for abnormal proteins for the action of ATP-dependent nonelastic proteases (Hershko and Ciechanover 1982)) also appears in PHF (Mori et al 1987) and it has been suggested the protease is attached directly to tau (Iqbal and Grundke-Iqbal 1991). Amyloid A4 protein and its precursor has been identified in NFT isolates (Masters et al 1985 and

Yamaguchi et al 1990 respectively), although it has been argued that the amyloid A4 protein from the NFT is/is not the same as amyloid A4 protein in SP (Muller-Hill and Beyrether 1989). Finally, other cytoskeleton proteins microtubule associated protein 2 (MAP 2) and MAP 5 and casein kinase II have been identified in PHF (Katzman and Saitoh 1991). PHF tau and MAP 5 seem to be hyper phosphorylated, Ksiezak-Reding (1990) (cited by Kosik 1991) has estimated there to be four times more phosphate in PHF tau compared to normal brain tau.

1.1.4.2 SENILE PLAQUES

SP are complex structures which have a range of appearances from diffuse plaques to classical (or mature) plaques, burnt-out plaques are thought to have reached their final stage of development. Diffuse plaques are usually the most numerous type (Mann and Jones 1990) and are virtually indistinguishable from surrounding brain tissue (Yamaguchi 1991). There is no association with abnormal neurites or glial cells and amyloid fibrils appear to be minimal in content (Hardy and Allsop 1991). Mature plaques are spherical (approximately 100 μm in diameter) and contain the amyloid A4 protein which is packed into a dense central core (amyloid fibrils polymerise, condense and crystallise to form an amyloid plaque core). Other constituents of mature plaques include several cellular and extra cellular elements: aluminium in the inorganic form aluminosilicate (Candy et al 1986), altered neurites (themselves containing PHF), glial processes, microglial cells (Joachin and Selkoe 1989) astrocytes (Ulrich 1990), and ferritin (Grundke-Iqbal et al 1990). The dominant histological feature of burnt out plaques is an amyloid core with a few barely distinguishable neurites (Perry 1986). Virtually all plaques show gradients of density (the greatest density being internally) which implies the increase in plaque size is due to the diffusion or coalescence of the amyloid protein (Benes et al 1989). It is believed the different plaque appearances reflect different evolutionary stages (Selkoe 1991, Hardy and Allsop 1991).

The amyloid A4 protein (A4) consists of 42 amino acids, is a fragment of a larger 92 KD amyloid precursor protein (APP) (Kang et al 1987), and derived by proteolytic cleavage (Ishiura 1991). The gene for APP is located on the long arm of chromosome 21. It is a transmembrane protein with a short intracellular C-terminal tail and a much larger extracellular N-terminal segment. The A4 region is found close to the extracellular membrane and is partially buried in the membrane (Hardy and Allsop 1991). The production of A4 from its precursor is subject to intense examination. Normally, APP is broken down into products which do not form the A4 protein because cleavage occurs within the A4 protein sequence, close to the outer membrane. The enzyme responsible for this is APP secretase (Sisodia et al 1990). Golde et al (1992) have suggested that an alternative cleavage mechanism is responsible for A4 protein production. They describe an endosomal-lysosomal pathway which results in the cleavage of intact A4 that will precipitate and form SP.

1.1.5 NEUROCHEMISTRY

Numerous neurochemical changes are found in AD cortical brain sections post mortem. These are especially prominent in the cholinergic neurotransmitter system (in particular choline acetyltransferase (CAT)) and to a lesser degree in other cortical neurotransmitter systems such as the serotoninergic and noradrenergic systems (Corain et al 1990). Many of these changes can be experimentally reproduced by exposing animals to minute concentrations of Al (Zubenko and Hanin 1989 and Gulya et al 1990). These and other biochemical affects of Al are described in sections 1.2.4.

1.1.6 ORIGINS AND RISK FACTORS ASSOCIATED WITH ALZHEIMER'S DISEASE

AD is due in at least some patients, to a genetic defect. Nevertheless, the cause of AD in the majority of the patients is still unknown. Some researchers believe it is due to a slow viral infection (as in the dementing illnesses Kuru and Creutzfeldt-Jacob disease) but there is little credence in this hypothesis. Many scientists believe an environmental toxin is responsible for AD (Al is implicated by the majority of workers and there is much evidence to support a hypothesis in which Al has an active role in AD).

Whatever the cause of AD, there are various factors that have been associated with an increased risk of developing the disease. These include: severe head trauma (from accidents or dementia pugilistica of boxers), exposure to Al, the sex of an individual, and most importantly, old age. Other risk factors such as thyroid disease in woman, smoking, and alcoholism have been suggested but not confirmed. It is also argued by some that Parkinson's disease is a risk factor in the development of AD.

1.1.6.1 FAMILIAL ALZHEIMER'S DISEASE

The ability for AD to be passed from generation to generation was noted as early as 1929 by Flugel. Studies confirm an increased risk to the immediate families of an afflicted person (Goate et al 1989, Mayeux et al 1991). Such family studies strongly suggests an autosomal dominant form of Mendelian inheritance (Farrer et al 1990). When AD results from this apparent genetic link it is known as familial AD (or FAD).

Neuropathological hallmarks of AD are found in Downs syndrome, a known genetic disorder of chromosome 21. This suggests the genetic defect responsible for AD may be on chromosome 21. Genetic linkage studies on this chromosome by using early-onset families confirmed linkage. The disease locus was found to be centromeric to the locus D21S1/S11 on the long arm of chromosome 21 (St George-Hyslop et al 1987). These findings have been corroborated by Goate et al (1989). However, two further groups have failed to find a linkage at the disease locus found by St George-Hyslop and Goate (Pericak-Vance et al 1988 and Tanzi et al 1988 cited by St George-Hyslop et al 1990). This has lead to the assumption that the disease may be genetically heterogeneous (St George-Hyslop et al 1990) with AD being caused by

different genetic defects on different chromosomes (Selkoe 1991).

The gene for APP is also located on chromosome 21 and recently, Goate et al (1991) have identified a gene mutation for APP in two families which results in FAD. Since the original findings further families with FAD have been shown to carry the same mutation (Hardy et al 1991).

1.2 Al AND ALZHEIMER'S DISEASE

1.2.1 INTRODUCTION

Aluminium (Al) is a ubiquitous metal which represents approximately 8% of the earth's crust making it the third most abundant element after oxygen and silicon, and the most abundant metal. As metallic Al is too reactive to be unbound naturally, it is found only in complex compounds such as oxides in the form of bauxite and as silicates in the form of micas and feldspars (Martin 1991). Al is increasingly used in industrial and domestic products. This process of utilisation has lead to a large increase in the expose to Al for most people.

As early as 1814, reports were published concerning Al toxicity to animals (Orfila 1814, cited by Altmann 1991). Nevertheless, Al was considered an innocuous element until the middle of 1970 (Sorenson et al 1974). It is now clear that Al intoxication is responsible for inducing several pathological states which include: the fatal neurological disease dialysis encephalopathy (Alfrey 1986), fracturing osteomalacia (Parkinson et al 1979), and anemia (Kaiser and Schwartz 1985). There is considerable evidence from a number of sources which implicates Al as having a major role in AD, many of the toxic effects of Al being in some way related to the clinical or neuropathological observations in AD. The causal relationship between dialysis encephalopathy and Al lead many scientists to examine the links between AD and Al, therefore it is appropriate to discuss dialysis encephalopathy further.

1.2.2 DIALYSIS ENCEPHALOPATHY

Maintenance haemodialysis therapy emerged as a successful treatment of patients with end stage renal disease during 1961. Soon after the introduction of this treatment, Kerr et al (1966) noted that classical symptoms of uremia (anemia, neuropathy, hyperparathyroidism, and matastic calcification) occurred more frequently in uraemic patients receiving dialysis compared to those which were not.

In 1972, a distinctive neurological disease, dialysis encephalopathy (dialysis dementia) was described by Alfrey et al (1972) in the USA. It was subsequently reported by others worldwide (Mahuraker et al 1973, Platts et al 1973, Barratt and Lawrence 1975, Burks et al 1976). Following three to seven years on haemodialysis treatment, the first clinical symptoms of the disorder were evident, usually as a speech disorder. Other

clinical features included: directional disorientation, twitches, dyspraxia, motor apraxia, episodes of muteness, personality changes and vivid visual, and auditory hallucinations (Alfrey 1986). The symptoms would often fluctuate during the early stages of the disease but were always worse after dialysis treatment. With the progression of the disease the features became persistent until the patient was immobile and mute. Coma and death usually followed six to nine months after the first clinical symptoms appeared.

After exclusion of other possible causes of dialysis encephalopathy (a slow viral infection as in Creutzfeldt-Jacob disease and normal pressure hydrocephalus), researchers turned to the possibility of an environmental toxin as a cause of the disease. After analytical problems were encountered and overcome, Al was identified in the brain grey matter of patients who died from dialysis encephalopathy. Significantly higher concentrations of Al were found in those patients (24.98 μg/g) compared to dialysis patients who died from other causes (6.5 μg/g) or non-uremic patients (2.18 μg/g) (Alfrey et al 1976). Cartier et al (1978), McDermott et al (1978), and Crapper et al (1980) confirmed these original observations. Furthermore, Al accumulation was observed in virtually all other tissues of patients with dialysis encephalopathy compared to the tissues of other dialysis patients (Alfrey et al 1980). Addition evidence which linked Al to the pathogenesis of dialysis encephalopathy was obtained by epidemiological studies in Europe (Wing 1980). Platts et al (1977), Ward et al (1978), and Davison et al (1982) correlated high Al water content (water is used in the preparation of the dialysate) to an increased frequency of dialysis encephalopathy. When Al was removed from the tap water by reverse osmosis (prior to preparation of the dialysate), dialysis encephalopathy was largely eradicated (Alfrey 1980, Davison et al 1982, Sideman and Manor 1982). An acceptable Al concentration in water for the preparation of dialysate was set at 10 μg/l and it was thought this was an adequate precaution (Alfrey 1986). Effective chelation therapy using desferrioxamine in haemodialysis patients to remove Al resulted in beneficial effects to the patient (Ackrill et al 1980, Arze et al 1981, Milne et al 1983) and this was generally accepted as a final piece of supporting evidence which confirmed the central role Al plays in dialysis encephalopathy.

A reduction in dialysate Al concentration to the standard set at 10 μg/l has failed to eradicate the disease completely. Heaf and Nelson (1984) have described similar symptoms to those observed in demented patients during the 1970's with an Al dialysate concentration of 1 μg/l. Similarly, Alfrey (1986) described three patients who developed a dialysis encephalopathy when dialysate Al had been consistently less than 10 μg/l. The link between both reports was the chronic use of orally administered Al containing phosphate binding gels by all patients. Uraemic, non dialysed children have also displayed symptoms of dialysis encephalopathy. Again, Al containing phosphate binding gels were used at concentrations greater than 100 mg/Kg/day (Milne et al

1983).

As Al has been shown to be the causative agent in dialysis encephalopathy, Winney et al (1986) suggested the monitoring of serum Al in haemodialysis to identify patients at risk from dementia. Chazan et al (1991) have shown a correlation between serum Al concentration and mortality in patients undergoing long term haemodialysis who do not show overt Al toxicity. A significant correlation appeared when serum Al exceeded 40 µg/l, the mortality rate rose by 18% at 41 - 59 µg/l and progressively increased to 60% at 200 µg/l.

1.2.3 BRAIN AL LEVELS IN ALZHEIMER'S DISEASE

Despite serum Al levels being within normal limits (mean 7.1 c.f. 5.9 µg/l) (Shore and Wyatt 1983), AD patients have elevated levels of Al occurring within the hippocampus (7.5 c.f. 2.7 mg/Kg dry weight) and the cerebral cortex (9.1 c.f. 2.8 mg/Kg dry weight) (Ward and Mason 1986). It is these brain areas where mental functioning is abnormal in AD. Al has also been detected both in SP (Candy et al 1986, Edwardson et al 1991) and NFT (Crapper et al 1973, Perl and Brody 1980).

1.2.4 BIOCHEMICAL EFFECTS OF Al

Once Al enters the cell (via Tf or a low molecular weight species), it is possible that it could co-exist in the labile Fe pool of the cystol (May and Williams cited by Birchall and Chappell 1988) or bind to ligands which have stronger binding constants than those of the Al complexes within the labile pool (e.g. citrate) (Birchall and Chappell 1988). Intracellular Al is most commonly found bound to phosphate or in the active centre of metalloenzymes. The consequences of such binding are expressed in altered second messenger systems, enzymes, and neurotransmitters. Furthermore, Al has been shown to bind to the phosphate groups of ADP and ATP, and to DNA in the cell nucleus, although Martin (1991) disputes this.

1.2.4.1 Al AND NEUROTRANSMITTERS

Al is known to affect both the synthetic and metabolic enzymes of the cholinergic neurotransmitter system, as well as the adrenergic and amino acid neurotransmitter systems (Boegman and Bates 1984). Table 1.1 illustrates the effects Al administration has upon the neurotransmitters of the brain in experimental rabbits. In comparison to AD there are many similarities in the actions of Al, but differences do exist.

At least 60 neurochemical reactions are altered in the presence of Al, these include: toxic affects on the membrane, nuclear transcription processes, and cytoskeletal functions (Crapper McLachlan et al 1991). A review of the Al induced neurochemical effects is given by Crapper McLachlan.

Neurotransmitter	AD	Al induced neurodegeneration
acetylcholine	decreased	decreased
serotonin	decreased	decreased
noradrenaline	decreased	decreased
glutamate	decreased	decreased
aspartate	decreased or unchanged	decreased
taurine	unchanged	decreased
GABA	decreased	unchanged
somatostatin	decreased	unchanged
neuropeptide Y	decreased or unchanged	unchanged
substance P	decreased or unchanged	decreased

Table 1.1 Neurochemical effects of Al toxicity compared to AD.
Taken from Beal et al (1989)

1.2.4.2 Al AND TETRAHYDROBIOPTERIN METABOLISM

Al directly affects neurotransmitter synthesis by affecting the metabolism of tetrahydrobiopterin (BH_4). BH_4 is an essential co-factor in the in vivo hydroxylation of phenylalanine, tyrosine, and tryptophan during the formation of the catecholamine (dopamine and noradrenaline) and indolamine (serotonin) neurotransmitters (Katoh and Sueoka 1986). It is now understood that there is a defect in the processing of BH_4 in AD sufferers, which consequently affects catecholamine and indolamine synthesis. The CSF neopterin levels are unchanged, but biopterin levels are decreased in AD. This indicates the failure of BH_4 synthesis is with dihydroneopterin triphosphate (DHNTP), the precursor of BH_4 (Hamon and Blair 1987) and it is in this second stage of BH_4 production that the Mg requiring enzyme pyruvoyl tetrahydropterin synthetase is faulty. The addition of Al to an in vitro human temporal cortex preparation from a control subject resulted in the decreased biosynthesis of BH_4. A similar action was observed in untreated preparations from AD patients (Cowburn et al 1990).

BH_4 is oxidised to quinonoid dihydrobiopterin qBH_2 (in its capacity as a co-factor) during the production of neurotransmitter substances. The conversion of qBH_2 to 7,8-dihydrobiopterin (7,8-BH_2) ensues, and would be lost from the cell if it were not for the 'salvage' action of dihydropteridine reductase (DHPR) which reduces BH_2 to BH_4. Therefore DHPR is an enzyme which is essential for the maintenance of BH_4 concentrations in the brain. Altmann et al (1987a) have shown a reduction of DHPR activity in haemodialysis patients who had modest (15 - 190 µg/l) plasma Al concentrations and that Al concentrations inversely correlated with brain DHPR

(Altmann et al 1987b). This has lead the group to suggest Al may underline the inhibition of DHPR activity which disturbs neurotransmitter metabolism (Altmann et al 1989).

1.2.4.3 MAGNESIUM REQUIRING ENZYMES

The ionic radius of Al^{3+} is similar to that of Mg^{2+} (0.66 Å), therefore Al^{3+} is most likely to compete against this cation for ligands. It is possible that where ever Mg^{2+} is present as a co-factor for an enzyme reaction, Al^{3+} can interfere. Al^{3+} has a very slow ligand exchange rate which is 10^5 slower than Mg^{2+} (Martin 1991), which is one reason why when Al^{3+} is present as a substituted co-factor in enzymic reactions, these reactions are inhibited. During a metalloenzyme reaction, it is common for the metal ion to change its valency state. Another mechanism of inhibition exhibited by Al^{3+}, is in its failure to change its valency, thus inhibiting the reaction. Approximately 300 enzymes are known to require Mg^{2+} as a co-factor and Al within the brain can inhibit a number of Mg dependent enzyme systems (Ebel and Gunther 1980, Garfinkel and Garfinkel 1985, Trapp 1986).

For example, hexokinase (a Mg-ATP dependent enzyme and responsible for the first stage of glucose phosphorylation) has been found to be competitively inhibited by Al-ATP (Trapp 1986). Activity of brain hexokinase in Al fed rats (100 μM in drinking water) was reduced to 73% of the activity found in controls after a year (Cho and Joshi 1988). The activity of superoxide dismutase (SOD) requires the presence of Cu and Zn, and it has been demonstrated that Al inhibits the enzyme in vitro (Shankin-Kestenbaun et al 1989). It is thought Al occupies the site for either Zn or Cu in the active centre of the enzyme, thus altering its structure and activity. Sodium-potassium ATPase is another enzyme which is inhibited in the presence of Al (Trapp 1986) and is important to correct neuronal function (Cowburn et al 1990). The sodium pump within the cell membrane is dependent upon sodium-potassium ATPase as a source of energy. This regulates the ratio of potassium/sodium in a variety of cells and maintains the correct electrochemical environment in neurons and other cells. The enzyme is also required for the mechanism of synaptic transmission and conductance along axons. Adenylate cyclase (another Mg dependent enzyme) activity has also been shown to increase in the presence of Al, while the Ca-calmodulin dependent phosphodiesterase is inhibited (Richardt et al 1985). Both could contribute to raising the concentration of 3', 5'-cyclic adenosine monophosphate (cAMP) in the brain (Johnson and Jope 1987).

1.2.4.4 Al AND SECONDARY MESSENGERS

The secondary messengers cAMP (Cowburn et al 1990) and inositol triphosphate (IP_3) (Cochran et al 1990) have shown altered behaviour when Al is present. An increase in cAMP or IP_3 results in increased phosphorylation of proteins and activation of other enzymes via the enzyme protein kinase. Proteins which are associated with

microtubules (tau and microtubule-associated protein 2 (MAP 2)) and neurofilaments have been shown to be phosphorylated by protein kinase (Johnson and Jope 1986 and Theurkauf and Vallee 1983 respectively). Therefore, chronic exposure to Al would raise cAMP levels which may induce abnormally phosphorylated microtubules and neurofilaments. Lee et al (1991) have shown the presence of abnormally phosphorylated tau in paired helical filaments (which are the basis of NFT).

1.2.4.5 ALTERATIONS IN VISUAL EVOKED POTENTIALS BY Al

Visual evoked potentials (VEP) are generated as a result of transient changes in the electrical activity of the brain in response to an external stimulus. The most common stimuli used to record VEPs are visual flash and checkerboard reversal patterns because they produce relatively large and reproducible potentials.

Harding et al (1985) demonstrated that in patients with clinically diagnosed pre senile dementia (compared to patients with cortical atrophy but no dementia), there was a slowing of the major positive (P2) component of the VEP to flash stimulation: the VEP to pattern reversal stimulation (P100) was of normal latency. These findings were corroborated by Wright et al (1986). Therefore, it has been suggested that VEPs can be used as a diagnostic marker for AD because a characteristic alteration of the normal P2-P100 latency exists in such people (Wright et al 1986).

Altmann et al (1989) measured VEPs in a group of ten haemodialysis patients without overt Al toxicity (mean serum Al concentration was 72 $\mu g/l \pm 37.9$). They observed a delay in the flash stimulated potential, whilst the pattern stimulated potential was within the normal range. There was a significant correlation between the oral Al intake of a patient and the difference in the flash pattern times. It was suggested that alterations in VEPs were related to Al intoxication rather than any psychomotor function (which was also examined).

1.2.5 EPIDEMIOLOGICAL LINKS BETWEEN Al AND ALZHEIMER'S DISEASE

Seven independent epidemiological studies have concluded that an increased water Al concentration is a risk factor in AD (Crapper McLachlan 1991 and references therein) and only one has failed to find any such relationship (Wettstein et al 1991). Although each of the studies were open to criticism on a number of grounds, this would serve only to reduce the chance of a positive correlation (Edwardson et al 1991). The fact a positive correlation was found between AD and water by the majority of researchers suggests that the possibility of the relationship occurring by chance was very small (Crapper McLachlan 1991).

Another epidemiological study by Graves et al (1990) has demonstrated an association between Al containing antiperspirants and AD, with a trend towards increased risk with

increased frequency of use. The authors were cautious in interpreting their results, but noted they were biologically plausible.

The addition of DFO to AD patients and the subsequent decrease in the rate of progression of deterioration those taking the drug (McLachlan et al 1991) indicates the role of Al in the brains of AD patients to be more than just coincidental. DFO removes both Fe and Al from the body. However, Crapper McLachlan found Fe levels unaltered in the brain of patients and it was therefore assumed the effect of the drug was due to the Al being removed from the brain.

1.2.6 OCCUPATIONAL EXPOSURE TO Al AND NEUROTOXICITY

Spofforth (1921) was the first to describe the toxic effects of Al in an industrial context. The case report focussed on a metal worker who was exposed to Al and developed encephalopathy. No further reports on Al induced encephalopathy were published until in 1962, when a factory worker was documented as having a neurological disorder which was probably attributed to inhalation of Al dust (McLaughlin et al 1962). Prior to this, the only known toxic effects of Al were in pulmonary fibrosis in Al workers.

Al has now been associated with several conditions including osteoflourosis, lung and bladder cancer, asthma, and chronic lung disease (Longstreth et al 1985 and references therein). In the same report by Longstreth, neurological disorders in three workers at an Al smelting plant were discussed. The illnesses were related to occupational exposure and no conclusive evidence for a role for Al was presented. The authors did however state, 'It is theoretically possible that long term low level exposure to Al could lead to neurotoxicity.' Finally, Canadian miners who were exposed to McIntyre powder (a finely ground Al and Al oxide which was used as a prophylactic agent against silicosis) performed less well in standard cognitive tests than did miners who were not exposed to McIntyre powder (Rifat et al 1990). There was an increased probability that an impaired performance was given in the tests when exposure to the powder was increased.

Significantly increased serum Al concentration have been found by Rollin et al (1991) in Al exposed foundry workers, although an increase in urinary Al was not evident. This suggested accumulation of the metal was occurring. Goran et al (1991) also found increased blood and urinary Al in workers exposed to Al flake powder and also concluded that Al accumulation in various organs was occurring.

1.3 OTHER Al ASSOCIATED DISORDERS

1.3.1 Al AND OTHER NEURODEGENERATIVE DISEASES

AD is not the only neurodegenerative disease to be associated with Al. There are strong

indications that Al is implicated in the etiology of Parkinsonism dementia and amyotrophic lateral sclerosis (AL.) of Guam (Perl et al 1982). Elevated levels of Al, Mn, and Fe in the soil and drinking water of the island were identified along with lower levels of Mg and Ca (Garruto et al 1984). This was coupled to an increase in the prevalence of ALS (for further details see section 5.1.5.4). Individuals with Down's syndrome have shown neurochemical (Godridge et al 1987) and neuropathological (Mann 1988) changes identical to those of AD and Al has been identified in SP from Down's syndrome brains (Edwardson et al 1991).

1.3.2 Al AND BONE DISEASE

Al associated osteomalacia is distinct from the general cause of osteodystrophy (a disturbance in Ca and phosphate metabolism) because it is untreatable with vitamin D (Hodsman et al 1981). Osteomalacia was first described by Schorr (1968) in patients undergoing haemodialysis and Parsons et al (1971) were the first to identify an increased Al content of bone in such people. It was observed that there was a good correlation between osteomalacia and dialysis dementia in areas where Al was found in the dialysate (Ward et al 1978, Parkinson et al 1979), and between osteodystrophy (osteomalacia and osteitis fibrosa) and high Al deposition in the bone (Cournot-Witmer et al 1981, Winterburg et al 1990). Histochemical staining has shown Al deposition at the junction between osteiod and mineralised bone (Ebina et al 1991).

1.3.3 Al INDUCED ANAEMIA

Anaemia is frequently associated with chronic renal failure which involves the increased destruction and decreased production of red blood cells (Erslev 1983). The cause of anaemia in such patients is multifactorial, but Al is an important factor which affects it (Kaiser and Schwartz 1985). Elliott et al (1978) described a causal relationship between anaemia and Al toxicity in which haemoglobin concentrations were reduced prior to encephalopathy. Short et al (1980) and O'Hare and Murnaghan (1982) provided supporting evidence for a causative role for Al in anaemia. Short et al (1980) described a severe, microcytic hypochromic non-iron deficient anaemia in haemodialysis patients who all had elevated plasma Al concentrations. The majority of patients showed clinical and histological evidence of Al toxicity. O'Hare and Murnaghan (1982) reported an increase in haemoglobin with a concomitant fall in dialysate and subsequent fall in plasma Al concentrations in dialysis patients. No new cases of any Al associated disease were found after the introduction of water treatment.

The exact pathogenic mechanism of Al induced anaemia has yet to be discovered, although several theories have been postulated.

1.4 Al ENTRY INTO THE BODY

Al may enter our bodies by a variety of ways, although the oral route of administration

(i.e. via the gastrointestinal tract) is by far the most significant in normal healthy people. Other routes of Al entry include the lungs, nose, and possibly the skin. In haemodialysis patients and in patients undergoing parenteral nutrient, Al may enter systemic circulation directly as a result of their mechanism of treatment.

Inhalation of dust is unavoidable and as Al is such a ubiquitous element there is no doubt Al is also inhaled. Evidence from occupational exposure (see section 1.2.6) clearly demonstrates Al toxicity from this mode of entry into the body. Perl and Good (1987) have shown the direct uptake of Al into the brain via the nasal-olfactory pathways in the rabbits exposed intranasally. The skin is frequently exposed to Al in the form of antiperspirants and cosmetics and Graves et al (1990) have demonstrated an association between Al containing antiperspirants and AD.

As previously mentioned, the primary route of Al entry into systemic circulation is via the gastrointestinal tract. A source of Al encountered by most people would be through the use of medicines. Al salts are found in antacids, phosphate binders, buffered aspirins, vaccines, and allergen injections (Lione 1985). Al may enter the body via ingestion through food which has been cooked or kept in an Al container. This may not just be limited to the home, but to the food industry using Al in large scale processes. Al may occur directly in foods in the form of sodium aluminium phosphate or as a decoration on the surface of cakes.

One source of Al that all are exposed to is Al in the water supply. In many parts of the country aluminium sulphate is routinely to added to water to remove particulate matter and substances causing colour. It also aids effective and long-lasting disinfection. Another function of Al is to improve the appearance of the water. Although most of the added Al is removed in the later stages of treatment, some of the metal remains in the water and is available for absorption by humans. The European Community maximum admissible concentration (EC MAC) for Al in drinking water is 0.2 mg/l, this however is not based on toxicological data but aesthetic considerations (Gardner and Gunn 1991). Typical Al concentrations in the UK drinking water are between 50 - 150 µg/l, deviations from this range will occur if pH control is poor or if the water is particularly dirty (Gardner and Gunn 1991).

The Camelford incident (6th July 1988) indicated the danger of Al compounds when in contact with the human body. The delivery of twenty tonnes of aluminium sulphate (8% solution) into the wrong storage tank caused devastating consequences. Al was recorded at 620 mg/l, sulphate concentrations reached 4500 mg/l, and the pH was as low as 3.9 - 5.0 (Second Report of the Lowermoor Incident Health Advisory Group, 1991). Subsequently, the South West Water Authority received many complaints about the water, which included: sickness, blisters, mouth ulcers, sore throats, irritations of the skin, and blue water (Milne 1989). As a secondary consequence copper levels

increased dramatically due to the acid water attacking copper pipes, releasing the element into the water supply. Up to 9 mg/l from 'cold' taps and 22.5 mg/l from 'hot' taps were registered which was in excess of 3 mg/l allowed by the EC MAC (Second Report of the Lowermoor Incident Health Advisory Group, 1991).

1.5 Al ABSORPTION

The average daily intake of Al in a normal healthy adult has been shown to be 3 - 20 mg (Wilhelm et al 1990), but can vary considerably with the diet, food source, and medication. Lione (1985), estimated Al intake to be reach 5 g in patients on antacids or phosphate binders. It is generally accepted that virtually all absorbed Al is subject to elimination in the urine ($\approx$ 99%), but excretion in the bile ($\approx$ 0.1%) has also been noted (Molitoris et al 1989). Increased plasma Al levels and urinary Al excretion after administration of high oral doses of Al in humans clearly demonstrates gastrointestinal absorption (Yokel and McNamara 1988, Molitoris et al 1989) and it is therefore an important area of study.

Al exists predominantly in uncomplexed forms in water and as such it is possible to be absorbed more readily in the gastrointestinal tract. It is one of the major sources of Al in normal people. The low pH of the stomach and proximal duodenum are most likely to be the main areas of absorption because the Al species are soluble in this pH range. The increased pH of the distal regions of the small intestine will induce Al insolubility and hence reduced absorption.

Factor	effect	reference
Physical		
citrate	enhancement	Slanina et al (1984)
silicic acid	possible inhibition	Birchall (1991b)
iron	enhancement	van de Voet and de Wolff (1986)
	inhibition	Blaehr et al (1986)
fluoride	inhibition	Lote and Saunders (1991)
pH	dependent on pH	Martin (1986)
calcium	inhibition	Lote and Saunders (1991)
Physiological		
PTH	enhancement	Mayor and Burnatowska-Hledin (1983)
	inhibition	Hell et al (1989)
Vitamin D_1	enhancement	Burnatowska-Hledin et al (1984)
Pathophysiological		
Uremia	enhancement	Alfrey et al (1980)

Table 1.2 Factors effecting gastrointestinal absorption of Al
Adapted from Lote and Saunders (1991)

The exact mechanism(s) of gastrointestinal absorption remains unidentified (Wilhelm et al 1990, Lote and Saunders 1991), although certain factors are known to influence this process. It is excepted that Al transfers across the gastrointestinal tract and reaches systemic circulation. Therefore a brief summary (in the form of table 1.2) will suffice to discuss this aspect of Al absorption. It must be remembered that the chemical speciation of Al is of vital importance to its absorption, which can be effected by dietary, physiological, and pathophysiological parameters.

1.6 Al SOLUTION CHEMISTRY

Al exhibits only one valency state (the trivalent Al^{3+} ion) and has an ionic radius of 0.54 Å. It has a hydration number of 6 and remains unhydrolysed below pH 3 (Baes and Mesmer 1976).

The species of Al^{3+} formed in water is dependent upon metal ion concentration and pH, both being important when considering interactions with various physiological ligands. At relatively high concentrations of Al, polynuclear species can exist as a result of extensive hydrolysis. However, as biological systems are exposed to relatively low Al concentrations, these species will not exist and therefore they will not be discussed further.

At micromolar concentrations of Al that are usually seen in biology, only mononuclear species are formed. These reactions occur rapidly and reversibly. In acidic solutions (pH < 5), Al(III) exists as the octahedral hexahydrated ion $[Al(H_2O)_6]^{3+}$ which is normally abbreviated to Al^{3+}. As a solution becomes more basic $[Al(H_2O)_5(OH)]^{2+}$, $[Al(H_2O)_3(OH)_3]$, and $[Al(H_2O)_4(OH)_2]^{+}$ are formed due to successive deprotonations until, at neutral pH an $Al(OH)_3$ precipitate is formed. The precipitate redissolves in basic solutions and at physiological pH 7.4, the predominant species is the tetrahedral aluminate ion $Al(OH)_4^-$ (Martin 1988). The solution chemistry of Ga and Fe is remarkably similar to that of Al, with the exception of Fe having two valency states. These similarities results in similarities in distribution and transport of Al and Fe in the body (Crumbliss and Garrison 1988) and by implication Ga would share similar distribution and transport patterns. A summary of the species existing at varying pH and Al and Ga concentrations is given in Baes and Mesmer (1976).

1.7 Al SPECIATION IN THE HUMAN BODY

In human plasma, Al is primarily bound to the Fe binding protein transferrin (Tf) (Trapp 1983, Rahman et al 1985, Rahman et al 1986 and Martin 1987). Al^{3+} and Fe^{3+} have similar ionic radii (0.54 and 0.65 Å respectively) which allows Al to fit in the binding sites on the Tf molecule. Although Fe-Tf binding is 10^9 times stronger (Martin 1991) than that of Al-Tf, the protein has approximately 70% of the Fe binding

sites empty. Therefore, Al can bind to the protein without competition because there are sufficient sites for both metals to bind without competition (in normal Al and Fe plasma concentration ranges). Al has also been reported to be bound to other high molecular weight proteins such as albumin (Gardiner et al 1984, Bertholf et al 1984, and Glick 1991) and ferritin (Fleming and Joshi 1987, Joshi and Zimmerman 1988).

It has been shown that not all plasma Al is bound to high molecular weight proteins (Gardiner et al 1984 and van Ginkel et al 1990). It also exists in complex with low molecular weight ligands which are most likely to be oxygenated and negatively charged (Martin 1991). Generally, Al cations form strong complexes with ligands such as carboxylates, phosphates, nucleotides and polynucleotides (Martin 1986). Martin (1986, 1987) has suggested on the basis of stability constants, that citrate (in the form of a tricarboxylate anion at pH < 6 and at a concentration of 10^{-4} M in plasma) would be most likely to bind Al. Although in plasma, Ca^{2+} is usually bound to citrate, Al^{3+} would easily displace it (Martin 1991). Al-citrate binding in plasma has been confirmed experimentally by van Ginkel et al (1990) using the rat.

1.8 TRANSFERRIN

Once Al enters systemic circulation, the majority of the metal is bound by Tf. It is therefore appropriate to discuss this protein further.

Transferrins (originally named siderophilin, meaning Fe loving) are proteins that reversibly bind Fe and are found in biological fluids. The name transferrin (Tf) was derived by Holmberg and Laurell (1946) in recognition of the function of the protein in relation to Fe transport.

Although Tf (seroTf) found in the blood is the most common (delivering Fe to the majority of body tissues), other forms of Tf occur which transport Fe to areas where plasma is restricted. These include mucosal Tf (derived from biliary secretions of plasma Tf), testicular Tf, and a Tf of the central nervous system (Huebers and Finch 1987 and references therein). Apart from these Tfs, three other groups of closely related proteins exist. Alderton et al (1946) discovered the Fe binding and antibacterial properties of egg white were due to ovotransferrin (conalbumin), which is a major protein of egg white and is also found in bird and reptile oviduct secretions. Lactoferrin (lacto transferrin) is found in milk and other extracellular secretions such as: colostrum, tears, pancreatic juice, and gastrointestinal secretions (Aisen and Listowsky 1980). Finally, p97 is an integral cell surface glycoprotein and a human malignant melanoma associated antigen (Brown et al 1982).

The molecular weight of Tf is calculated to be 79 570 D (Huebers and Finch 1987, Welsh 1990), but more commonly it is quoted as ≈ 80 KD. Other Tfs range in molecular weight from 77 - 82 KD (Welsh 1990). The human Tf gene is known to be

located on chromosome three, at position q21-25. The cDNA base sequence has also been characterised (Yang et al 1984). Production of Tf is primarily found in the hepatocytes of the liver, although other sites do produce local Tf (see previous paragraph). Tf is a monomeric glycoprotein (adult mean plasma concentration 3.75 x 10^{-5} M, 95% confidence limits 2.45 - 5.73 x 10^{-5} M) of 679 amino acid residues. It is folded into two homologous globular lobes and further subdivided into domains surrounding a cleft (Aisen 1989) giving rise to a prolate ellipsoid shape. Each lobe is capable of binding an Fe atom (see fig 1.1).

There are three forms of Fe bearing Tf: diferric Tf in which both binding sites are occupied, monoferric A Tf (also known as C-terminal Tf or Tf-Fe) in which a single Fe atom is bound at the acid stable C-terminal, and monoferric B Tf (N-terminal Tf or Fe-Tf) in which the acid labile N-terminal is occupied with Fe (Vogel et al 1989, de Jong et al 1990). Tf also exists in a state where both binding sites are vacant, this is known as apo Tf. The stability constants for ion-Tf binding of three metals of interest are given below. These are optimum conditions and deviations in pH and bicarbonate concentration affect the stability constants.

Ion	Ionic radius (nm)	K_1	Log K_1	K_2	Log K_2
Al^{3+}	0.054	7.9×10^{12}	12.9	2.0×10^{12}	12.3
Ga^{3+}	0.062	2.0×10^{20}	20.3	2.0×10^{19}	19.3
Fe^{3+}	0.065	3.16×10^{22}	22.5	2.5×10^{21}	21.4

conditions: pH 7.4, bicarbonate concentration 27 mmol/l

Table 1.3 Stability constants and atomic radii of three metals which bind to transferrin.
Adapted from Martin et al 1987.

The distribution of Fe in the different binding sites has received much attention. This is mainly due to the understanding of the molecular physiology of Fe transport it would reveal (Zak and Aisen 1986). However, many conflicting results have been published. For example, Huebers et al (1984) and Beguin et al (1988) described random occupancy of binding sites in normal subjects and patients with a variety of haematological disorders, while Zak and Aisen (1986) and Vogel et al (1989) showed the distribution of Fe to be non-random.

Measurements of the amount of Fe bound to Tf is known as the total iron binding capacity (TIBC), in conjunction with measured total plasma Fe to obtain Tf saturation (plasma Fe/TIBC) these are used as a clinical tool to assess the human metabolic status (International Committee for Standardisation in Haematology, 1978). At any one time

only ≈ 30% of the binding sites are full (i.e 30% Tf saturation) in normal individuals. Given a Tf concentration of 3g/l, the total Fe binding capacity of Tf would be 4.2 mg/l based on the fact that each Tf molecule can bind two Fe ions. Deviations from the standard 30% Tf saturation can occur either by a reduction in the concentration of Tf or an increase in concentration of Fe. Physiological factors which alter transferrin saturation are reviewed by Huebers and Finch (1987).

Using crystallographic evidence from human lactoferrin (Anderson et al 1987), the actual Fe binding sites appear to be buried quite deeply in each lobe 10 Å from the protein surface in the clefts (see fig 1.1). This can explain in part, the great stability constant of the Fe-Tf complex (Baker et al 1987). The protein ligands involved in Fe binding at both sites in lactoferrin are: three anionic oxygen atoms, the phenolate oxygens of two tyrosines, a carboxylate oxygen from aspartic acid, and one neutral nitrogen, the imidazole nitrogen from a single histidine. On the N-lobe these are Asp-61, Tyr-93, Try-191, and His-252 (C-lobe; Asp-404,Try-447, Try-540, and His-609) (see fig 1.2) (Baker et al 1987). Arg-121 is situated so as to provide anchorage for the (bi)carbonate anion.

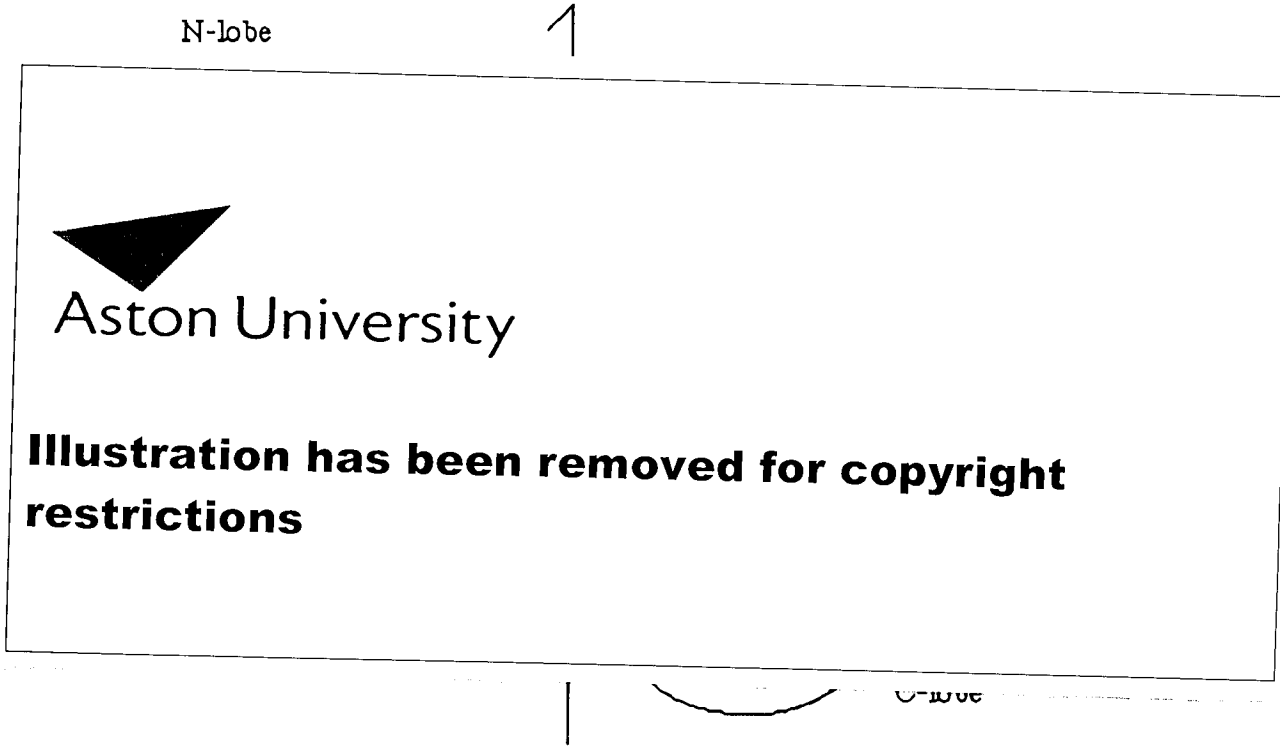

Fig 1.1 Simplified diagram of the human lactoferrin molecule.
The relative orientation of the two lobes is related by a twofold screw axis (arrow).
Each of the domains within the lobe is labelled one and two.
Taken from Baker et al (1987).

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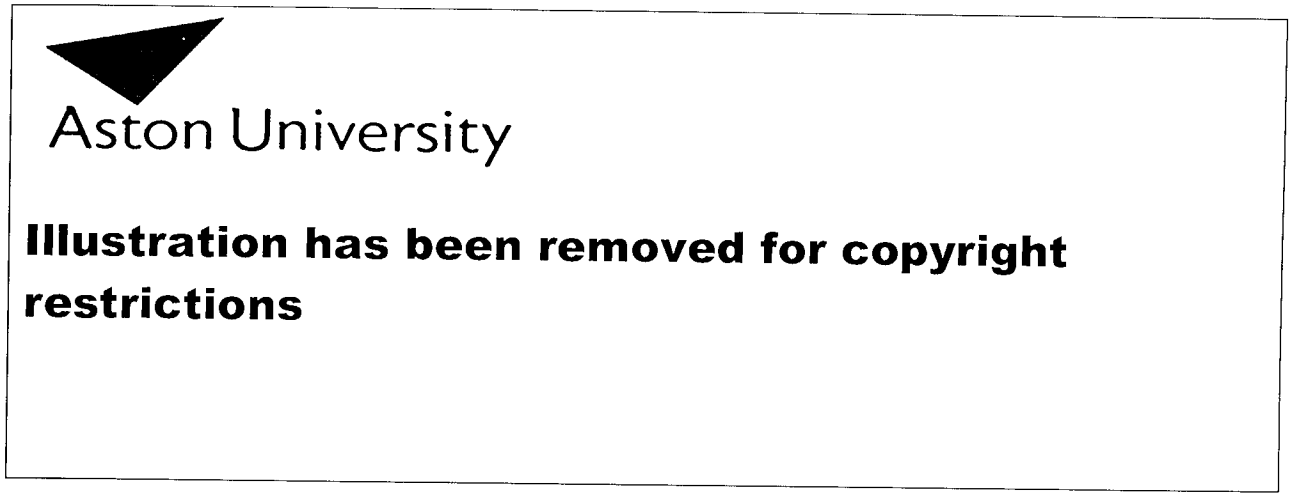


Fig 1.2 Iron binding site on the N-lobe of human lactoferrin.
Taken from Baker et al (1987).

For Fe to bind to Tf , the (bi)carbonate anion is essential (Schade et al 1949, Aisen et al 1969), but no definitive observations have been made to distinguish whether bicarbonate or carbonate is the active species physiologically. Carbonate has been shown to be the binding anion in one lobe of Tf when Cu is bound (Dr. R. Evans, personal communication, 1992), but there is still uncertainty about the exact anion involved in Fe, Al, or Ga binding.

X-ray crystallography of lactoferrin (Baker et al 1987) shows there to be a bulge of electron density in each cleft which could be attributed to the (bi)carbonate anion, coordinated water molecules or both (see fig 1.2). In the absence of (bi)carbonate, the anion's function in metal binding may be replaced only by a limited class of carboxylic acids (Harris and Aisen 1989 and references therein). Due to the nature of the relationship of the anion and Fe during metal binding, (bi)carbonate is known as a synergistic anion (Anderson et al 1990, Marques et al 1990). Without bicarbonate, binding of Ga is weak and to a very small degree (Farrar et al 1990). In the presence of bicarbonate, in vitro binding is more physiologically representative (Hodgkins et al 1991, Farrar et al 1991a).

Although Fe is the most important metal in relation to Tf, it is known that Tf can bind a wide range of other metals. Clinicians exploit this fact and use ^{111}In as a tracer of metabolic processes (Beamish and Brown 1974) and ^{67}Ga as a tumour imaging agent

(Sephton and De Abrew 1990). Apart from Fe, no other metal binding to Tf has any physiological significance with the exception of perhaps zinc (Evans 1976, Morgan 1981). Other ions which are able to bind to Tf include metals from the first row of the transition elements, the lanthanides and actinides (Al^{3+}, Cd^{3+}, Co^{3+}, Cr^{3+}, Cu^{3+}, Er^{3+}, Eu^{3+}, Hf^{4+}, Ho^{3+}, Nd^{3+}, Ni^{2+}, Mn^{3+}, Pr^{3+}, Pt^{3+}, Pu^{4+}, Sc^{3+}, Tb^{3+}, Th^{4+}, TI^{3+}, V^{3+} and VO^{2+}) (Harris and Aisen 1989 and references therein). Despite in vitro binding occurring with some ions, it should not be assumed in vivo binding would also occur. Copper, for example will bind to Tf in vitro, but no copper has been demonstrated in Tf which was precipitated (by antibodies) out of normal plasma, despite the presence of copper in concentrations relative to those of Fe and free binding sites on the protein being available (Huebers and Finch 1987). This is explicable because the majority (95%) of Cu in vivo is bound to ceruloplasmin (Sarkar 1988).

Fe is of major importance to virtually all forms of life. Ferric Fe is the predominant form of the metal under physiological conditions, primarily because it is bound to Tf in its trivalent state and ferrous Fe is readily oxidised to ferric Fe under the extracellular conditions found near most aerobic cells (Griffiths 1987). However, the free ferric ion (Fe^{3+}) is reduced to ferrous Fe during its sequestration from ferritin. Fe is found mainly in the form of haemoglobin in the body, although significant amounts are present in myglobin and in complex with enzymes where acts as a co-factor for the basic metabolic oxidation and reduction reactions. Storage of intracellular Fe is by the protein ferritin, primarily in hepatocytes of the liver, which to a certain degree can limit its availability (Harrison and Lilley 1989).

Fe absorption occurs mainly in the duodenum and proximal jejunum and subject to a variety of factors (Woods et al 1990). Uptake through the brush border intestinal mucosa involves the proteins Tf and ferritin where it can be released into the circulation (Wapnir 1990) or it may enter the body as ionised Fe or Fe bound to Haem (Huebers and Finch 1987). Once Fe is bound to Tf, the protein takes up its functional role of Fe transportation, e.g. delivery of Fe to developing red blood cells in bone marrow for incorporation into the haemoglobin molecule.

Cellular uptake of Fe from Tf is well characterised and can occur via the Tf receptor-mediated endocytosis of Tf where the Fe is taken into cells via acidic vesicles and apoTf is released into the extracellular fluid (Morgan and Appleton 1969, Van Renswoude et al 1982, Cole and Glass 1983, Page et al 1984). Nevertheless, it is argued by some that the quantitative importance of this mechanism is not total as other mechanisms have been proposed. Page et al 1984 described an Fe uptake system based on fluid phase endocytosis, Grohlich et al 1979 put forward a diffusion based theory, and Cole and Glass 1983 and Veldman et al 1986 proposed that Fe is released at the cell surface. More recently, Sun et al (1987) and Thorstensen and Romslo (1988) have provided evidence of an NADH diferric Tf reductase enzyme. Fe enters the cell in a

reduced state, prior to translocation through the plasma membrane. Cooperative proton and electron fluxes labilise the ferric Fe from Tf and reduction of this ion is at the expense of the conversion of NADH to NAD^+.

Other roles of Tf include: antimicrobial activity, possibly as a regulatory agent in Fe metabolism, and in protecting against the toxic effects of free Fe (Harris and Aisen 1989). Tf has also been reported to be a catalyst in the reduction of superoxide to form the hydroxyl free radical (McCord and Day 1978, Motohashi and Mori 1983) and as a growth factor (Trowbridge and Osmary 1981).

1.9 Ga AS A SUBSTITUTE IN Al STUDIES

The ubiquitous nature of Al means contamination is a major factor in measurements of the metal. Al is a constituent of laboratory glass ware and even found in ultra pure acids. Al is known to adsorb onto the sides of both plastic and glass ware, its release being dependent upon the container material and sample within (Cornelius and Schutyser 1984 cited by Farrar 1988). Therefore, there are many problems to overcome if the measurement of Al in human tissue samples is to be performed accurately. For example, aliquots of serum sent to various laboratories reported Al levels between 2 and 150 µg/l (Adan et al 1986).

Research into the mechanisms of absorption and metabolism of Al are extremely difficult, partly because of the analytical problems associated with accurate measurements of Al, but also because there is no suitable radioisotope available (there are two isotopes of Al, both gamma emitting; ^{26}Al (half life = 7×10^5 years) and ^{28}Al (half life = 2.27 minutes)). The usefulness of radioisotopes in biomedical studies are numerous: when using gamma emitting isotopes, there is no need for sample preparation, detection limits are often in the sub picogram region, and the differentiation between endogenous matter and exogenous additions is possible.

Farrar (1988) has examined the viability of an isotope of Ga as a marker for Al in tissue uptake and distribution studies in the rat and found it to be suitable. The isotope of Ga used by Farrar was ^{67}Ga, which is a gamma emitting isotope with a half-life of 78 hours and readily available commercially. Ga is a member of Group 3 of the periodic table (as is Al) and therefore has very similar hydrolysis and speciation chemistry to Al (for a review see Baes and Mesmer 1976 and Farrar 1988). ^{67}Ga has since been used successfully in plasma Al speciation studies (Farrar et al 1990, Brammer et al 1990, and Hodgkins et al 1991), Tf-receptor positive cell uptake studies in vitro (Chen et al 1982), and Tf mediated Al transport to the brain (Pullen et al 1990). It has also been accepted as an appropriate analogue of Al by Day et al (1991) and Edwardson et al (1991).

1.10 STUDY OBJECTIVES

It has been shown the serum Al concentration in AD is similar to controls (Shore and Wyatt 1983) and Al can be delivered to the brain by Tf, at a rate controlled by the number of available Tf receptors. In AD, the Tf receptors are not enhanced in the cerebral cortex compared to controls (Morris et al 1987). Furthermore, Fe accumulation does not correlate with the number of Tf receptors in experimental rats (Hill et al 1985). Consequently, the delivery of Al to the brain by a Tf/Tf receptor mechanism seems to be limited, and provides a baseline of accumulation in both controls and AD (Farrar et al 1991a). Therefore, it is possible the increased entry of Al into the brain of AD patients is due to altered Al speciation in the circulatory system (Farrar et al 1990) in a similar manner to gastrointestinal absorption of Al which is dependent upon the species of Al present in the gastrointestinal tract (Farrar et al 1988).

The basis of work by Farrar et al (1990) was to elucidate a possible mechanism of Al accumulation in the brain of AD and Down's syndrome patients. The group demonstrated the plasma distribution of Ga (as an analogue of Al) was different in AD and Down's syndrome compared to healthy controls, haemodialysis and stroke dementia patients. Ga was bound to a high molecular weight species, identified as transferrin and to a low molecular weight species (unidentified by Farrar) in all groups studied, but the relative amount of Ga bound to the low molecular weight species was enhanced in the AD and Down's syndrome groups. Ga (and Al) bound to Tf was seen to be a relatively non-toxic, neuroprotective species, while metals which are bound to a low molecular weight species are potentially neurotoxic. Therefore, in AD and Down's syndrome the demonstration of an increased proportion of Ga in a low molecular weight species represented a possible mechanism of Al accumulation via enhanced uptake into the brain in the two disease groups. Criticism of the work by McGregor et al (1991) and Taylor et al (1991) followed which centred upon the low binding figures obtained by Farrar. The differences demonstrated by Farrar and coworkers were relative. This was the key observation and the lack of a physiologically representative buffer was irrelevant (Farrar et al 1992). It was acknowledged the that binding was atypical and they were aware this did not reflect absolute binding since in vivo Tf binding is known to take place to a much greater degree.

The cause of Wilson's disease is due to Cu toxicity and the similarity between this disorder and AD and Al are good. This well documented disorder can serve as an illustration of the clinical effects a metal can cause when it is present in a different species (or in an increased amount) to what is normally present in the body. Wilson's disease (hepatolenticular degeneration) is a rare autosomal recessively inherited disease, characterised by a disturbance in copper metabolism which leads to accumulation of and toxicity due to Cu in multiple organs including the liver and brain (Scheinberg and Sternlieb 1984). In Wilson's disease, ceruloplasmin (ceruloplasmin is the main metal binding protein of Cu and is thought to have a role in Cu transport because 95% of the

metal is bound to the protein (Sarkar 1988)) concentration is low but the Cu level in serum is normal. However, the fraction of Cu which is not bound by ceruloplasmin is increased (Sarkar 1988). The non-ceruloplasmin bound fraction of Cu is in a low molecular weight species which accumulates in various organs including the brain and liver.

Another example of metal toxicity due to altered speciation in the plasma is in the rare condition atransferrinaemia, where the patient has little or no endogenous Tf. Accumulation of Fe occurs in the heart, pancreas, and liver. Finally, there is at least one example of reduced plasma Fe-Tf binding in a subject who has an variant form of Tf (Dr. R. Evans personal communication, 1992).

The purpose of this thesis was to reinvestigate the initial observations of Farrar et al (1990) and to further the original work. There were four main study objectives: to replicate the original work of Farrar, to refine the gel-filtration chromatography technique with respect to physiological conditions encountered in vivo, to gain a better understanding of Ga-Tf binding and factors affecting the equilibrium between the high and low molecular weight species, and to compare Ga-Tf binding in a number of disease and control groups.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

To reduce the possibility of trace metal contamination, plastic ware was used wherever possible.

All chemicals used were ANALAR grade unless otherwise stated and were supplied by: BDH Ltd., Poole, UK, Sigma Chemical Company Ltd., Poole , UK, Fisons Scientific Apparatus, Loughborough, UK, and Aldrich Chemical Company, Gillingham, UK. Radioisotopes were obtained from Amersham International PLC, Amersham, UK.

Fasted adult male Wistar rats (grade four, 250 grams) were used in all appropriate experiments (Bantin & Kingman, Hull, UK). Animals were fed on a rat and mouse breeding diet (code 422) (Pilsbury Ltd., Birmingham, U.K.) with water *ad libitum* and kept in the University animal house at 20^{o}c in a 12 hour light / 12 hour dark cycle. Prior to experiments, rats were starved overnight in cages with grid bottoms to prevent coprophagia. Access to water was allowed. All experiments were performed within the limitations of the project and personal licences held by the research group and myself.

2.2 USE OF RADIOCHEMICALS

As Al has no appropriate radionuclides, the chemically similar radioisotope ^{67}Ga (half life seventy eight hours) was used as a suitable marker for Al binding in plasma (see section 1.9). ^{67}Ga was supplied as a carrier free $GaCl_3$ in 0.04 M HCl. Samples containing ^{67}Ga were counted for gamma emissions over the energy range 50-420 KeV for sixty seconds and automatically corrected for radioactive decay. (LKB Compugamma Counter, Pharmacia-LKB, Milton Keynes, UK).

Radioactive [1,5-^{14}C] citric acid was supplied as an aqueous solution containing 2% ethanol. The tracer was used to aid identification of the low molecular weight species produced in chromatographs from G75 columns. A 1 ml aliquot from a sample fraction was taken and 5 mls of Optiphase 'Hisafe II' scintillation fluid added (FSA laboratory Supplies, Loughborough, UK). Samples were counted for beta emissions over the energy window range 0-655 for five minutes and C.P.M. obtained (Beckman LS7500, Beckman Instruments Inc., USA).

2.3 METHODS

2.3.1 PLASMA PREPARATION

Blood was obtained from control and experimental groups after ethical agreements were reached. Samples were collected into lithium heparin tubes to prevent clotting and spun down to obtain plasma (2000 x g for fifteen minutes at room temperature, MSE minor centrifuge, MSE Ltd., Crawley, UK). Plasma samples were stored at -20 ^{o}C prior to use.

2.3.2 GEL-FILTRATION CHROMATOGRAPHY

Gel filtration chromatography is a technique commonly used which separates solutes depending upon molecular size. It has been successfully utilised to study the interactions of Al (and Ga) with human blood plasma (Lonnerdal 1980, Cochran et al 1983, Bertholf et al 1985, Trapp 1986).

The speciation of Ga in human and rat blood plasma was resolved by gel-filtration chromatography using Sephadex G75 beads in a 60 cm x 1.6 cm glass column (Amicon Wright Ltd., Stowehouse, UK). Dry beads were swollen in excess elution buffer (25 mM Tris-HCl, 100 mM NaCl, pH 7.4 (Cochran et al 1984)) for 48 hours then degassed for 30 minutes prior to packing at a flow rate of 2 mls/minute. The column was stabilised by running elution buffer through for three hours at a flow rate of 1 ml/minute and calibrated using a Dextran Blue (void volume)/tritiated water (exclusion volume) solution. A single 5 ml sample solution was loaded onto the column and 50 x 6 ml fractions were collected using an LKB Redirac 2112 fraction collector (LKB-Pharmacia Ltd., Milton Keynes, UK). The void volume was determined visually and found to be at fraction seven. Measurement of the exclusion volume was by beta emissions and found to be at fraction 21.

Plasma (1800 µl) was incubated in vitro with 1-5 µCi ^{67}Ga and a 'cold' $Ga(NO_3)_3$ solution to give a final concentration of 1.5×10^{-7} M. After incubation in a shaking water bath (60 oscillations/minute) for one hour, plasma was applied to the column. The concentration of Ga chosen was similar to levels of Al observed in serum of normal individuals and those suffering from AD (Shore and Wyatt 1983). 50 x 6 ml fractions were collected and counted for gamma emissions. The percentage of Ga bound to a peak was derived from the recovered activity from the column. Recovery of radioactivity was in the order of 85 - 95% and the columns were cleared of any activity (and mobile species) by a prolonged wash out period prior to further Ga speciation analysis.

2.3.3 ALTERATIONS TO THE ELUTION BUFFER

Experiments were performed which altered the electrolytic composition of the buffer. When a different buffer solution was used, the column was flushed through with the new buffer for five hours.

Additions of sodium bicarbonate to the standard buffer in varying concentrations were made and alterations to the elution profile noted. Buffer solutions containing bicarbonate were not degassed and were replaced at three hour intervals due to bicarbonate in solution being unstable (CO_2 was readily lost to the environment). The pH was of the elution buffer (containing 25 mM bicarbonate) was also altered by

adding either concentrated HCl or NaOH.

2.3.4 ALTERATION OF THE ELECTROLYTIC COMPOSITION OF PLASMA

Although the principal role of Tf is to bind Fe, it is known that unoccupied binding sites on the Tf molecule can accept other di and trivalent metals. To assess the impact of various trace metals on Ga-Tf, the following metals were added to plasma in known concentrations and the pH altered to 7.4 if necessary: Fe, Cu, Zn, Mg, Mn, Li, Al, and Ga. The sample was incubated in a shaking water bath (sixty oscillations/minute) for two hours, then Ga was added (as described in 2.3.2) and further incubated for an hour. Subsequently, the plasma sample was applied to a G75 column for speciation analysis.

2.3.5 G150 AND G15 CHROMATOGRAPHY COLUMNS

Additional gel-filtration chromatography columns were used with Sephadex G150 and G15 beads. Column preparation was as described above. The running conditions were identical except: using G150 beads the flow rate was decreased to 0.5 ml/minute and with G15 beads the flow rate was increased to 2 mls/ minute.

G150 columns were employed to further resolve the first peak found on G75 columns. After column calibration, the void volume was found to be at fraction seven and the exclusion volume to be fraction twenty two. Plasma was prepared and applied onto the column as described above. 50 x 6 ml fractions were collected and counted for gamma emissions.

To identify the second peak observed on G75 columns when plasma samples were analysed, G15 columns were used. Calibration of the column gave fraction nine as the void volume and fraction nineteen as the exclusion volume. Further calibration of the column was performed using solutions of gallium: nitrate, citrate, silicate, and phosphate. 6 ml solutions were prepared with the addition of the radiolabel and incubation in a shaking water bath for an hour (unless otherwise stated) at 37^{o}C before being applied to the column. The stability of the calibration gallium species was determined by running sample solutions through a G75 column prior to application on a G15 column. The Ga species was isolated from the G75 column by examining gamma emissions and the fraction with the highest activity was re-chromatographed on the G15 column.

The second peak (low molecular weight species) obtained from plasma samples applied on a G75 column was re-chromatographed on a G15 column. The identification and isolation of the second peak was described above. Because the size of the second peak was small in control subjects (using an elution buffer containing 25 mM HCO_3^-),

plasma was pre incubated with 2 x 10^{-4} M $FeCl_3$ (see section 2.4.5 for details) to block all the potential Ga binding sites of Tf, thus producing an artificially large second peak. The low molecular weight species from plasma samples were also examined which had been run on a G75 column containing no HCO_3^-.

As ^{67}Ga has beta activity in the middle of the ^{14}C window, duel label experiments were not possible. As an alternative, experiments were performed as follows. One solution contained 'hot' Ga and 'cold' citrate and the other contained 'cold' Ga and 'hot' citrate. Sample solutions of Ga citrate at various concentrations were prepared as described above and applied to a G15 column. The fractions from the column which contained 'hot' Ga were counted for gamma emissions and those which contained 'hot' citrate were counted for beta emissions. Comparison of the peak positions provided by the two isotopes gave positive identification to the species yielded on the columns.

2.4 FENTON'S TYPE REACTIONS

Production of hydroxyl free radicals is possible by the addition of hydrogen peroxide to a ferrous salt. This species is highly reactive and has the potential to damage many proteins including Tf.

Ferrous chloride (various concentrations) and 1% v/v of a 60% H_2O_2 solution were incubated in 900 µl of plasma for two hours in a shaking water bath (sixty oscillations/minute) at 37°C. Incubation of Ga (as previously described) followed and then the sample was applied to the G75 column. The effect of H_2O_2 alone and the ferrous salt were used as appropriate controls for this set of experiments.

2.5 NUCLEAR MAGNETIC RESONANCE (NMR)

NMR spectra were measured using a JOEL JNM-GX400 FT NMR spectrometer equipped with a 10 mm multinuclear broad-band probe operating at 104.05 MHz for ^{27}Al. Wilmad 733-5PQ tubes were used for the measurements because of there low Al content. Wilmad 513-PP tubes were found to have a too high Al content to obtain useful spectra. Spectra were acquired over a 40 000 Hz sweep width with a 90° observation pulse. External $[Al(H_2O)_6]^{3+}$ was used as a chemical shift standard with its ^{27}Al resonance taken to be 0 ppm. Other relevant experimental parameters are given in figure captions.

The detection limit of ^{27}Al NMR (2 x 10^{-5} M) is such that endogenous Al (≈ 2.5 x 10^{-7} M) cannot be detected. Since the identity of low molecular weight Al species was of interest, the binding capacity of Tf was fully saturated with $Al(NO_3)_3$ to give a final concentration of 10^{-4} M (Peters 1985). Addition of 10^{-4} M $Al(NO_3)_3$ leaves approximately 4 - 6 x 10^{-5} M in the low molecular weight pool. 10% v/v D_2O was

used as the solvent, this was necessary for field frequency locking. The sample was incubated in a shaking water bath at 37°C for an hour and the pH of the plasma was noted (and if necessary altered to 7.4.) The sample was then scanned by NMR. After analysis, the pH of the solution was determined once again.

Occasionally, when a spectra from a plasma sample had been obtained the proteins within the sample were removed by the addition of 10% v/v of 60% perchloric acid and centrifugation for twenty minutes at 5 000 x g. The supernatant was decanted off and further analysed. Solutions of Al, Al citrate, and Al bicarbonate were studied at various pH values and spectra obtained.

2.6 DATA HANDLING

Where data is presented, the mean, standard deviation (SD), and number of observations (N) are given. The two tailed, unpaired Student's t-test was predominantly used throughout this thesis (this statistic tests for differences between two sets of means drawn from independent populations). Other appropriate statistical analysis was performed as necessary (see appendix 1).

CHAPTER THREE

Ga SPECIATION IN NORMAL PLASMA AND FACTORS AFFECTING IT

3.1. INTRODUCTION

Al analysis is difficult, partly due to its ubiquitous nature, but the lack of a suitable radioisotope has been the greatest drawback in the study of this metal and its compounds. Therefore, Ga was used as a marker for Al (see section 1.9). It should be remembered that Ga, not Al has been used, but one can infer the action of Al by implication.

3.1.1 AIMS OF THE CHAPTER

The aim of this chapter was to investigate the binding of Ga in human blood plasma in 'normal' (i.e.control) subjects. Farrar et al (1990) have illustrated Ga binding species in vitro. As a basis for future work, the observations of Farrar were tested and developments in the methodology carried out. Factors which can alter the speciation of Ga were reviewed and the identification of both Ga species was attempted.

3.2 Ga SPECIATION IN CONTROL BLOOD PLASMA

3.2.1 METHOD

see section 2.3.2

The method of Farrar et al (1990) was followed with no modifications. Plasma used in these experiments was obtained from eighteen volunteers at Aston University. The ratio of male to female was 13:5 and the mean age was 24.8 years $\pm$ 3.55.

3.2.2 RESULTS

The elution profile (fig 3.1) obtained from a G75 column illustrates the division of Ga species in plasma. There was a bimodal distribution of Ga which was present in all the subjects studied. The relative positions of the two peaks remained constant in all experiments. Peak one (the high molecular weight species (HMWS)) eluted at fraction ten, while the second peak was observed at fractions 22 to 24. The HMWS was believed to be protein bound and eluted close to the void volume. Therefore, one can assume the molecular weight was approximately 80 kilo Daltons (KD). Accordingly, the species is believed to be the Fe binding protein Tf. In the second peak, Ga was bound to a low molecular weight species (LMWS). The mean percentage Ga binding to Tf (as a percentage of the recovered activity) was 13.3 $\pm$ 6.1 (N=18). Recovered activity was measured by a set of standards (taken from the plasma sample prior to chromatographic analysis) of known activity and volume. Recoveries were found to be in the order of 85 - 95% in all samples. The results were in agreement with those of Farrar et al (1990) (17.1% $\pm$ 7.4 (N=22)) and Brammer et al (1990) (15.5% $\pm$ 7.3 (N=10)).

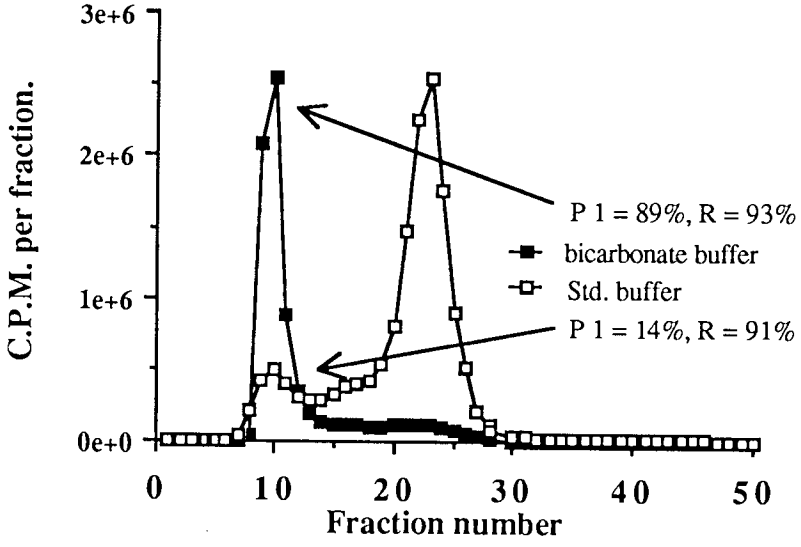


Fig 3.1 Ga speciation in control blood plasma using standard and bicarbonate (25 mM) buffers, pH 7.4, on a G75 column.

3.3 MODIFICATION OF THE ELUTION BUFFER

3.3.1 INTRODUCTION

Previous chromatographic work (section 3.2) used a standard Tris-HCl buffer. It was noted that using this buffering system, the percentage of Ga bound Tf was rather small and represented an unphysiological situation; more Ga should be bound to Tf in vitro due to the large numbers of empty binding sites accessible on the proteins' molecules. Recent measurements of Tf saturation by Farrar et al (1990) demonstrated that up to 60% of the binding sites were unoccupied.

For Tf to function correctly it was noted as early as 1949 by Schade et al that bicarbonate was an essential anion, this was later confirmed by Aisen et al (1969). Bicarbonate binds concomitantly with Fe in the Tf molecule (one HCO_3^-: one Fe, Baker et al 1987), withdrawal of bicarbonate from Tf resulted in some metal binding but to a very small degree. This is shown in the results of Farrar et al (1990), Brammer et al (1990), and those described section 3.2.

3.3.2 METHOD

see section 2.3.3

Plasma was separated on a G75 column as previously described, except the concentration of bicarbonate in the elution buffer was altered to give a final concentration of 0 (std.), 2.5 x 10^{-3}, 0.01, 0.025, 0.05, 0.1, and 0.25 M.

3.3.3 RESULTS

Fig. 3.2 reveals that maximal binding of Ga to peak one was at 0.025 M bicarbonate. This value is similar to that found in healthy, human plasma (0.021-0.028 M). At this concentration there was less variation in the distribution of results compared to other concentrations of the anion (shown by the error bars in fig 3.2). The results show that even with maximal Ga binding to Tf there is still a small percentage of Ga that is present in a LMWS.

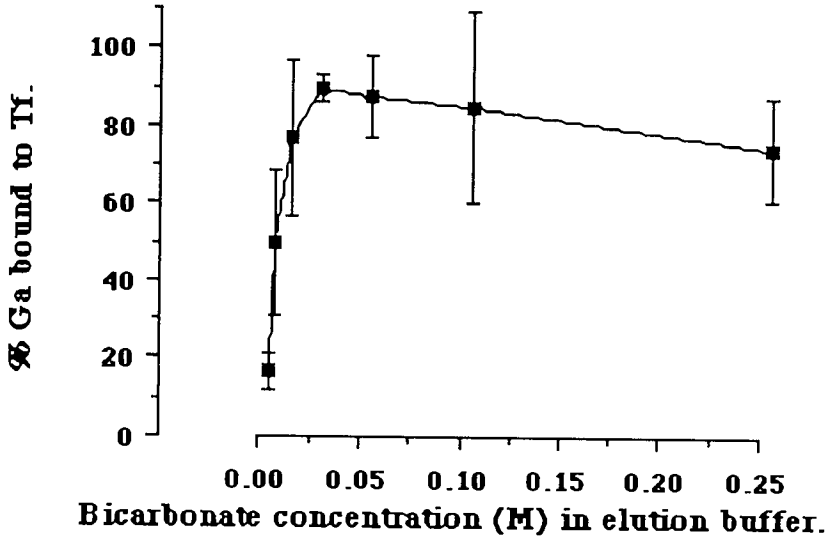


Fig 3.2 Ga-Tf binding as a function of bicarbonate concentration in the elution buffer.

Bicarbonate concentration (M)	% Ga-Tf	N	Bicarbonate concentration (M)	% Ga-Tf	N
0	13.75 ± 4.6	6	0.05	84.7 ± 10.3	6
2.5×10^{-3}	47.0 ± 19.2	6	0.1	82.2 ± 24.6	6
0.01	74.2 ± 20	6	0.25	71.5 ± 13.4	6
0.025	86.8 ± 3.6	6			

Each plasma sample was run at each bicarbonate concentration

Table 3.1 The effect of bicarbonate concentration on Ga-Tf binding.

3.4 Ga SPECIATION IN CONTROL BLOOD PLASMA USING 25 mM BICARBONATE IN THE ELUTION BUFFER

3.4.1 METHOD

See section 2.3.3

Plasma used in these experiments was obtained from fifteen volunteers at Aston University. The ratio of male to female was 7:8 and the mean age was 25.3 years ± 5.1. Samples were run on a G75 column with an elution buffer containing 25 mM bicarbonate (approximate physiological concentration) at pH 7.4.

3.4.2 RESULTS

The binding of Ga to Tf increased dramatically with the inclusion of bicarbonate (fig 3.1) from a mean value of 13.3% ± 6.1 to 85.7% ± 5.0. This was significant at the 0.1% level (Student's unpaired t-test). This clearly indicated that bicarbonate was a vital component in the binding of Ga to the HMWS in human blood plasma. It provided further evidence that peak one was indeed Tf, as the concomitant binding of bicarbonate and Fe is a unique property of Tf (Baker et al 1987). The observation that bicarbonate increased Ga-Tf binding was important because it showed that the experimental conditions created were representing a much more physiological scenario.

3.5 ADJUSTMENT TO Ga INCUBATION TIME

3.5.1 METHOD

see section 2.3.2

An incubation time of one hour was thought sufficient for Ga binding to occur and an equilibrium to be reached. To elucidate the chronology of Ga binding and to identify the effects of a longer incubation, the time was increased to 24 hours. All other parameters were kept constant.

3.5.2 RESULTS

In all three samples studied, the binding of Ga to Tf was complete, i.e. peak one was 100%. Initially, the uptake of Ga into the binding sites may be rapid. It is possible as the binding sites begin to fill, the rate at which Ga binds decreases. Only after prolonged incubation does one see 100% Ga-Tf binding. The complete binding of Ga to Tf was not unexpected because of the large excess of available binding sites compared to the concentration of Ga in the plasma.

3.6 MODIFICATION TO THE pH OF THE ELUTION BUFFER AND PLASMA

3.6.1 INTRODUCTION

At physiological pH, Fe and Ga are strongly bound to Tf. However, deviations from this pH alter the tertiary protein structure of Tf and therefore its binding properties (de Jong et al 1990).

3.6.2 METHOD

Using an elution buffer containing 25 mM bicarbonate, the pH was altered (using concentrated HCl) and if necessary the plasma pH was adjusted to the experimental value. The column was equilibrated to the new pH by running buffer through for five hours (section 2.3.3). A bicarbonate buffer was used because with the increase in metal binding to peak one observed with this anion, any alteration to metal binding properties of Tf would be exaggerated compared to the use of the standard buffer. It was also important to reproduce as closely as possible a physiological environment (with the exception of experimental parameters) for Tf to function.

3.6.3 RESULTS

Maximal Ga-Tf binding was observed at (physiological) pH 7.4. Upon entering an acidic or alkali environment, Ga binding decreased and the coefficience of variance about the mean increased (fig 3.3). Regression analysis of the results showed a second order polynomial to produce the best and most significant correlation ($R = 0.96$, $p = 0.005$).

pH of the buffer	6.0	6.5	7.0	7.4	7.7	8.0	8.3
% Ga bound to Tf	56.9	83.9	83.5	88.8	85.9	79.4	65.4
± SD	12.2	3.3	6.1	3.0	7.4	14.1	17.1
N	6	11	5	6	6	10	6

Table 3.2 The effect of pH upon Ga-Tf binding.

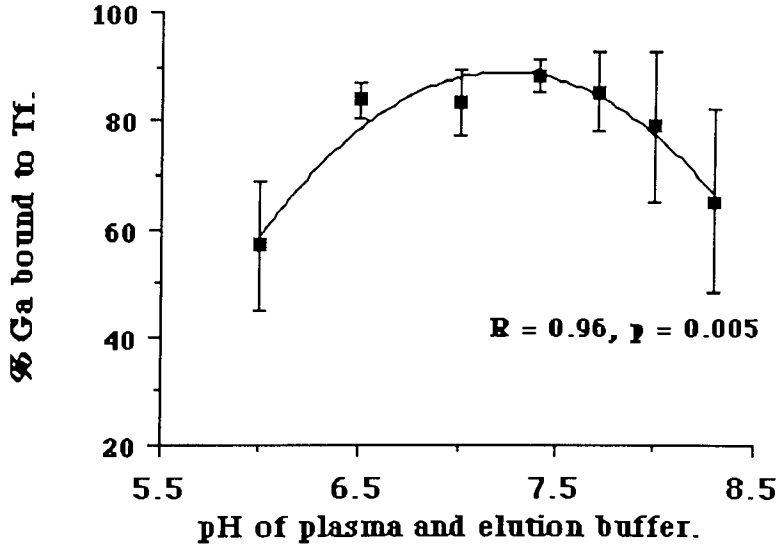


Fig 3.3 Ga-Tf binding in control plasma at varying pH values.

3.7 STORAGE OF PLASMA

It is possible the chemical speciation of Ga in plasma as resolved by gel filtration chromatography was due to the condition of the plasma prior to use (e.g.aging). Plasma was obtained from three control subjects and stored in the fridge (+4 ^{o}C) or freezer (-18 ^{o}C) for one, two, or seven days and applied to a G75 column which was bicarbonate free. The results from stored plasma samples were compared to fresh plasma. Table 3.3 indicated that short term storage of plasma (by freezing or refrigeration) will reproduce the same Ga speciation within the plasma. It was therefore reasonable to use stored plasma samples rather than obtaining fresh plasma for analysis. These observations were an important segment of quality control within the experiments.

	2 hours	24 hours		48 hours		1 week	
	fresh	R	F	R	F	R	F
% Ga bound to Tf.	18.1	15.6	16.7	18.2	16.4	14.9	17.3
± SD	3.64	2.7	2.7	1.63	0.64	3.18	2.7

R = refrigerated plasma F = frozen plasma

results courtesy of Dr. G. Farrar

Table 3.3 Comparison of Ga-Tf binding in plasma which was stored under different conditions.

3.8 FURTHER Ga SPECIATION IN HUMAN PLASMA

3.8.1.INTRODUCTION

Ga speciation in plasma was found to be physiologically representative in a Tris-HCl buffer containing 25 mM bicarbonate at pH 7.4. It was therefore appropriate to use this buffer in all subsequent work.

3.8.2 METHOD

See section 2.4.3

Section 3.3.1 details the analysis of eighteen plasma samples obtained from 'young' control subjects (volunteers from Aston University). To complement the analysis of this control group, other controls were studied. These were: another 'young' group, a hospitalised non-demented group, and a group of people from the community (where age and sex matching was possible). The groups will be defined as: Wolverhampton volunteers, hospital volunteers and community volunteers.

Eleven plasma samples from Wolverhampton volunteers were obtained from Dr. R. Davie. All volunteers were healthy males, the mean age of the volunteers was 28.5 years ± 7.6. Twenty two (M/F - 10/14) aged volunteers were supplied by Dr. S. Sturman, Lecturer in Neurology, Smethwick Neurology and Neurosurgical Hospital, Smethwick. All were inpatients at the hospital and were not known to be suffering from any dementing disorder prior to inclusion in this control group. The mean age of the group was 61.7 years ± 11.6. Fifty seven plasma samples (M/F - 26/31) were confidentially acquired from a group of people complaining of short term memory loss (to a minor degree). All subjects were not known to be clinically demented and lived within the community. The mean age of the group was 41.8 years ± 16.8.

3.8.2 RESULTS

Unlike the results from section 3.4, the percentage of Ga bound to Tf in some samples was not high. Segregation of the results into two groups was possible: those who had 'high' Ga-Tf binding and those who had 'low' Ga-Tf binding. The division between the two groups was taken to be 50% Ga-Tf binding (after analysis of the distribution of Ga-Tf binding results). Figs 3.4 and 3.5 shows the clear differentiation between the two sub populations.

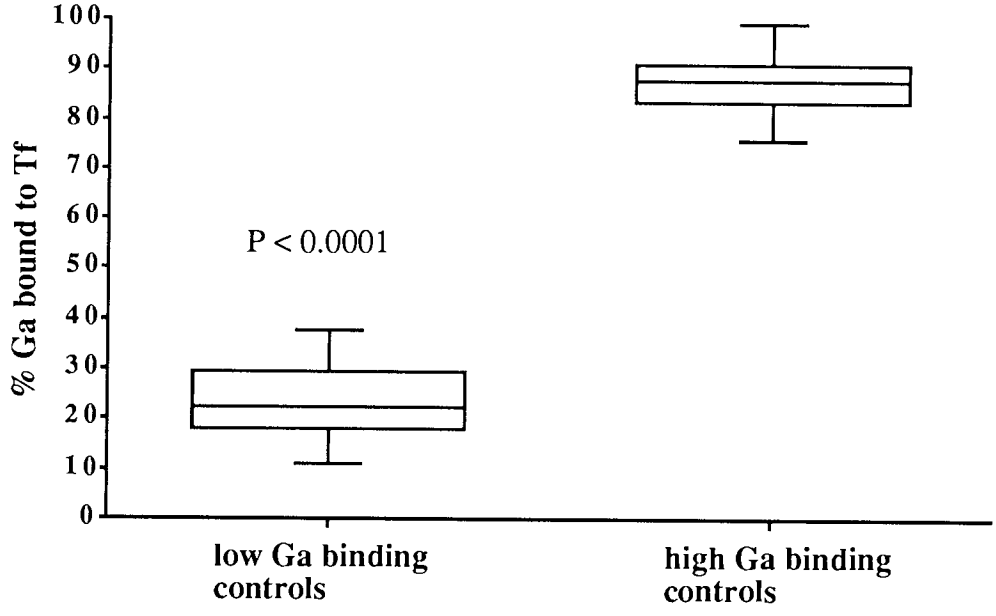


Fig 3.4 Percentile plots (10, 25, 50, 75, 90th) of Ga-Tf binding in high Ga binding (N=86) and low Ga binding (N=21) controls.

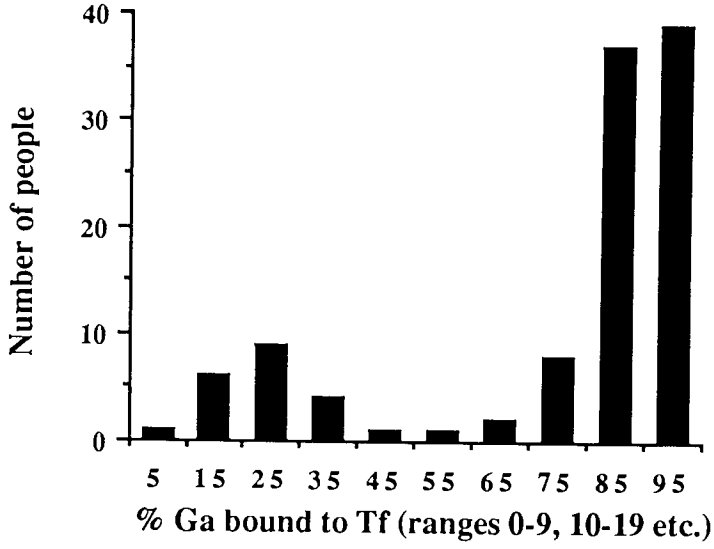


Fig 3.5 The bimodal distribution of Ga-Tf binding in the control groups

Table 3.4 summarises the Ga-Tf binding results from the three independent control groups. In combining all the data to form a large control group, it is interesting to see that the results from individual groups match closely to those from other groups differentiated by age and geographical location. When comparing Ga binding in a control group to that of an experimental group, it would be appropriate to use the 'high' Ga binding people because that is representative of a normal in vivo situation.

Group Age	Aston and W'ton young	Hospital old	Community varied	All varied
M/F	18/8	10/14	26/31	54/53
Mean age	26.0 ± 5.8	62 ± 11.6	41.8 ± 16.8	42.2 ± 18.2
age range	18-45	41-82	5-70	5-82
% Ga-Tf	69.05 ± 29.5	64.8 ± 28.1	81.2 ± 23.0	74.6 ± 26.6
(all)	N=27	N=24	N=57	N=107
% Ga-Tf	83.7 ± 9.9	83.1 ± 8.8	89.4 ± 6.2	86.9 ± 8.2
(high)	N=20	N=16	N=50	N=86
% Ga-Tf	20.2 ± 11.8	28.0 ± 11.0	22.4 ± 8.5	23.9 ± 10.0
(low)	N=6	N=8	N=7	N=21

Table 3.4 Summary of Ga-Tf binding results in the four control groups.

Plasma from the Wolverhampton volunteers produced a mean Ga-Tf binding of 46.2% ± 33.1 (N=11). All samples except one (Ga-Tf peak 100%), produced a bimodal distribution of Ga in the elution profile. However, the size of the Tf peak in the subjects varied considerably (unlike Aston controls). The high binding group had a mean Ga-Tf binding of 77.4% ± 17.9 (N=5). This was similar to the value obtained from Aston controls (section 3.4). Low Ga binders had a mean binding of Ga to Tf of 20.2% ± 11.8 (N=6). This was significantly different from the high binding and the Aston University group ($p < 0.001$).

The hospital volunteers had a mean Ga-Tf of 64.8% ± 28.1 (N=24). Again the division between high and low binders was evident. This could not be attributed to age or sex differences. High Ga binders had a mean Ga-Tf of 83.1% ± 8.8 (N=16) while low binders had a mean value of 28.0% ± 8.8 (N=9). Both these values fit data in the respective binding groups present in the other sets.

Plasma from the community group gave the following results: as a whole the percentage mean Ga binding to Tf was 81.2 ± 23.0 (N=57). This was not significantly different from the high binders in the previous groups. The large SD indicated that there was a wide spread of data points and it was therefore possible to split the values into high and low binders. For high binders the mean Ga-Tf was 89.4% ± 6.2 (N=50) and for the low group the value was 22.4% ± 6.5 (N=7). Age and sex differences between the groups was not evident.

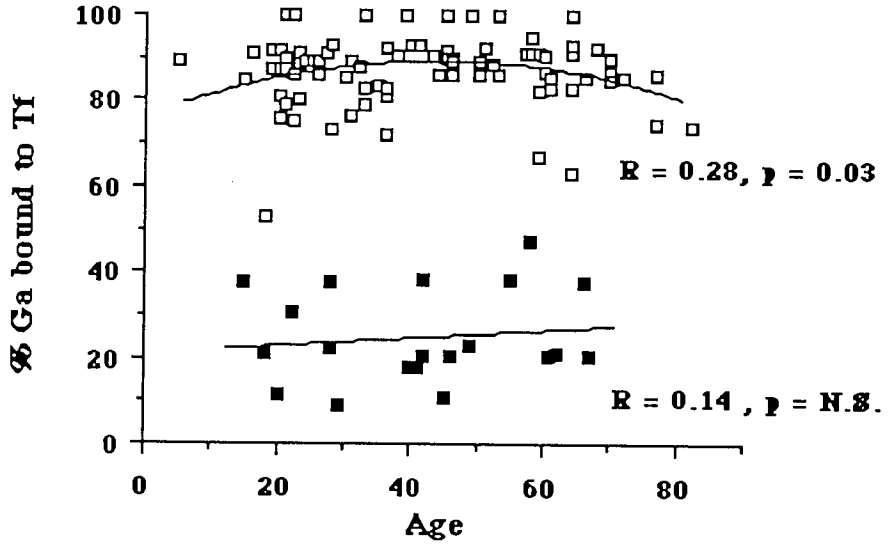


Fig 3.6 Ga-Tf binding on a G75 column with a 25 mM bicarbonate buffer at pH 7.4 in the control groups.

Fig 3.6 shows the distinct segregation of the sub groups within the control group. The high control group had a significant curvilinear relationship between age and Ga

binding ($p < 0.03$). In distinguishing the high control group by decades (see table 3.5), Ga-Tf binding increased with age (coupled to a decrease in the coefficience of variance of the results) until maximal Ga-Tf binding was reached in the fourth decade. Subsequently, Ga-Tf binding within the groups declined with age and the coefficience of variance increased. There were no statistical differences between Ga-Tf binding in any decade, just the curvilinear trend observed with age. The low control group had no age related changes to Ga-Tf binding. The mean Ga-Tf binding remaining similar in each decade.

Age range	0 - 9	10 - 19	20 - 29	30 - 39	40 - 49	50 - 59	60 - 69	70 +
High binders								
% Ga-Tf	89.2	82.4	84.9	86.0	91.8	88.9	86.7	83.0
± SD	-	14.7	8.6	8.0	4.9	7.9	9.3	5.8
N	1	6	21	14	14	12	11	8
Low binders								
% Ga-Tf	-	14.5	22.0	-	22.4	42.4	24.6	-
± SD	-	2.4	12.1	-	6.3	6.3	8.0	-
N	-	2	5	-	8	2	4	-

Table 3.5 Ga-Tf binding in the control groups spilt by age.

When the high control group was spilt by sex, no statistical differences were found. Although, females had a slightly higher value for Ga-Tf binding and less coefficience of variance in the results (88.2 ± 5.7 (N=44) c.f. 85.6 ± 10.1 (N=42)). Plasma Fe levels are slightly higher in males compared to females (mean 1.8×10^{-5} c.f. 1.65×10^{-5} M) which could explain why there was reduced Ga-Tf binding in males (less available binding sites). Taking the female group and dividing them into two on the basis whether they were pre or post menopausal (age division taken as forty five), there were no differences in the Ga-Tf binding results (87.8 ± 5.3 (N=23) c.f. 88.6 ± 5.9 (N=21) respectively). This division was examined because during menstruation women are known to loose up to 30% of total body Fe compared to the intermenstrum period (Geigy scientific tables). Sex differentiation in the low binding group produced similar results to the high binding group with females having a slightly higher Ga-Tf binding mean than did men (28.3 ± 10.6 (N=9) c.f. 20.6 ± 8.6 (N=12)).

	Male	Female	Female (> 45)	Female (45 +)
high % Ga-Tf binding	85.6 ± 10.1 (N=42)	88.2 ± 5.7 (N=44)	87.8 ± 5.3 (N=31)	88.6 ± 5.9 (N=23)
low % Ga-Tf binding	20.6 ± 8.6 (N=12)	28.3 ± 10.6 (N=9)	20.5 ± 6.1 (N=4)	34.6 ± 9.5 (N=4)

Table 3.6 Ga-Tf binding in the control group split by sex.

Five plasma samples taken from the low binding group were analysed on a G75 column with a bicarbonate free buffer (section 3.2). The mean percentage Ga-Tf binding was 7.0 ± 3.5, this was significantly different ($p < 0.01$) from the Aston control results of section 3.2 (13.3% ± 6.0). This further categorises the low binding group as different from other control subjects.

3.9 G150 COLUMN CHROMATOGRAPHY

3.9.1 INTRODUCTION

Although Tf is believed to be the major protein which binds Ga, it is thought that albumin can bind the metal as well. The use of Sephadex G150 beads may resolve the speciation of Ga bound to the first peak (assuming Ga is bound to more than one protein). Sephadex G75 beads which were used previously will separate species of molecular weight 75 KD or less, species of molecular weight greater than 75 KD will be eluted off in the void volume. The use of G150 beads will enable a more discrete separation of larger molecular weight species bound to Ga on the gel-filtration column.

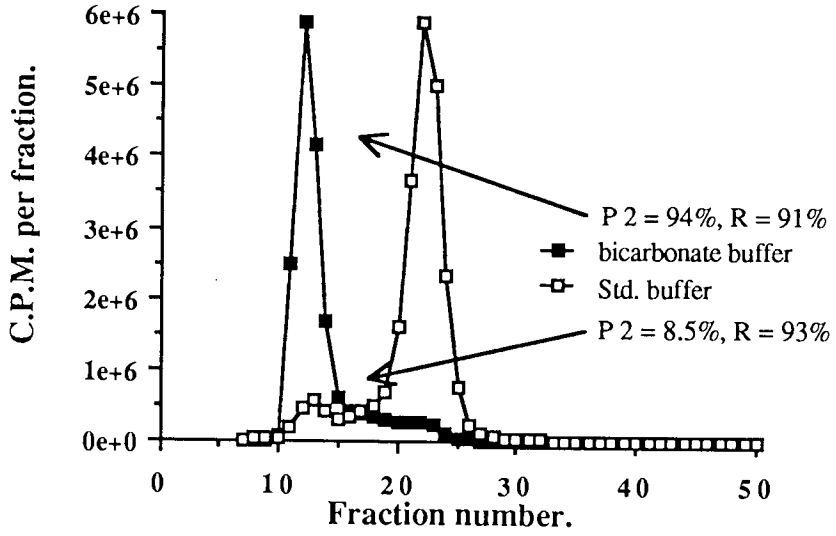
3.9.2 METHOD

see section 2.3.5

3.9.3 RESULTS

Plasma applied to a G150 column (which included the standard and 25 mM HCO_3^- buffers) showed the presence of three Ga species (fig 3.7). The first peak at fraction eight was minute (< 1%) and its size was independent of bicarbonate inclusion in the buffer. The second peak eluted at fraction twelve. In the bicarbonate buffer, this was the predominant species (Ga-Tf binding 94%), while in the standard buffer this peak was not (Ga-Tf binding 8.5%). Due to the increased binding observed with this peak in the presence of bicarbonate, one can assume this was the Tf bound Ga species. A third peak was present in both chromatographs and this represents a low molecular weight Ga bound species which has been observed on G75 columns. Tf molecular weight ≈

80 KD) was assumed to be the second peak, therefore the molecular weight of the first species would be greater than 80 KD because the largest species elute off the column first, followed by species of lesser molecular weight.



N.B. Peak 1 is too small to be observed on the chromatograph

Fig 3.7 Ga speciation in plasma using a G150 column with and without a 25 mM bicarbonate buffer at pH 7.4

3.10 IDENTIFICATION OF PEAK TWO FROM G75 COLUMNS

3.10.1 INTRODUCTION

Peak one from a G75 column is generally accepted as being Tf. However, the use of a G75 column does not resolve the identity of the second peak. There is a substantial amount of literature which implicates the LMWS of Al to be citrate. It is know that an increase in Al absorption occurs if citrate is present in the gastrointestinal tract (Slanina et al 1984, Slanina et al 1985, Farrar 1988). At a concentration of 10^{-4} M in blood plasma citrate would be bound by Ca^{2+} or Mg^{2+}, however Al would readily displace the Ca or any other alkaline earth metal ions (Martin 1991). Examination of the stability constants of Al show Tf and citrate to be the most likely species to bind Al in vivo (Martin 1986, Martin 1987) and in a recent study by van Ginkel et al (1990), they have confirmed the HMWS in rat serum to be Tf, while the LMWS was identified as citrate.

Using a G15 column any species of molecular weight greater than or equal to 15 KD would be found in the void volume. Species which are of molecular weight less than this would be found in subsequent fractions.

3.10.2 CALIBRATION SOLUTIONS

3.10.2.1 METHOD

see section 2.3.5

3.10.2.2 RESULTS

Fig 3.8 shows the elution profiles of various Ga-low molecular weight ligand solutions. The concentration of Ga in all solutions was 10^{-4} M and there was a ten fold excess of the ligand (10^{-3} M), except for citrate where there was a thousand fold excess (10^{-1} M). The fraction position and shape of the peaks are the important feature of the chromatographs.

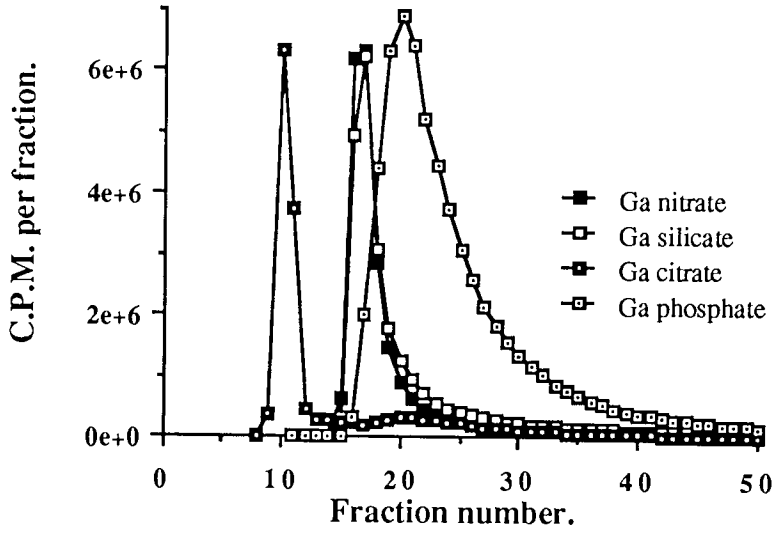


Fig. 3.8 Ga calibration solutions for a G15 column.

In Ga citrate solutions where the citrate concentration was in ten fold excess (10^{-3} M) compared to the 10^{-4} M Ga, chromatographs were produced with the peak at fraction ten greatly diminished (4.5% c.f 91% peak respectively). This was observed again when the concentration of Ga was reduced to 10^{-6} M and there was a one hundred fold excess of citrate (10^{-4} M) (< 1.0% c.f. 91%). The citrate peak at fraction ten therefore, was not only dependent upon the ratio of Ga to citrate but also on the absolute concentration of the metal and ligand.

3.10.3 Ga CITRATE STABILITY

3.10.3.1 METHOD

see section 2.3.5

To determine the stability of the Ga species applied to the G15 column (and to

reproduce conditions which the plasma Ga-LMWS encounter), solutions of Ga citrate were applied to a G75 column, recovered and applied to a G15 column.

3.10.3.2 RESULTS

Table 3.7 shows that in all concentrations of Ga and citrate (except 10^{-4} M Ga and 10^{-1} M citrate) the peak at position ten was negligible.

Ga and citrate concentrations	Peak at fraction 10	Second peak	Peak position
Ga 1.5 x 10^{-7} M citrate 10^{-3} M	-	100%	21
Ga 10^{-4} M citrate 10^{-1} M	91%	8%	22
Ga 10^{-4} M citrate 10^{-2} M	1.1%	89.9%	19
Ga 10^{-4} M citrate 10^{-3} M	0.75%	99.25%	20
Ga 10^{-4} M citrate 10^{-4} M	-	100%	21
Ga 10^{-6} M citrate 10^{-3} M	0.4%	99.6%	22

Number of observations at each point = 1

Table 3.7 Comparison of the elution positions of Ga citrate solutions.

At a physiological citrate concentration (10^{-3} M) and the concentration of Ga added to the plasma (1.5 x 10^{-7} M), a peak at fraction ten was not present. The failure to produce a large citrate peak at fraction ten in the described circumstances was not due to insufficient incubation time of the solutions, 24 hour incubations were carried out which produced identical results to the one hour incubations. The majority of the Ga was attributed to a peak at fraction twenty, which was the position of phosphate in the calibration of the column. It was possible that due to the relative abundance of phosphate (from the buffering solution) over citrate, that Ga was preferentially binding to the former. Alternatively, the Ga citrate peak at lower concentration ratios may indeed shift and elute at fraction twenty.

3.10.4 ANALYSIS OF THE Ga CITRATE PEAK USING ^{14}C

3.10.4.1 METHOD

see section 2.3.5

Identification of the Ga citrate peak was unresolved using the above technique. It was therefore decided to use a ^{14}C labelled citrate species to show at what fraction the Ga citrate species eluted off the G15 column.

3.10.4.2 RESULTS

Separate solutions of Ga and citrate applied to a G15 column and then counted for beta activity gave elution positions of; fraction seventeen (single peak) for a 10^{-4} M Ga solution, fraction ten (single peak) for a 10^{-1} M citrate solution, and a minor peak at fraction eleven (major peak at fraction twenty) for a 10^{-3} M citrate solution (see fig 3.9).

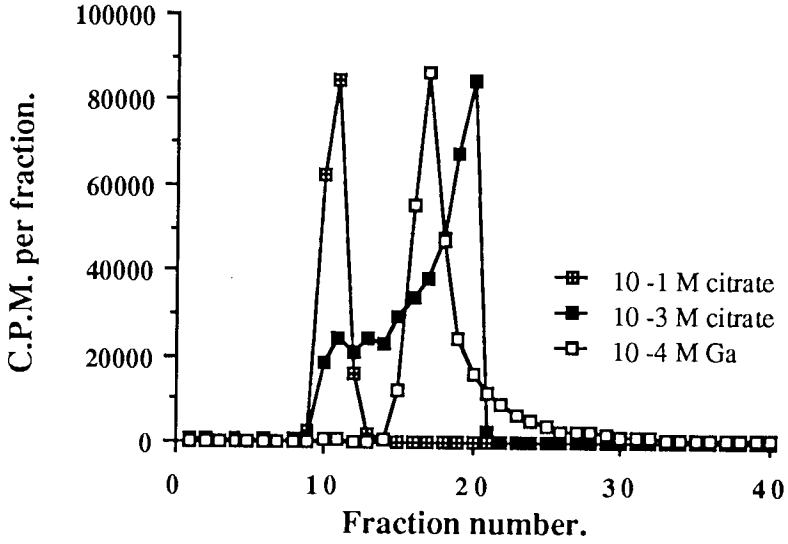


Fig 3.9 Calibration solutions (using beta activity) on a G15 column.

Using a 10^{-4} M Ga and 10^{-1} M citrate solution with 'hot' Ga, a peak (98%) was observed at fraction ten (as measured by a gamma and beta counter). The same solution with 'hot' citrate (as measured by a beta counter) produced a peak (97%) at an identical position. These results are synonymous with those from the previous section. Therefore, one can conclude that a Ga citrate peak at fraction ten was observed with concentrations used.

A peak at fraction ten (100%) was seen with a 10^{-4} M Ga and 10^{-3} M citrate solution using 'hot' citrate, while an identical solution containing 'hot' Ga produced a minute peak (> 1%) at the same fraction. The second peak (using 'hot' Ga) was seen at

fraction sixteen (this was the elution position of Ga nitrate). Using the same concentrations, a dual label solution was used. When examining beta emissions (Ga and citrate active) a peak (7%) was present at fraction ten, while gamma emissions (Ga only) gave a peak at fraction ten of 1.5%. The second peak occurred at fraction sixteen with both types of emissions. These results indicate that Ga and citrate in solution at the given concentrations do not bind greatly. Both appear to elute off the column at different, specific positions.

	b activity ^{14}C peak at 10	g activity ^{67}Ga peak at 10	second peak	position
Ga 10^{-4} M citrate 10-1 M	97%	98%	-	-
Ga 10^{-4} M citrate 10-3 M	100%	>1%	<99%	16
	b activity $^{14}C + ^{67}Ga$	g activity $^{14}C + ^{67}Ga$	second peak	position
Ga 10^{-4} M citrate 10-3 M	7%	-	93%	16
Ga 10^{-4} M citrate 10-3 M	-	1.5	98.5%	16

Table 3.8 Comparison of the elution positions of Ga citrate using gamma and beta emissions.

3.10.5 IDENTIFICATION OF PEAK TWO FROM PLASMA

3.10.5.1 METHOD

see section 2.3.5

When using a bicarbonate buffer, the majority of Ga is bound to Tf. Consequently, very little is in the low molecular weight form (in normal 'high' binders). Because so little was bound in this region, there was only a small amount of radioactivity associated with the peak. This was insufficient to produce further chromatographs from a G15 column, so an artificially large peak two was created by filling all the available Tf binding sites with Fe. The bicarbonate buffer was used to present a physiological

environment for the Tf.

3.10.5.2 RESULTS

Using a G75 column with a 25 mM bicarbonate buffer, five plasma samples (taken from the Aston control group) which were pre incubated for two hours with a 2 x 10^{-4} M $FeCl_3$ solution, produced a mean Ga-Tf binding of 2.7% $\pm$ 0.95. This indicated that virtually all the binding sites on the Tf molecules were occupied with the addition of Fe, therefore Ga in the plasma was present as a low molecular weight species. The fraction with the highest activity in peak two was re-eluted immediately on a G15 column. In all five samples there was a peak (< 1%) at fraction ten and a second peak between fractions twenty one and twenty four.

Subsequently, four of the five plasma samples were run on a G75 column using the standard buffer to see if Fe had altered the speciation of Ga in the plasma. A peak one of 20.8% +/- 4.5 (N=4) was obtained from the G75 column, this was in the range one would expect using this chromatographic system. Once again, the fraction with the highest activity in peak two was applied to a G15 column. In two of the four samples, a small peak of < 1% at fraction ten was observed (as before) and peak two occurred at fractions twenty two and twenty four. However, the other two samples produced a major peak at fraction ten (95 and 100%, the former had a peak two at fraction twenty four). To confirm this was a LMWS and not protein bound Ga from the void volume, fraction ten from the G15 column was re-eluted on a G75 column. Both fractions eluted at position twenty which identified the species as low molecular weight. Repetition of the experiment with the same samples failed to reproduce the earlier results.

With one plasma sample which gave a large peak at fraction ten (as described above), peak two from the G75 column failed to produce a large peak at fraction ten on a G15 column. However, using the next elution fraction from peak two and incubating it for twenty four hours at room temperature, a peak of 89% was seen at fraction ten. The second peak was at fraction twenty two. By the modification of the protocol to allow peak two from the G75 column to incubate at room temperature for twenty four hours, a dominant peak at fraction ten was always seen.

Further G15 column work identifying peak two off a G75 column was carried out using plasma taken from volunteers of control groups other than Aston. With eight samples from the aged group, the peak at fraction ten was always less than 1% when a fraction was applied straight onto the G15 column. The second peak was seen at fractions twenty two to twenty five. When fractions were incubated for twenty four hours and then applied to a G15 column, no change in the elution pattern was observed except on one occasion when a peak of 34% at fraction ten was present. Six samples from the Wolverhampton group gave results previously described, no major peak at

fraction ten.

3.11 CHANGES TO THE ELECTROLYTIC COMPOSITION OF PLASMA

3.11.1 INTRODUCTION

Tf is known to bind other charged cations besides Fe (Aisen et al 1969, Huebers and Finch 1987, de Jong et al 1990). It is probable that an equilibrium exists between Ga-Tf binding and the concentration of trace metals in the blood. The following set of experiments will examine the potential role of trace metal status in Ga-Tf binding.

3.11.2 METHOD

see section 2.3.4

3.11.3 RESULTS

Figs 3.10 and 3.11 and table 3.9 show the detrimental effects four trace metals have on Ga-Tf binding, thus forcing Ga into a low molecular weight pool.

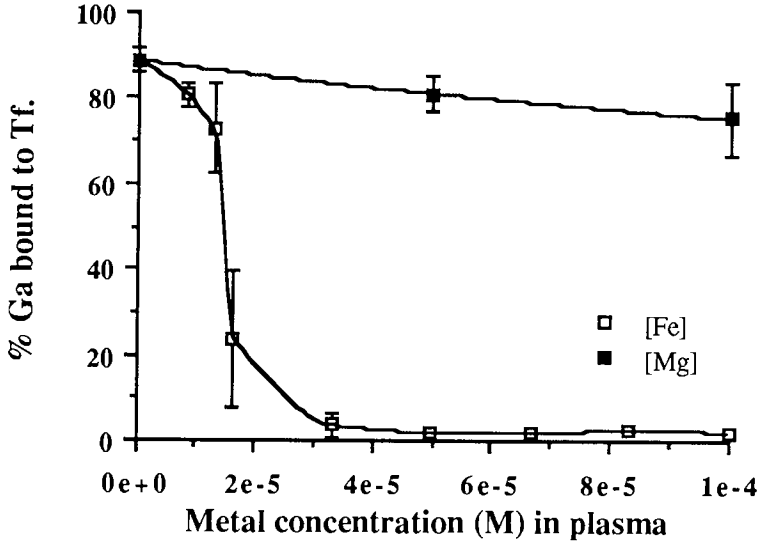


Fig 3.10 Ga binding to Tf in the presence of added ferric and magnesium chloride.

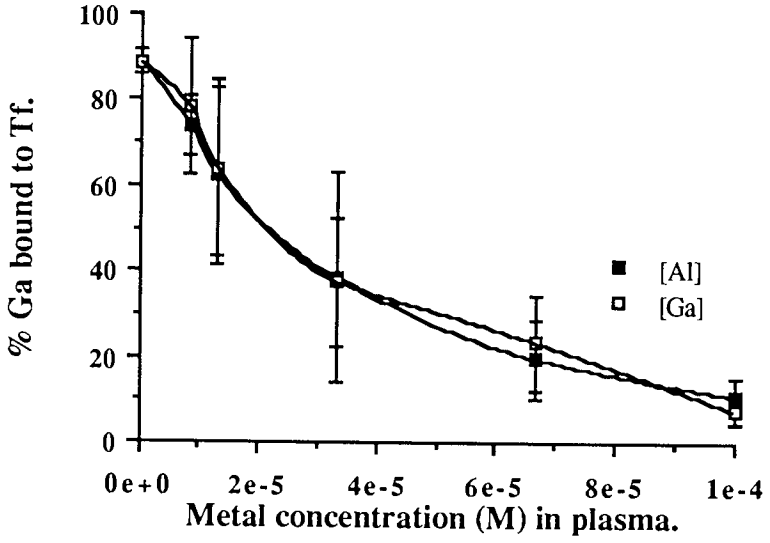


Fig 3.10 Ga binding to Tf in the presence of added aluminium and gallium nitrate.

Concentration	Fe^{3+}	Al^{3+}	Ga^{3+}	Mg^{2+}
10^{-4} M	2.0 ± 0.4	10.9 ± 4.6	7.4 ± 2.8	75.5 ± 8.4
8.3 x 10^{-5} M	2.6 ± 0.8			
6.6 x 10^{-5} M	1.7 ± 0.3	19.5 ± 9.5	23.3 ± 11.4	
5 x 10^{-5} M	1.6 ± 0.4			81.2 ± 4.3
3.3 x 10^{-5} M	3.6 ± 3.0	38.5 ± 24.5	37.5 ± 15.0	
1.6 x 10^{-5} M	23.8 ± 15.8			
1.3 x 10^{-5} M	72.4 ± 10.4	62.3 ± 20.8	63.9 ± 20.8	
8.3 x 10^{-6} M	80.7 ± 3.0	73.8 ± 7.1	78.3 ± 16.0	
0 M	88.8 ± 3.0	88.8 ± 3.0	88.8 ± 3.0	88.8 ± 3.0

Results are expressed as a percentage of Ga bound to Tf.
Number of observations at each concentration = 6

Table 3.9 Comparison of trace metal concentrations which adversely affect Ga-Tf binding.

Fe produces a sigmoidal concentration curve: an increase in Fe concentration decreases Ga-Tf binding. Subtle alterations in the Fe concentration range 1 - 2 x 10^{-5} M produced dramatic changes to the proportion of Ga bound to Tf. It should be remembered that the total plasma Fe will differ from added Fe. Recent measurements in control subjects gave a plasma Fe concentration of 2.75 x 10^{-5} M ± 4.9 x 10^{-6} (Farrar et al 1990). At the critical concentration range of Fe, the SDs of the means are large and variable. This could be due to an individuals own Fe and Tf plasma concentration prior to extraneous

Fe being added. Large concentrations of Mg had little effect on the overall percentage of Ga bound to Tf. Whether Ga replaces the Mg ion or Mg binds minimally to Tf was unclear. Al and Ga produced comparable results with respect to the size of peak in the high molecular weight region. An increase in added metal concentration would decrease Ga-Tf binding. The uniform effect of Al and Ga concentration was important because it reinforces the belief that using Ga as a marker for Al speciation in the plasma is valid.

Four other trace metal ions were used to determine an influence on Ga-Tf binding. These were: Zn^{2+} (as zinc sulphate), Cu^{2+} (as copper sulphate), Li^{1+} (as lithium sulphate), and Mn^{2+} (as manganese chloride) (see table 3.10). Mn^{2+} caused Ga bonding to decrease in a similar fashion to that observed when Mg was used. The three remaining metals (even at concentrations of 10^{-4} M) did not effect Ga binding.

concentration	Zn^{2+}	Cu^{2+}	Li^{1+}	Mn^{2+}
1×10^{-4} M	88.0	88.6	90.3	77.8
5×10^{-5} M	88.0	85.2	91.3	73.8

Results are expressed as a percentage of Ga bound to Tf.
Single observations were made as the results were well defined
Table 3.10 Additional trace metals which affect Ga-Tf binding.

3.12 FENTON REACTIONS

3.12.1 INTRODUCTION

Free radical formation from oxidative biochemical reactions under physiological conditions is well accepted. It is believed that these molecules damage proteins, lipids, and DNA (Halliwell and Gutteridge 1984, Mello-Filho and Meneghini 1984). Using ferrous (Fe^{2+}) Fe as a source of electrons, hydroxyl free radicals can be formed in the presence of hydrogen peroxide as shown in the Fenton reaction below.

$$H_2O_2 + Fe^{2+} \text{ —— } \cdot OH + OH^- + Fe^{3+}$$

Under physiological conditions, hydroxyl radicals are most likely to be produced by a Fentons reaction as opposed to a Haber-Weiss reaction which is very slow (see below) (Olanow 1990).

$$H_2O_2 + O_2^- \text{ —— } O_2 + OH^- + \cdot OH$$

Formation of hydrogen peroxide occurs from the action of superoxide dismutase on the superoxide free radical.

It is possible the hydroxyl free radicals which are generated from this reaction are available for oxidative protein damage to the Tf molecule, while ferric (Fe^{3+}) Fe which is produced would be available for binding.

3.12.2 METHOD

See section 2.5

3.12.3 RESULTS

Addition of 1% v/v - 60% hydrogen peroxide to plasma prior to Ga incubation did not reduced Ga-Tf binding. It follows therefore that any reduction in Ga binding would be due to other experimental parameters.

In deciding the concentration of ferrous Fe to use, it was important to consider the steep concentration curve (fig 3.10) that Fe produces after incubation with plasma in vitro. Using a 10^{-4} M ferrous chloride solution with 1% v/v hydrogen peroxide the Ga-Tf peak was (duplicate value given in brackets) 3.5% (1.95%). Although Ga-Tf binding was reduced by a large amount, it was not possible to distinguish between the effects of the ferrous salt and from the possible oxidative attack on Tf (thereby preventing Ga binding) from the hydroxyl radicals produced in the Fentons reaction.

Owing to the inability to differentiate between Fe and free radical attack upon Ga-Tf binding at relatively high Fe concentrations, a number of solutions containing ferrous Fe were used (see table 3.6).

$[Fe^{2+}]$	1×10^{-4}	8.3×10^{-6}	3×10^{-6}	5×10^{-7}	1×10^{-7}
(Fe^{2+})					
% bound Ga-Tf	2.4	6.5	72.3	81.5	80.3
($Fe^{2+} + H_2O_2$)					
% bound Ga-Tf	3.5	21	46.1	69.4	87

Single observations were made during these preliminary experiments.

Table 3.11 Comparison of Ga-Tf binding after Fentons reactions in plasma with varying concentrations of ferrous Fe.

As plasma Fe concentrations increased, the quantity of Ga bound to Tf was reduced (table 3.11). A similar observation was described in section 3.11. A further decrease in

Ga binding was noted with Fe concentrations of 3×10^{-6} and 5×10^{-7} M when hydrogen peroxide was added to the plasma. At these two concentrations it was assumed detectable oxidation of Tf was occurring which prevented Ga binding.

A concentration of 3×10^{-6} M ferrous Fe was used to replicate the experiment with different plasma samples to see if the initial observations were reproducible and to increase the sample size (table 3.11). Three duplicate samples were used. A mean Ga-Tf binding of 41.7% $\pm$ 33.2 was noted with ferrous Fe (ferric Fe at the same concentration causes a comparable decrease in Ga binding) and a mean binding of 35.7% $\pm$ 21.8 was observed with ferrous Fe and hydrogen peroxide. This was not statistically significant. A reduction in Ga-Tf binding occurred three times when Fe and hydrogen peroxide were combined, in the other three samples the Ga-Tf peak increased slightly. Large SD values indicated the variation in Ga-Tf binding in response to the addition of Fe to plasma was broad. At the concentration of Fe used in the experiment, the decrease in Ga binding was known to be varied (fig 3.10).

To try and eliminate the large variation in Ga-Tf binding, a concentration of 10^{-6} M ferrous Fe was used (table 3.12). With this concentration of Fe, Ga-Tf binding was reduced to 45.5% $\pm$ 19.4 (N=5). The addition of Fe and hydrogen peroxide to an identical plasma sample reduced the Ga-Tf peak further to 23.5% $\pm$ 14.8. This secondary depression of Ga-Tf binding was statistically significant (p=0.46). In all but one sample Ga binding fell.

	3×10^{-6} M Fe			10^{-6} M Fe	
Sample	Fe^{2+}	$Fe^{2+} + H_2O_2$	Sample	Fe^{2+}	$Fe^{2+} + H_2O$
J.H.	72.7	46.1	J.H.	28.0	8.6
	79.2	74.0	P.H.	35.0	36.0
P.H.	61.1	11.4	M.P.	36.9	7.6
	26.6	30.O	K.H.	75.0	26.4
M.P.	4.6	26.0	G.F.	57.7	39.0
	10.O	27.0			
				46.5 $\pm$ 19.4	23.5 $\pm$ 14.8
	41.7 $\pm$ 33.2	35.7 $\pm$ 21.8			

Results are expressed as the percentage Ga bound to Tf.

Table 3.12 Free radical oxidation of Tf at two ferrous Fe concentrations.

fraction ten, any other ratio or absolute concentration would result in a large peak at fraction nineteen to twenty two and a very small peak at fraction ten. The use of radio-labelled citrate demonstrated that a Ga-citrate species was not present to any great degree unless in the ratio and concentration stated above. It appears therefore, Ga was binding preferentially to another (possibly phosphate as the elution positions were similar) species.

Although it was obvious a Ga-citate species was only present to a very small degree (Ga-citrate taken as a peak at fraction 10) in simple solutions, the identification of the second peak from plasma samples ran on a G75 column was attempted. Initially an artificially large peak was produced by blocking all available Tf binding sites with Fe. Results showed a minute peak at fraction ten (< 1%) and a predominant peak at fraction twenty two to twenty four. It was possible any excess Fe which did not bind to Tf was preferentially bound to citrate, therefore artificially producing a new Ga species. Further experiments without Fe additions showed a predominant citrate peak in two of four samples. However, those results were not reproducible. Modification of the protocol to allow samples to incubate at 37^{o}C for 24 hours induced a large citrate peak in all samples studied. This may have been achieved by the alteration of an equilibrium within the sample solution.

In conclusion, the second peak from plasma samples applied to a G75 column and subsequently to a G15 column has shown the presence of a citrate peak in all plasma samples studied. This was not however, the predominant Ga binding species (except for two sample which were unreproducible and when an increased incubation time was allowed). The major Ga binding species eluted off the column at between fraction nineteen to twenty four. The exact identity of this peak remains unidentified, although a phosphate species has tentatively been suggested.

3.13.2 IMPLICATIONS OF Ga SPECIATION RESULTS

The section above detailed Ga speciation in human plasma subsequent to gel-filtration chromatography. Accordingly, it is appropriate to discuss the relevance of the Ga species found. As described in section 1.8 and above, Tf is accepted as the major Ga and Al carrier protein. Any metal which is bound to Tf can be assumed to be in a nontoxic species. The binding constants of Al to Tf are greater than that for any other species in plasma (Martin 1991), thus ligand exchange would only occur with species at higher concentrations than Tf with relatively similar binding constants. Al present as a LMWS (identified partially as citrate) would represent a possible neurotoxic species.

Accumulation of Al in the brain has been demonstrated in people suffering from AD and normal non-demented controls. The normal mechanism of Al accumulation is mediated via Tf-Tf receptor exchange, the rate of internalisation of Al being regulated

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Accumulation of Al in the brain has been demonstrated in people suffering from AD and normal non-demented controls. The normal mechanism of Al accumulation is mediated via Tf-Tf receptor exchange, the rate of internalisation of Al being regulated

by the number of Tf receptors on the cell surface. In turn, the expression of Tf receptors would be governed by the need for Fe in the cell (Huebers and Finch 1987). Pullen et al (1990) have shown the uptake of Ga into the brain of rats using a Tf mediated mechanism and it has been shown accumulation correlates well with the distribution of Tf receptors (Morris et al (1989). This mechanism is believed to be a slow process in which Edwardson et al (1991) have calculated 8 μg/year of Al would be deposited in the brain. The group reported this was consistent with an observed concentration of Al in the brain after a life time of exposure. Thus, the Tf mediated mechanism of Al accumulation appears to be limited and provides a baseline level of accumulation in all people.

The LMWS of Ga would represent a much greater neurotoxic threat that an Al-Tf complex. It has been suggested an Al-citrate species would cross the blood brain barrier (Farrar et al 1990). This would be in a random, undiscriminating fashion and would occur more rapidly than Tf mediated accumulation of Al. In support of this argument, Hill et al (1985) demonstrated that in rats with an Fe overload, accumulation of the metal was not necessarily occurring in areas of the highest Tf receptor density and Morris et al (1987) have shown the numbers of Tf receptors in the cerebral cortex of brains from AD patients are not increased compared to controls.

3.13.3 BICARBONATE BUFFER STUDIES

Following publication of the work by Farrar et al (1990), there were criticisms of the work which included the unphysiological environment to which Tf was exposed (McGregor et al 1991 and Taylor et al 1991). The basis behind their arguments were that in the system used, less than 20% of Ga was bound to Tf and an unphysiological environment was being produced. Accordingly, it was appropriate to study the effects of bicarbonate on Ga-Tf binding in the system originally devised by Farrar.

In a chromatographic system which contained a physiological concentration of bicarbonate (25 mM), the relative proportion of Ga bound to the two peaks altered. Ga was bound mainly to Tf (≈ 85%) and just a small percentage (≈ 15%) of the Ga was bound by the LMWS (this was an inverse observation to those described above). The large increase in the first peak upon the addition of bicarbonate provided further evidence that the HMWS was indeed Tf and it also provided a more representative in vitro view of an in vivo situation.

Using a bicarbonate buffer, control groups of various ages and geographical locations were shown to be similar with respect to Ga-Tf binding. In combining all four control groups to create a single large control group (which would include both healthy and hospitalised people), it was obvious there was a bimodal distribution in the ability of Tf to bind Ga. Of the 107 subjects studied, 80% had normal Ga binding (mean Ga-Tf

binding 86.9% ± 8.2 (N=86)), the other 20% subjects had very much lower Ga-Tf binding values (mean Ga-Tf binding 23.9% ± 10.0 (N=21)). The sub group of low Ga binders were suggested to be at greater risk from metal neurotoxicity because there is an increased amount of Ga bound to a LMWS.

Examination of the control group by age and sex showed altered Ga-Tf binding with age, but similar binding in both sexes. In the high binding group, there was a significant second order polynomial correlation with age. Maximal binding was seen in the fourth decade. No such trends were apparent in the low binding group (most probably due to a small sample size). Although males and females had similar binding values, females had slightly more Ga bound to Tf than did males (in both the high and low binding groups). This was probably a reflection in the Fe status of both sexes (males have more plasma Fe than do females).

The proportion of Ga bound to Tf can be influenced by many factors. It has already been shown that bicarbonate concentration has a huge impact on the size of the Tf peak. If one examines Ga-Tf binding in the presence of bicarbonate (fig 3.2) there is a sharp decrease in Ga binding once a physiological level of bicarbonate is lost. In disease conditions such as diabetic acidosis, lactacidosis, and ammonium chloride intoxication, it is known the concentration of bicarbonate in the blood is reduced to or below 5 mM (Geigy scientific tables vol 4). The consequence of this would to impair the ability of Tf to bind Ga, this would lead to an increase in the neurotoxic LMWS. This could increase the deposition of the metals in the brain.

Trace metals alter Ga-Tf binding. However, some are more potent than others (Fe, Al and Ga show most inhibition) and a shift in pH has been shown to reduce Ga binding. Free radical oxidative attack on Tf has been demonstrated (oxidative damage also generally increases with age), this again would reduce Ga-Tf binding. Thus, it appears there is a delicate equilibrium between the amount of Ga bound to the high and low molecular weight species which can be moved by any one of the aforementioned factors, or a combination of factors.

CHAPTER FOUR

NEONATES

4.1 INTRODUCTION

Parenteral nutrition involves the administration of nutrients through a large vein. It is used in patients with disorders of the gastrointestinal tract and has been successfully used for over thirty years. The long term use of parenteral nutrition has been associated with a deficiency in micronutrients and osteodystrophy (Klein et al 1980). High serum, urine and bone Al levels have been recorded in patients undergoing parenteral nutrition treatment (Klein et al 1982), with classical symptoms of Al induced osteomalacia also being observed (Sherrard et al 1983).

Al exposure to neonates has been given much attention over the last few years. There have been reports on elevated plasma, urine and bone Al levels in premature infants receiving intravenous therapy (Sedman et al 1985). It has been documented that fatal Al toxicity (Al present in infant formula) has occurred in uraemic preterm infants (Freundlich et al 1985) where brain Al levels were elevated. The body burden of Al in some neonates is greater than that of others and of normal adults. This is as a result of prolonged parenteral nutrition (Sedman et al 1985, Koo et al 1986, Bozynski et al 1989) or an impaired gastrointestinal function coupled with impaired renal function (Hewitt et al 1987). Not only are neonates vulnerable to Al accumulation, but also children and young adults with chronic renal failure who are receiving dialysis therapy (Salusky et al 1991). Tsou et al (1991) have shown in infants with normal renal function, raised plasma Al levels (approximately nine times greater than controls) after taking Al containing antacids for at least one week.

Al is derived mainly from parenteral solutions or the diet of the neonate. The amount of Al ingested from milk varies greatly depending on whether the infant is breast-fed or formula-fed and whether their diet includes solids. Al concentrations below 1.85×10^{-7} M and up to 1.67×10^{-6} M have been observed in mothers' milk (Sedman et al 1985, Freundlich et al 1985, Koo 1988), while a cows' milk based infant formula contained 5.19×10^{-7} M to 2.09×10^{-5} M Al, and a soy based infant formula was shown to have $1.69 - 8.69 \times 10^{-5}$ M Al (Koo 1988). Litov et al (1989) suggest "Aluminium several fold greater than that found in human milk" is of "No clinical relevance" but this has been challenged by Goyens and Brasseur (1990).

4.1.1 AIMS OF THE CHAPTER

It appears infants are particularly sensitive to the toxic effects of Al (Koch et al 1985, McGraw et al 1986, Robinson et al 1987, Bishop et al 1989, Klein 1991). This could be due to the altered speciation of Al in the body, which in turn could be explained by an abnormal Tf function in the neonate. Therefore, the aim of this chapter was to investigate any such Tf differences which may be present in the neonate compared to normal adults.

4.2 METHODS

4.2.1 PLASMA PREPARATION

Plasma samples were obtained courtesy of Dr. P. Altmann, Senior Registrar in Nephrology, The Royal London Hospital, Whitechapel, London. Blood was drawn from the placental fetal venous circulation (umbilical cord) of forty two newborn babies immediately after delivery and plasma obtained (see section 2.3.1). There was no possibility of contamination from maternal blood. Occasionally the sample haemolysed, in which case plasma haemoglobin concentration was measured.

4.2.2 Ga SPECIATION

see section 2.3.2, 2.3.3 and 2.3.5

Ga speciation studies were carried out in neonatal plasma on a G75 gel-filtration chromatography column. The method of Farrar et al (1990) was used with the following modifications: (i) initial studies with control plasma indicated no Ga-Tf binding differences when a reduced volume of plasma was applied the G75 column (Hodgkins et al 1991). As the plasma of neonates was difficult to obtain in large quantities and no binding differences were evident with the use of smaller volumes of plasma, all neonatal Ga speciation studies used 900 µl of plasma and (ii) although the majority of the neonatal studies were performed with the standard buffer, eleven samples were further analysed using an elution buffer containing 25 mM bicarbonate.

Using the LMWS (second peak) from the G75 column, the identification of the Ga species was attempted in four of the neonate samples by being re-chromatographed on a G15 column.

4.2.3 IRON ASSAY

Plasma iron concentrations were measured by the Guanidine/Ferrozine method (JT Baker BV). The mean adult Fe plasma levels for male and female are 1.8 and 1.65 x 10^{-5} M respectively (95% confidence interval (CI): 1 - 2.7 x 10^{-5} M).

4.2.4 Tf ASSAY

Tf levels in the plasma were measured using immunonephelometry on Technicon Instruments DPA1 (Waterson et al 1990). The mean adult Tf plasma concentration is 3.75 x 10^{-5} M (95% CI: 2.45 - 5.73 x 10^{-5} M). Tf Fe saturation is the ratio between plasma Fe and total Fe binding capacity (TIBC) (itself derived from the Tf concentration, one mol of Tf is able to bind two mol of Fe), normally this is approximately 30% (range: 20 - 40%).

4.2.5 PLASMA HAEMOGLOBIN

Haemoglobin in the plasma was measured using the cyanohaemoglobin method on a Technicon H2 analyser. This was necessary so the plasma Fe concentration could be corrected for the contribution of Fe derived from the haem molecule. Each haemoglobin molecule contains four Fe atoms. Therefore, a plasma haemoglobin concentration of 1.5×10^{-6} M contributes 7.1×10^{-6} M of Fe.

note: Fe, Tf, and haemoglobin measurements were performed at: The Royal London Hospital, Whitechapel, London under the supervision of Dr. P. Altmann.

4.3 RESULTS

The control group used to compare to the neonates in this chapter was not those used in chapter 3 with the exception of the bicarbonate studies, where data was taken from the previous chapter. The new adult control group was made up from twenty two (M/F 11/11) volunteers from Aston University. The mean age was 37.5 years $\pm$ 19.4 (range 20 - 73). Ga speciation, Fe and Tf measurements were performed by Farrar et al (1990), who have kindly let me use their data.

As seen in the results of the control group, the Ga distribution in neonatal plasma was bimodal. The two observed peaks eluted in identical positions to that of controls in all experiments (peak one Tf, peak two probable citrate) and recovery of radioactivity was approximately 80 - 90%. The mean Ga-Tf binding (using a bicarbonate free buffer) in the neonate plasma was 6.9% $\pm$ 4.4 (N=39). This was significantly lower ($p < 0.0001$) than control adult Ga-Tf binding which was 17.1% $\pm$ 7.1 (N=22). When 25 mM bicarbonate was introduced to the buffering system and Ga-Tf binding examined, the neonates showed no response. Ga-Tf binding remained low (8.3% $\pm$ 6.1 (N=11)), while the adult control Ga-Tf binding significantly increased (88.2% $\pm$ 5.4 (N=10), $p < 0.0001$).

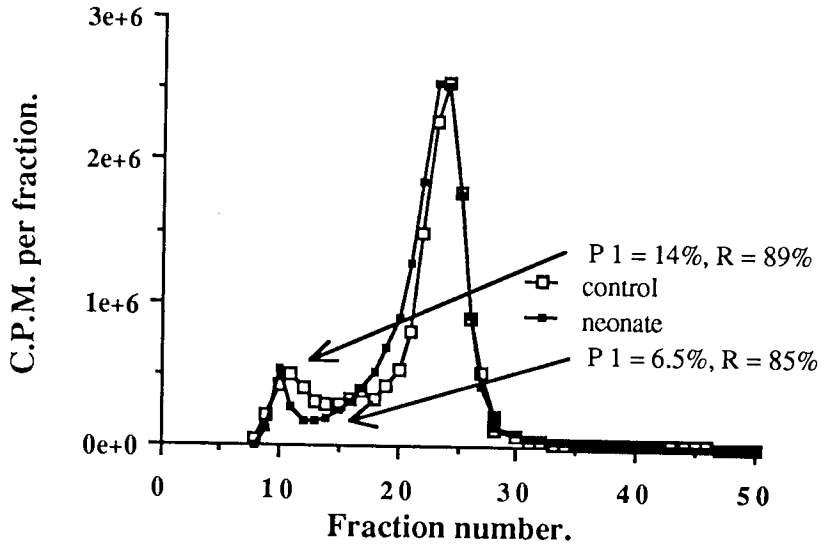


Fig 4.1 Comparison of Ga-Tf binding in neonatal and adult control plasma on a G75 column with a bicarbonate free buffer at pH 7.4

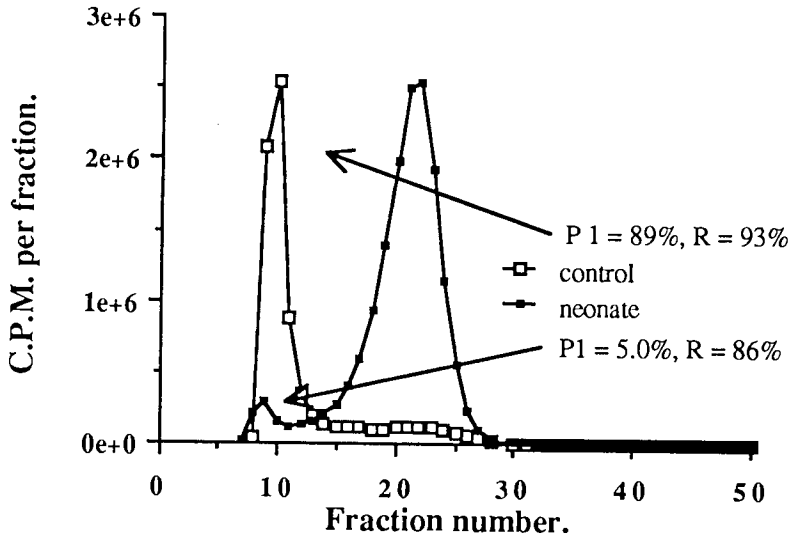


Fig 4.2 Comparison of Ga-Tf binding in neonatal and adult control plasma on a G75 column with a 25 mM bicarbonate buffer at pH 7.4

Fig 4.1 and 4.2 show the effect on the elution profiles of neonate and control plasma chromatographed on G75 columns with and without 25 mM bicarbonate.

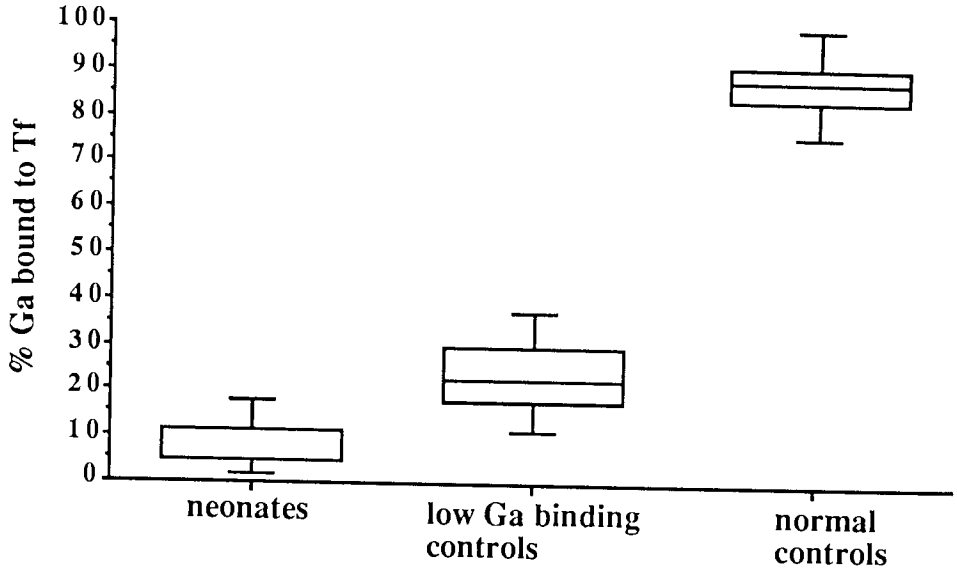


Fig 4.3 Percentile plots (10, 25, 50, 75, 90th percentiles) of Ga-Tf binding in neonates (N=11), low Ga binding controls (N=21), and normal adult controls (N=86) using a 25 mM elution buffer.

Fig 4.3 shows the great difference between neonatal and normal adult Ga-Tf binding and the similarity between neonatal and the low Ga binding control group.

The second peak from the elution profile of four neonate samples (from a G75 column) was isolated (described in section 2.3.5) and re-chromatographed on a G15 column to identify the LMWS to which Ga was bound. In two of the samples, a large peak at fraction ten was seen (100% and 86%). From evidence provided in earlier studies (chapter three) this was believed to be citrate. The other two samples had a minute peak at fraction ten (> 1%) and a second peak at twenty three and twenty four. Radioactive recoveries were between 90 - 95%.

Plasma Tf concentrations in the neonate were within the normal adult range although they were reduced (2.56×10^{-5} M $\pm$ 6.3×10^{-6} c.f. 3.73×10^{-5} M $\pm$ 8.7×10^{-6}), this difference in Tf concentration was significant ($p < 0.0001$). Neonatal Tf Fe saturation (corrected for haemolysis) was 86% $\pm$ 38 which was significantly higher ($p < 0.0001$) than the adult control value of 39% $\pm$ 29.0. There was a significant correlation between Tf Fe saturation and Ga-Tf binding when all the data was examined ($R = 0.49$, $p = 0.0004$) (fig 4.4). When the eleven neonates were studied using a bicarbonate free and a bicarbonate containing buffer, the correlation between Ga-Tf binding and Tf Fe saturation increased (fig 4.5 and 4.6). Plasma Fe concentrations in the neonate were significantly elevated ($p < 0.005$) compared to controls (4.7×10^{-5} M $\pm$ 1.9×10^{-5} c.f. 2.76×10^{-5} M $\pm$ 1.9×10^{-5} respectively) but there was no relationship between Fe and Tf concentrations.

	Adult controls	Neonates
Ga-Tf binding (no bicarbonate)	17.1 ± 7.2 (N=22)	6.9 ± 4.4 (N=39)
Ga-Tf binding (25 mM bicarbonate)	88.2 ± 5.4 (N=10)	8.3 ± 6.1 (N=11)
% Tf Fe saturation	39.0 ± 29.0 (N=15)	86.0 ± 38.0 (N=41)
Tf concentration (M)	$3.73 \times 10^{-5} \pm 8.7 \times 10^{-6}$ (N=15)	$2.56 \times 10^{-5} \pm 6.3 \times 10^{-6}$ (N=42)
Fe concentration (M)	$2.76 \times 10^{-5} \pm 1.9 \times 10^{-5}$ (N=15)	$4.7 \times 10^{-5} \pm 1.9 \times 10^{-5}$ (N=41)

Table 4.1 Summary of Ga, Fe and Tf measurements in neonate and adult control plasma.

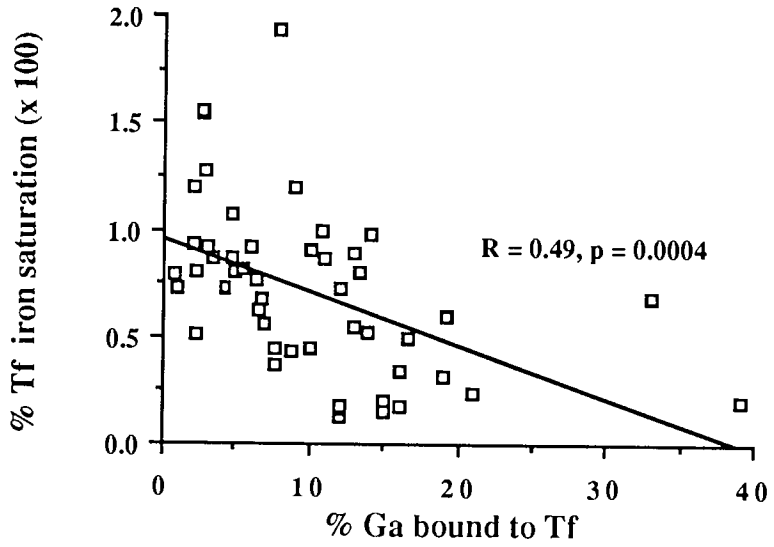


Fig 4.4 The relationship between Ga-Tf binding and Tf Fe saturation in neonates and adult controls (N=50).

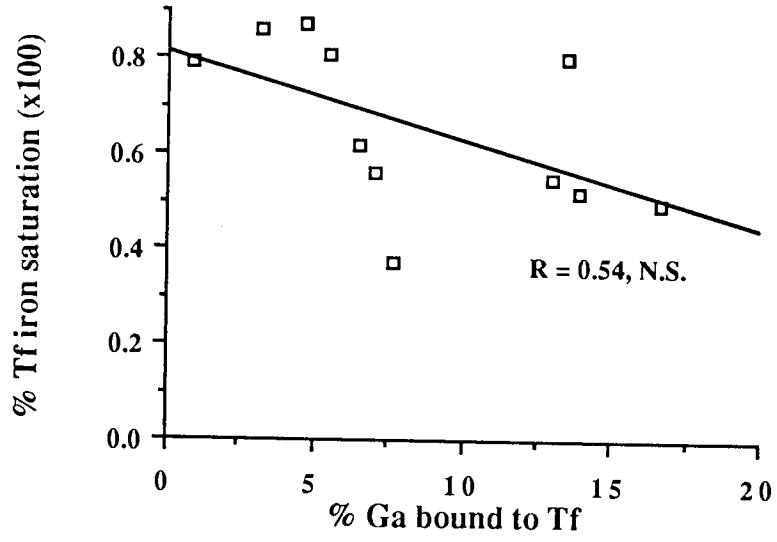


Fig 4.5 The relationship between Tf Fe saturation and Ga-Tf binding in eleven neonates using a standard elution buffer.

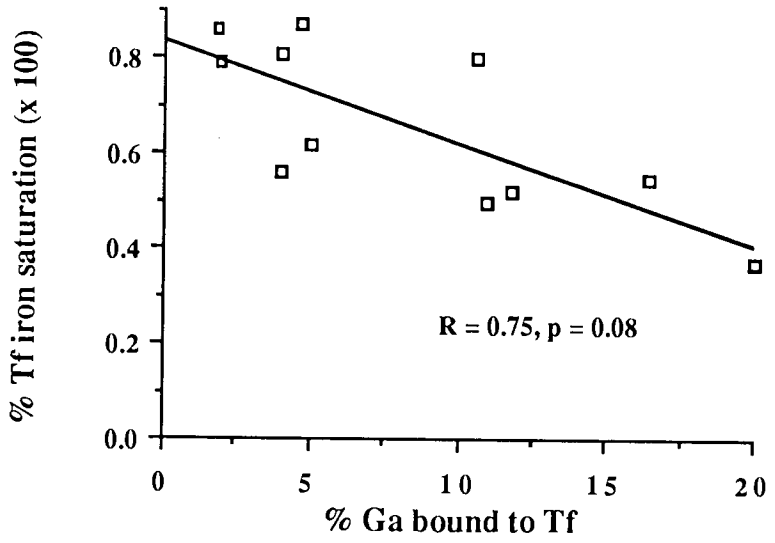


Fig 4.6 The relationship between Tf Fe saturation and Ga-Tf binding in eleven neonates using an elution buffer containing 25 mM bicarbonate.

4.4 DISCUSSION

Ga-Tf binding was much reduced in neonates compared to normal adult controls and was unaffected by the addition of bicarbonate to the buffering system. This was in complete contrast to the adult situation, where Ga-Tf rises in the presence of bicarbonate to at least four times that observed without bicarbonate. This has major implications for the newborn infant: with reduced binding to Tf, Al would be present bound to a LMWS in much greater amounts than compared to normal adults. As described previously, by binding metals such as Al, Tf acts as a detoxification

mechanism. The LMWS would however enhance transport of Al and thus toxicity by being able to penetrate across membranes such as the blood brain barrier (Farrar 1988).

The identity of the Ga binding ligands present in the second peak in neonatal plasma from a G75 column was found to be predominantly citrate in two of the four samples. In the other two samples, a citrate peak was only a minor species present in the elution profile, the other remaining unidentified, although a phosphate ligand could be possible. The consequence of such an Al species has previously being considered.

Reduced Tf levels in the neonate coupled with increased Fe concentrations was similar to that found by others (data available in Giegy scientific tables vol. 4) and inkeeping with the physiological requirements of the newborn (Altmann et al 1992). The high Fe saturation of neonatal Tf reflected high Fe stores. It may also represent a greater affinity of neonatal Tf for Fe than for maternal Tf and Fe. This could offer an insight into an Fe exchange mechanism between the fetus and mother via the placenta. Bentley (1985) (cited by Altmann 1992) has already suggested that this may be a Tf mediated process as there is a high concentration of Tf receptors on each side of the placental barrier. Furthermore, if luminal Tf in the neonate was of a similar type to that found in the plasma (highly saturated possibly due to the high Fe content of enteral feeds, or possessing an abnormally high affinity for Fe), then it would be less capable as acting as a detoxification mechanism for Al and subsequent absorption of the metal would increase.

The significant relationship between Ga-Tf binding and Tf Fe saturation (such a relationship was also found by Farrar et al 1990) indicates Tf Fe saturation was the main determining factor in Ga binding. This relationship was more evident when one examines the data with and without bicarbonate in the neonate samples (figs 4.5 and 4.6). When the chromatographic system contained bicarbonate there was a much tighter relationship between Tf Fe saturation and Ga-Tf binding. If all studies were performed using bicarbonate, it would be probable that there would have been an even greater correlation between Ga-Tf binding and Tf Fe saturation. It also reinforces the view that the bicarbonate system was more appropriate to use to compare to the in vivo situation.

These finding could offer a possible explanation to why neonates are particularly sensitive to Al toxicity. The high Al content of parenteral solutions in the neonate is evident, by bypassing the gastrointestinal tract which acts a barrier to Al absorption, the Al content in the plasma of neonates using these parenteral feeds increases. It has been demonstrated that Tf cannot bind Al to any great degree, this coupled with the relatively low glomerular filtration rates in infants up to six months of age would enhance the Al-LMWS, its toxic effects being able to be exerted. Similarly, neonates who take aluminium containing antacids or soy based infant formula diets will also be affected because of the reduced Al-Tf binding. It is known the permeability of the

gastrointestinal tract is greatest in the first few days of life (Bishop et al 1989) and although in these circumstances the Al would be partially removed by non absorption, there would still be significant levels of Al going into the blood.

Lucas et al (1992) have suggested that breast milk consumption in preterm infants during the first few weeks of life led to significant improvements in IQ compared to infants receiving no maternal milk. There was a dose-response relationship between the amount of maternal milk in the diet of the neonate and IQ, this implies that more formula feed was given to those infants who did not perform as well in the IQ tests. It is possible that the amount of Al ingested by the neonate (in the form of a formula feed) could have been a key source in the differences in IQ observed in these children.

CHAPTER FIVE

PARKINSON'S DISEASE

5.1 INTRODUCTION

5.1.1 ORIGINS

'Shaking Palsy' or 'Paralysis Agitans' was first described by Parkinson (1817). After criticism of the name in 1892 by Charcot, he proposed the term Parkinson's disease (PD).

During the early part of the twentieth century, the epidemic of von Economo's lethargic encephalitis (sleeping sickness) led to many cases of Parkinsonism, and it became clear that the then called PD was a collection of symptoms which resulted from a number of known and unknown causes. It is now accepted that PD (or primary Parkinsonism) should be used for those cases that are idiopathic. Secondary Parkinsonism refers to cases in which a causative agent is known (see table 5.1)

Causative agent	Examples
infections:	postencephalitic, luetic
toxins:	manganese compounds, carbon monoxide, carbon disulphide, cyanide, methanol, MPTP
drugs:	neuroleptic drugs (phenotiazines, haloperidol, reserpine) alpha-methyldopa
metabolic:	hypoparathyroidism and basal ganglia calcification, chronic hepatocerebral degeneration
vascular lesions	
brain tumours	
trauma	
syringomesencephalia	

Table 5.1 Factors known to cause secondary Parkinsonism.
Adapted from Heinonen (1989)

5.1.2 DIAGNOSIS AND CLINICAL FEATURES

PD is characterised by tremors, slowness of movement, and rigidity (Neal 1989). Infrequent, minimal tremors are the first sign of PD in the majority of cases. Initially, tremors are confined to just one hand, they subsequently spread to other limbs. Bradykinesia and akinesia are the slowing down of voluntary actions and an apparent difficulty to initiate movement respectively. In PD, these symptoms may be evident as a slowness of movement (which is apparent by a shuffling gait and lack of arm movement during walking). When the arms are affected, a loss in finger dexterity occurs which can lead to problems with handwriting and fine movements such as fastening buttons. Facial expressions and other spontaneous gestures are generally

impassive, a tenancy to maintain positions for an abnormal period of time, and a reduction in the blinking rate are evident. All of which produce a staring look and are early hypokinetic signs of PD.

5.1.3 EPIDEMIOLOGY

The incidence of PD in the Caucasian population has been estimated at between 0.07-0.2% (Marttila 1983). The age of onset and the incidence being the same for both men and women (Rajput 1984), although it is thought that non-whites living in Africa and Asian have a lower incidence of the disease (Heinonen 1989, Tanner 1989). The most common age at which PD symptoms occurs is fifty or over and the maximum rate of incidence is in the seventh decade of life. In this age group the prevalence rate is between 0.3% and 1.8% of the population (Heinonen 1989). The incidence of PD may be greater than is documented: there could be some undiagnosed patients in the community whose symptoms were put down to 'old age' and as many as 40% of all cases do not reach the hospital services (Marsden 1990).

Parkinsonism is a slow, progressive neurodegenerative disorder. Although surgery can remove tremors and rigidity in a small number of suitable cases, the main stay of treatment are drugs. These can alleviate the signs and symptoms of the disease, but it is only palliative. Before the introduction of levodopa, a study by Hoehn et al (1967) found the mortality rate of sufferers to be 2.9 times greater than that of the general population of the same age, sex, and race. Death was usually 9.4 years after the onset of the disease, although some lived for thirty or more years. Subsequent studies on patients who received levodopa treatment (Marttila et al 1977, Joseph et al 1978) have shown a reduction in the mortality rate to nearer that of the general population. However, a later study by Hardie et al (1984) showed the mortality rate of subjects who had been treated on levodopa for twelve years was in excess of 2.5 times that of the general population. A possible explanation for this was offered by Lees (1986). Although levodopa increased the quality of life and survival rates in the early years of treatment, a combination of diminished responsiveness and late side effects related to the progression of the disease lead to an increase in the mortality rate years later.

5.1.4 PATHOPHYSIOLOGY

In PD there is a selective degeneration of the neuromelanin containing dopaminergic neurons in the substantia nigra (Hirsch et al 1988) and of the nigrostriatal tract (Neal 1989) which results in a reduction of dopamine in the basal ganglia. It is generally accepted that a 50-60% reduction in dopaminergic neurons in the substantia nigra (Agid 1991) and an 80% reduction in striatal dopamine concentration (and thus basal ganglia concentration) is responsible for the major symptoms of the disease to be displayed (Heinonen 1989, Langston 1990, Agid 1991, Clough 1991, Nagatsu 1991). Dopaminergic neurons are continually lost during the course of PD. Riederer and Wuketich (1976) and Scherman et al (1989) have calculated the rate of loss of the

neurons in the substantia nigra to be twice that of normal subjects. In PD patients this was approximately 1% per year or ten neurons per day. Another characteristic pathological finding in the brains of PD patients are the presence of Lewy bodies (eosinophilic cytoplasmic inclusions) and these are accepted (in combination with degeneration of dopaminergic neurons) as conformation of the disorder post mortem (Gibb and Lees 1989). Lewy bodies are not found in all cases of PD and not confined to the disease either, they are present in some cases of symptom free PD, some AD cases, and in diffuse Lewy body disease.

During the symptom free period of PD, compensation for a reduced dopamine concentration will occur pre- and post-synaptically. The ratio of striatal homovanillic acid (the main metabolite of released dopamine) to dopamine increases, which indicates the remaining dopaminergic neurons increase their activity (Agid et al 1987, Neal 1989). Radiolabelled spiperone binding to dopamine receptors increases, suggesting an increase in the number of dopamine receptors and an enhanced post-synaptic response to dopamine (Neal 1989). Agid et al (1987) have demonstrated the post-synaptic dopamine receptors become hypersensitive.

The basal ganglia possess many cholinergic neurons. Normally the effects of acetylcholine (which has an excitatory role within the CNS) and dopamine (which has an inhibitory role in the CNS) are balanced. However, as the nigrostriatal tract progressively degenerates in PD, the cholinergic system becomes relatively overactive and is responsible for the positive signs of PD such as the tremor (Shimomura and Headley 1988). This has been shown by the administration of centrally acting cholinesterase inhibitors which exaggerate the tremor of PD and conversely the administration of centrally acting anticholinergics reverse the effects (Shimomura and Headley 1988).

5.1.5 ETIOLOGY

5.1.5.1 GENETICS

It had been thought for many years that there may be an hereditary factor which could contribute towards the cause of PD as at least 10-15% of cases had relatives who were similarly affected (Marsden 1990), but the frequency of cases has been reported the same in first degree relatives of both patients and controls (Agid 1991). By studying pairs of identical twins, evidence could be obtained in relation to a possible genetic link.

A survey of twins in Montreal (Rinne et al 1980 and Barbeau and Pourcher 1983) concluded there were four forms of PD: idiopathic, postencephalitic, symptomatic and genetic. The genetic form was split further into: a subgroup where transmission was via an autosomal recessive trait with characteristic akineto-rigidity, and a subgroup which was transmitted via a dominant inheritance pattern with group members having an

'essential' tremor. A study of 43 pairs of monozygotic and 19 pairs of dizygotic twins in America by Ward et al (1983) and in another similar survey by Marttila et al (1988a) in Finland found that for most patients a genetic consideration was unimportant. The age of onset and prevalence of PD amongst twins was found to be the same as in the general population (Marttila et al 1988a).

Marsden (1990) suggests that although the twin studies rule out any major role for simple mendelian genetics, more complex genetic factors may be involved. Although the concordance rates of mono- and dizygotic twins are very similar, Johnson et al (1990) do not rule out a genetic component to the disease. In early on-set PD where there are more familial cases (Alonso et al 1986), it is suggestive of a stronger genetic link in this group of patients (Agid 1991).

5.1.5.2 INFECTION INDUCED PARKINSON'S DISEASE

As well as genetic abnormalities, environmental factors which could be responsible for PD have been examined. Following the epidemic of the viral infection encephalitis lethargica after the first World War, many patients were left with clinical symptoms and neuropathological damage which was remarkably like that found in patients with PD. This was the basis to try and identify a viral cause for the disease. A number of infectious diseases can mimic Parkinsonism as an occasional complication. These include: syphilis, malaria, typhoid, coxsackie virus, and measles (Shimomura and Headley 1988). The possibility that a slow or latent virus (as in Jakob-Creutzfeld disease and Kuru) is responsible for PD is doubtful because the transfer of brain tissue of an affected patient to an animal will not result in the transfer of the disease (Gibbs and Gajdusk cited by Calne and Langston 1983). Furthermore, the histological staining of brains from PD patients do not resemble the characteristic findings of either a conventional or slow viral infection (Calne and Langston 1983).

5.1.5.3 CHEMICALLY INDUCED PARKINSON'S DISEASE

The discovery that 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) (which is a human neurotoxin that selectively destroys the substantia nigra) leads to symptoms of PD, which can be treated by conventional parkinsonian drugs was important. It lead many to believe environmental factors were responsible for, or at least contribute to idiopathic PD.

Chronic exposure to, and poisoning from Mn (usually in the form of Mn dioxide dust which enters systemic circulation via the respiratory tract) has been documented as early as the nineteenth century (Couper 1837). It was later reported that a Parkinson type syndrome was due to Mn exposure (Mena et al 1967, Balani et al 1967, Browning 1969, Recommended health-based limits in occupational exposure to heavy metals 1980, Manganese Environmental Health Criteria 17 1981, Donaldson et al 1982). Although Barbeau et al (1976), regard these symptoms as a better model for dystonia.

Mena et al (1967) first recognised Parkinsonian type symptoms in a group of Mn ore miners in the villages of Northern Chile. Other groups of workers known to be at risk from exposure to Mn compounds are; ore crushers, brown stone millers, processing plant or dry cell battery workers, and electric welders (Barbeau et al 1976). Neuropathologically, neurochemically, and clinically the similarities of idiopathic and Mn Parkinsonism are strong (Donaldson 1981, Donaldson et al 1982). There is a reduction in dopamine in the basal ganglia and of noradrenaline from the hypothalamus (Bernheimer et al 1973). A loss of neuromelanin pigmentation in the substantia nigra is present (Bernheimer et al 1973), which is due to degeneration of this area (Donaldson 1981). Akinesia, rigidity, and tremors are present as extrapyramidal symptoms and it is also possible to relieve the signs of both diseases by treatment with levodopa (Mena et al 1970). However, it does differ from Parkinsonism in that patients display transitory psychiatric symptoms for about a month prior to displaying the permanent neurological symptoms (Mena et al 1967, Donaldson et al 1982). The first psychiatric symptoms of Mn intoxication can appear suddenly and progress rapidly. Extreme fatigue, somnolence, and irritability are followed by behavioral disorders such as violent behaviour, compulsive acts, hallucinations, and emotional instability (laughing-crying episodes) (Barbeau et al 1976). All of which have helped coin the phrase "locura manganica" or manganese madness.

5.1.5.4 PARKINSON'S DISEASE in GUAM

There has been an unusually high incidence of Parkinsonism, Parkinsonism in association with dementia, and amyotrophic lateral sclerosis (ALS) in the indigenous population of the Western Pacific island of Guam (Kurland and Mulder 1954). A component of the local diet and traditional medicines (seeds of the false sago palm (Cycas circanalis)) was found to contain beta--N-methlyamino-L-alanine (a known neurotoxin) which caused Parkinsonism. The increased incidence of these neurodegenerative diseases continued even in people who had left the island for up to thirty years (Heinonen 1989). This fact, coupled with the decreased incidence of the diseases over the last few years (due to Westernisation) suggests that environmental factors are important in these diseases (Perl and Brody 1980, Perl 1985). The higher incidence of PD in industrialised countries and in rural areas (factors associated were: chemicals, heavy metals, pesticides, vegetable farming, well water, and wood pulp) found by Barbeau et al (1985) and Tanner (1989) has added extra weight to an environmental theory.

It seems likely from evidence in the literature that a totally genetic or an environmental factor is not responsible for idiopathic Parkinsonism. It does suggest though, a PD patient may be genetically determined to be susceptible to environmental toxins. This would result in dopaminergic neuronal death or premature aging because of the inability to deal with the toxin (Agid 1991, Marsden 1990).

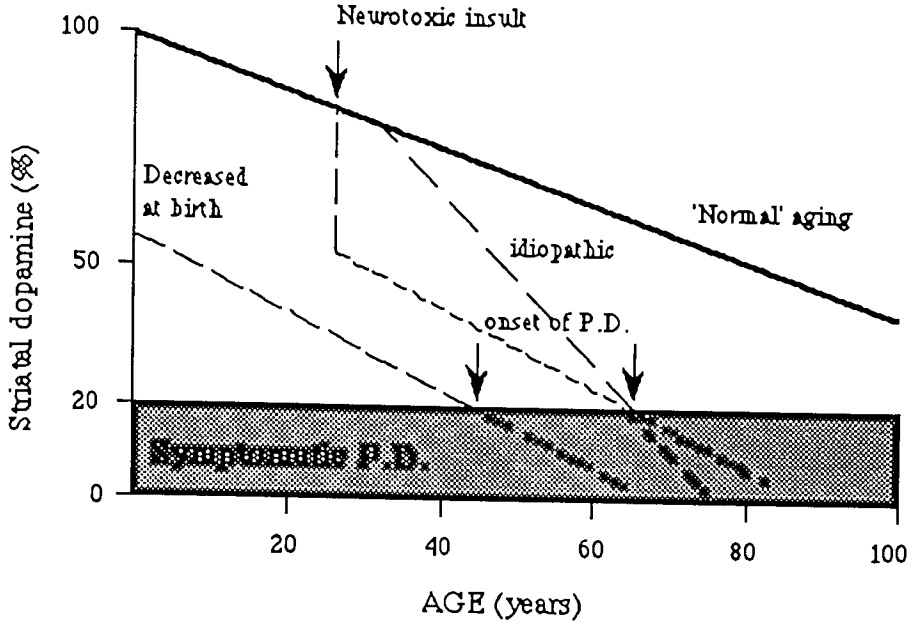


Fig 5.1 Dopamine loss in the nigrostriatal tract: possible theories which lead to PD. The bold line indicates the normal dopamine loss in the aging process. As it requires an 80% reduction in dopamine for symptoms of P.D. to be displayed, 'normal' people would need to live in the excess of 120 years for frank symptoms of PD to occur (Nagatsu 1991). The three dotted lines offer alternative routes into PD: dopamine concentration may be reduced at birth, an age related decline (as in normal people) results in Parkinsonism symptoms at a relatively early age. A neurotoxic insult may occur early in life which results in PD years later (e.g. environmental factors, MPTP). The idiopathic line represents a possible combination of genetic susceptibility combined with an environmental insult. In all circumstances the loss of dopamine from the nigrostriatal tract would be due to dopaminergic neuronal death and not the failure of an enzymic system.
Adapted from Langston (1990) and Nagatsu (1991)

5.1.6 OXIDATIVE DAMAGE in PARKINSON'S DISEASE

Free radical attack on the nigrostriatal dopaminergic system in PD may represent a mechanism which results in the symptoms of the disease (Graham et al 1978, Spina and Cohen 1989, Olanow 1990). The basis for this hypothesis is that for each mole of dopamine oxidised specifically by MAO B, a mole of hydrogen peroxide is generated. Both dopamine and MAO B are highly concentrated in the substantia nigra (Langston 1989 and Konradi et al 1989 respectively). Graham et al (1978) suggest that a life long injury to the dopaminergic neurons may occur from the by products of dopamine oxidation.

5.1.7 TREATMENT AND DRUG THERAPY

Correcting the balance between a depleted dopamine concentration and the relatively

overactive cholinergic system is the basis for treatment in some PD patients. Replacement therapy with levodopa will increase dopaminergic activity, while centrally acting anticholinergic drugs will decrease the activity of the cholinergic system. Treatment with anticholinergic drugs is usually only possible in the earlier stages of PD because as the disease progresses, the loss of dopaminergic activity is so large there is little possibility for regulatory adjustment. Dementia will occur in some patients with PD which is often associated with degeneration of cortical cholinergic neurons. The use of centrally acting anticholinergic drugs in this group of patients would be unacceptable.

Treatment of PD with dopamine proves unsuccessful because it does not pass through the blood brain barrier to any appreciable extent. The immediate precursor of dopamine, levodopa does so easily, where it is decarboxylated to dopamine and has been used since the late 1960's as the main stay of treatment in the majority of patients. Cotzias et al (1967) were the first group to report the effective therapy that resulted from high oral doses of levodopa, subsequently Marsden and Parkes (1977) and Lees (1986) confirmed the treatment of PD patients with levodopa was the most effective therapy available. Akinesia was usually the first symptom to be improve, followed by bradykinesia and tremors.

High oral doses of levodopa were needed because of wasting due to extracerebral metabolism. Such doses created adverse side effects which were mainly due to the widespread stimulation of dopamine receptors. Nausea and vomiting were common due to stimulation of the chemoreceptor trigger zone which lies outside the blood brain barrier. These problems were overcome by combining levodopa with a selective extracerebral decarboxylase inhibitor. This greatly decreased the effective dose of levodopa required (by reducing peripheral metabolism) with a concomitant reduction in adverse side effects (Boshes 1981).

Other drugs have been developed to treat sufferers of PD: Bromocriptine is a synthetic, directly acting D_2 agonist (there are two distinct types of dopamine receptor known as D_1 and D_2; the receptors of most significance in PD are D_2) which stimulates intact post synaptic receptors. Selegiline selectively inhibits monoamine oxidase type-B (MAO B) present in the brain. Dopamine is a substrate for MAO B and treatment with selegiline can reduce microsomal dopamine metabolism (therefore prolonging the action of dopamine in the brain by inhibition of the uptake by nerve endings and inhibition of the dopamine receptor) and allow patients to eat tyramine rich foods without having serious side effects (MAO A which metabolises tyramine is not inhibited by selegiline). Amantadine (originally designed as an antiviral agent) has anticholinergic and weak dopaminergic properties. It has temporary benefits, but tolerance to the drug soon occurs.

Long term treatment which produces a stable response from using levodopa can alter after five to ten years (the so called honeymoon period). The patient can experience an end of dose deterioration (the shortening of the effective time a dose of levodopa will work) or the gradual recurrence of akinesia. With time, a complication of levodopa therapy is an on-off effect. There are abrupt fluctuations in the patients response to the drug from being symptom free (on) to experiencing classical untreated PD symptoms (off). The exact mechanism of this is not known, but it is suggested it is due to a fluctuating levodopa plasma concentration or an alteration in the sensitivity of the receptors (Shimomura and Headley 1988). The latest preferred method of palliative treatment is to use a low dose of levodopa combined with a dopamine agonist such as bromocriptine (Marsden 1990).

Neurotransplantation or brain grafting is a surgical technique which has received much attention, not only for the possibility of a new treatment for PD, but from groups who are concerned with the ethics of using aborted fetal tissue. In laboratory animals (rat to rat, primate to primate, and human to rat (with immunosuppression)) grafting of fetal substantia nigra into the damaged striatum has resulted in the survival of the graft. It is able to synthesise and release dopamine, reinnervate host tissue, respond to host brain activity and restore some lost motor functions (Marsden 1990).

Implants of the adrenal gland (dopamine is produced in the chromaffin tissue within the gland) were attempted in a two relatively young PD patients and were associated with an improvement in the symptoms of the disease (Madrazo et al 1987). This procedure has been repeated over 300 times and the benefits (modest improvements) and draw backs (unacceptable morbidity and mortality) have been established (Lindvall 1989, Goetz et al 1989). Human fetal nigral grafts have also been attempted and there is evidence to suggest the grafts have survived in certain circumstances which produce a sustained improvement in the relief of the symptoms of PD (Lindvall et al 1990). Clough (1991) suggests this type of treatment would not benefit many sufferers due to the technical difficulties of the procedure.

5.1.8 AIMS OF THE CHAPTER

Fe accumulation in the brain of PD patients is localised in the substantia nigra and other brain areas which are not correlated to Tf mediated deposition (Sofic et al 1991 and Youdmin et al 1989 respectively). Al is also found in increased concentrations in Lewy bodies (a characteristic hallmark of PD) found in the substantia nigra (Hirsch et al 1991). From studies using control plasma and diseased groups, an alteration in bimodal distribution of Ga has been observed. Therefore it was the aim of this chapter to investigate the speciation Ga in medicated and unmedicated PD plasma to elucidate a possible mechanism of accumulation in the brain and to examine the consequences of such an accumulation.

5.2 METHODS

5.2.1 PATIENT DETAILS

PD plasma samples were obtained from: Dr. H. Pall, Consultant Neurologist, Queen Elizabeth Hospital, Edgbaston, Birmingham and Dr. S. Sturman, Lecturer in Neurology, Smethwick Neurology and Neurosurgical Hospital, Smethwick.

Blood was obtained from volunteers at the time of diagnosis of PD and before the commencement of drug therapy. All patients included in the experimental group displayed at least two of the three classical features of PD (tremors, rigidity, and bradykinesia) and subsequently responded to levodopa treatment. Plasma samples from treated patients received anti parkinsonian drugs for at least six weeks. Patients were starved from midnight until a blood sample was taken early in the morning (usually 08.00 - 09.00 hrs). Plasma was obtained from the blood and if not analysed immediately, frozen until needed (all samples were analysed within two weeks of receiving the blood).

5.2.2 GEL-FILTRATION CHROMATOGRAPHY

see section 2.3.2 and 2.3.5

5.3 RESULTS

The control group used in this chapter is defined as, all individuals who had 'high' Ga-Tf binding (as determined in chapter 3). Age/sex matching the PD patients was performed using the high Ga binding control group as a source. Individuals were age and sex matched in order of appearance on the control list (which was generated randomly). The 'low' Ga-Tf binding group of controls were included in appropriate places for comparative purposes.

Ga-Tf binding (using a 25 mM bicarbonate buffer) in the plasma of untreated PD patients was measured and compared to an age/sex matched control group, the control group as defined above, and the group of low Ga binders. As described previously, the Ga binding in plasma from PD patients was bimodal in all experiments and recoveries of radioisotope were 85% or above. Within the PD group, the two observed peaks eluted in similar positions and they were also similar to the elution positions in the control group.

There was a significant depression ($p < 0.0001$) in the Ga-Tf binding in plasma of untreated PD patients (31.5 ± 21.9 (N=32)) compared to the control group (86.9 ± 8.2 (N=86)) and the age/sex matched group (88.2 ± 8.0 (N=32)), but not compared to the low control group (23.9 ± 10.1 (N=21)) (see fig 5.2 and table 5.2). No differences in the Ga-Tf binding of males and females was evident in the untreated PD group.

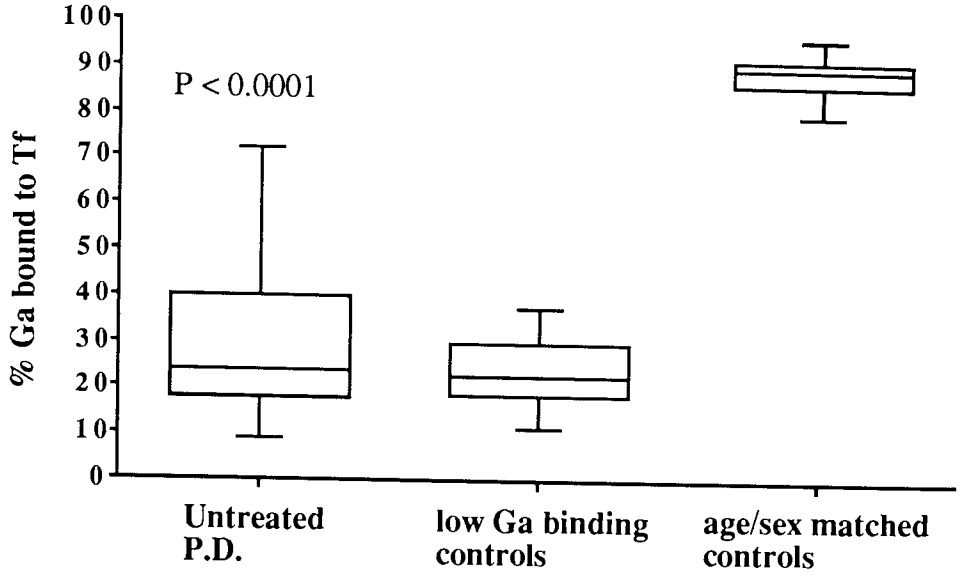


Fig 5.2 Percentile plots (10, 25, 50, 75, and 90th) of Ga-Tf binding in plasma from untreated P.D. patients (N=32), low Ga binding controls (N=21), and age and sex matched controls (N=32).

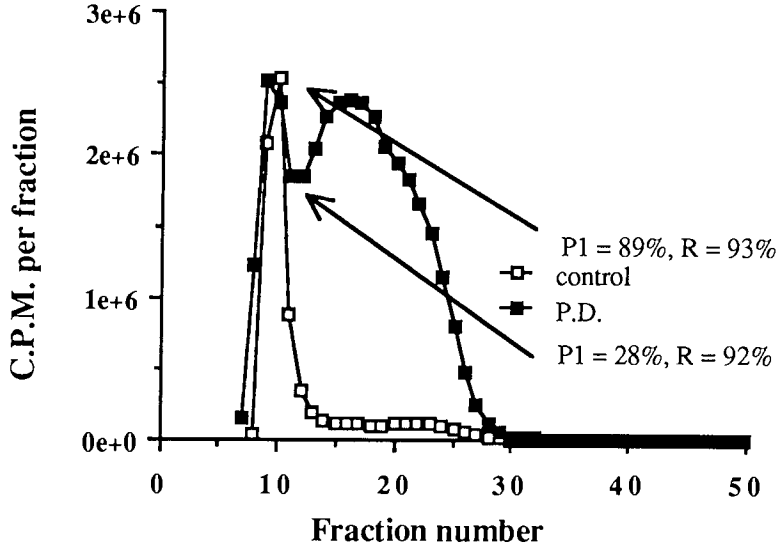


Fig 5.3 Comparison of Ga-Tf binding in PD and control plasma using a G75 column with a 25 mM bicarbonate buffer at pH 7.4

Fig 5.2 and fig 5.3 shows the great difference in Ga-Tf binding between the PD patients and age/sex matched controls, but there are no obvious differences (except an increased age of PD patients (significant at $p < 0.0001$) and in the coefficience of variance about the mean, the latter is not unusual in a diseased group) between PD and low Ga-Tf binding controls (fig 5.4).

It was possible to age match the PD patients with the low Ga binding control group to a limited degree using subjects within the age range 38 - 68 years (mean age: 56.8 years

± 8.9 c.f. 51.6 years ± 9.7 respectively). Using this new control group, there was no statistical difference between Ga-Tf binding in the PD and control groups (Ga-Tf binding 29.2% ± 21 (N=22) and 25.9% ± 9.5 (N=14) respectively). If one excludes the three high Ga-Tf binding results (see fig 5.4) in the age range stated, the Ga-Tf binding becomes almost identical in both groups with respect to both the mean and standard deviation about the mean (22.7% ± 11.1 (N=18) c.f 25.9% ± 9.5 (N=14)). Therefore, with the age matched low Ga binding control group the only difference between the two groups was the diagnosis of PD.

	untreated PD (N=32)	age/sex matched (N=32)	high controls (N=86)	low controls (N=21)
M/F	24/8	24/8	42/44	12/9
age (years)	61.8 ± 10.7	58.6 ± 12.1	42.2 ± 18.6	42 ± 16.2
age range	38-79	38-82	5-82	15-67
% Ga-Tf (HCO_3^-)	31.5 ± 21.9	88.2 ± 8.0	86.9 ± 8.2	23.9 ± 10.1
% Ga-Tf (std)	12.4 ± 4.0 (N=18)	-	13.3 ± 6.1 (N=18)	6.9 ± 3.5 (N=5)

results are expressed as: mean ± SD

Table 5.2 Ga-Tf binding with and without 25 mM bicarbonate in untreated PD, age/sex matched controls, the control group as a whole, and low Ga-Tf binding controls.

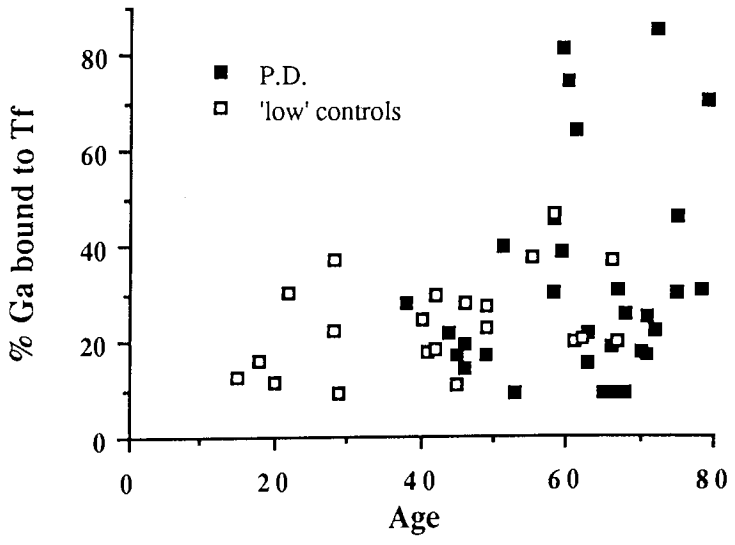


Fig 5.4 Ga-Tf binding in untreated PD and low Ga binding controls.

Using a gel-filtration chromatography system which did not contain 25 mM bicarbonate there was no difference in Ga-Tf binding between the control and the untreated PD group. This was in contrast to the observations made with AD and DS plasma presented in this thesis and by Farrar et al (1990). The Ga-Tf binding in the untreated PD group was significantly greater ($p < 0.01$) than that of the low Ga binding control group.

Using fractions obtained from untreated PD which had undergone separation by G75 gel-filtration chromatography (containing 25 mM bicarbonate), the identity of the LMWS (second peak) was attempted using a G15 column. In all but one sample, a minute peak (< 1%) was seen at fraction ten. The second peak eluted at fraction twenty two to twenty four. This was indicative of Ga binding to citrate (at least to a small degree) and was identical to the Ga binding on a G15 column in controls (see 3.10.5).

Further Ga-Tf binding observations were made (on a G75 column with and without 25 mM bicarbonate) in plasma from PD patients who had received drug therapy for at least six weeks. Using the standard buffer, there was no statistical difference in the Ga-Tf binding in treated and untreated PD plasma samples (12.0% ± 2.7 c.f. 12.4% ± 4.0 respectively) or in comparison to controls (13.3% ± 6.1). With 25 mM incorporated into the elution buffer the Ga-Tf binding rose from 12.0% ± 2.7 (N=7) to 74.4% ± 14.2 (N=13), which was significant ($p < 0.0001$), but it was still lower than the age/sex matched controls (88.2% ± 8.0 (N=32) ($p < 0.01$) (see table 5.4). In the three patients where pre and post treatment plasma was available the Ga-Tf binding rose from 18.7% ± 9.5 to 79.1% ± 5.1. and in only one patient was the binding less than 65%.

PD Patients:	untreated	untreated	treated	treated
Buffer:	HCO_3^-	standard	HCO_3^-	standard
% Ga bound to Tf	31.5 ± 21.9 (N=32)	12.4 ± 4.0 (N=18)	74.4 ± 14.2 (N=13)	12.0 ± 2.7 (N=7)

Table 5.3 Ga-Tf binding in treated and untreated PD patients.

group	% Ga-Tf binding	Fe (µg/l)	Tf (g/l)	TIBC (µg/l)	Fe sat.	Ferritin (µg/l)
PD (N=7)						
mean	22.14	774.5	2.55	3578.9	0.24	84.4
± SD	6.97	331.5	0.79	1110.3	0.13	70.8
controls (N=15)						
mean	ND	1540	2.98	-	0.39	63
± SD	-	1057	0.69	-	0.29	42.6
significance	-	$p < 0.05$	NS	-	NS	NS

Fe, Tf, and ferritin measurements in the control group were done by Farrar et al (1990) who have kindly let me use their data.

Table 5.4 Fe, Tf, and ferritin measurements in PD and control subjects.

Plasma Tf concentrations in the PD group were reduced compared to controls (2.55 µg/l ± 0.79 c.f. 2.98 µg/l ± 0.69 respectively), yet were within the normal adult range. There was no statistical difference between the two groups, despite a trend of lower Tf values in the PD group. Fe levels in the plasma of the PD group were significantly lower ($p < 0.05$) compared to controls (774.5 µg/l ± 331.5 c.f. 1540 µg/l ± 1057 respectively) which was reflected in the lower Tf saturation values. Ferritin levels were similar in both groups and a comparison between Ga-Tf binding was not possible because different elution buffers were used in each group. No correlation between any variables (Fe, Tf, TIBC, ferritin, Ga-Tf binding, age, and sex) were found in the PD group.

Chronic exposure to, and toxicity of Mn is known to lead to a Parkinsonian like disease state. This can be used to create a basic model for the development of idiopathic PD MPTP could also be used as a model, but the general population are exposed to Mn much more frequently (Mn is widely distributed in water, soil, and biological samples, it is present in all food stuffs and in the air in a respirable form) than to a designer drug. In this context, Mn is a more suitable model.

Mn (in the form of manganese dioxide (MnO_2), 'activated' manganese dioxide, and potassium permanganate) was incubated in control plasma at given concentrations (see table 5.4) for two hours at 37°C in a shaking water bath (procedure described in detail in section 2.3.4) before Ga incubation. After Ga was incubated for an hour, the plasma was applied to a G75 column containing 25 mM bicarbonate and elution profiles obtained.

Mn dioxide (which is sparingly soluble) was prepared as a suspension. The 'activated'

manganese dioxide was purchased from Sigma Chemical Company and was designed specifically to take part in oxidation reactions.

Potassium permanganate is a powerful oxidising agent which was shown to progressively inhibit Ga-Tf binding in vitro with an increase in concentration of the potassium salt. 'Activated' magnanese dioxide also showed the ability to detrimentally affect Ga-Tf binding by oxidative attack but to a lesser degree and normal manganese dioxide showed little effect at all on the binding of Ga to Tf.

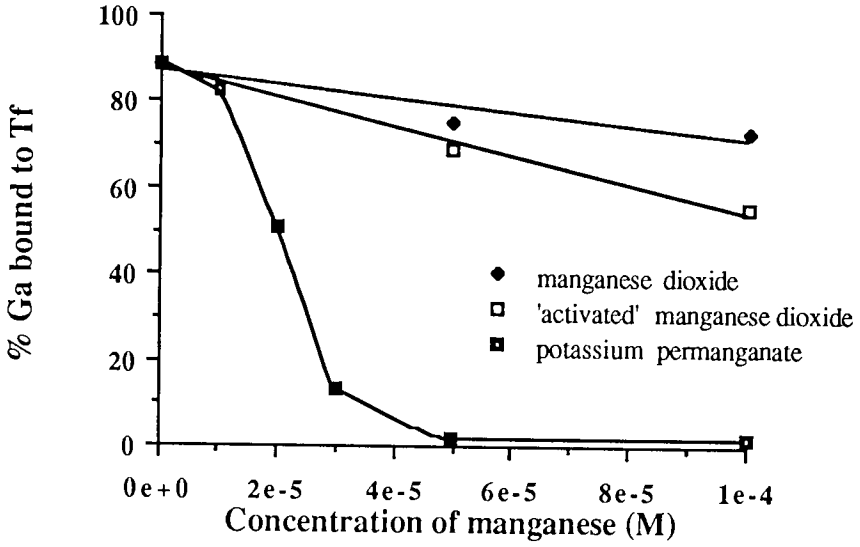


Fig 5.5 Ga-Tf binding in the presence of added manganese dioxide, 'activated' manganese dioxide and potassium permanganate.

	10^{-4} M	5 x 10^{-5} M	3 x 10^{-5} M	2 x 10^{-5} M	10^{-5} M
$KMnO_4$	1.7	2.0	13.4	50.7	82.5
MnO_2	74.0	76.0	-	-	-
'activated' MnO_2	55.9	69.4	-	-	-

results are expressed as the percentage of Ga bound to Tf

Table 5.5 Ga-Tf binding in control plasma in the presence of Mn.

Heafield et al (1990) reported that there is an increase in the cysteine/ sulphate ratio in the plasma of untreated PD (as well as in AD and MND) and it was suggested that it may lead to an interfered neural protein function and reduced xenobiotic detoxification. In an attempt to identify a causative agent which reduced the Ga-Tf binding in untreated PD plasma, cysteine was added to the plasma from a control subject to obtain a concentration which was double that expected in control plasma (normal cysteine

concentration 4.23 x 10^{-7} M/mg protein) (and in excess of that found by Heafield et al in untreated PD plasma). The cysteine was incubated in the plasma for two hours at 37°C in a shaking water bath and then applied to a G75 column containing 25 mM bicarbonate. The Ga-Tf binding was found to be unaltered by the addition of cysteine to the plasma.

5.4 DISCUSSION

The results show that after an in vitro incubation followed by gel-filtration chromatography using the standard elution buffer (which does not contain bicarbonate), there were no differences between Ga-Tf binding in PD and controls. This differed from the results of other diseased groups studied in this thesis (DS patients ($p < 0.02$) and AD patients ($p < 0.002$)) and those studied by Farrar et al (1990) ($p < 0.01$). Using a 25 mM bicarbonate buffer, significantly less Ga was bound to Tf in patients with untreated PD compared to normal controls, but not compared to control subjects with low Ga binding and was similar to other disease groups studied. Accumulation of Al which is known to occur in PD brains could be the result of the reduced metal binding to Tf.

The Ga-Tf binding results in the two buffering systems indicates an altered Ga-Tf binding scenario compared to AD, DS, and controls: binding in PD plasma was considered normal under bicarbonate free conditions but abnormal when bicarbonate was present. This represents a unique feature of PD plasma found in no other group studied and may possibly be exploited in a potential diagnostic test.

Using a G15 column to identify the LMWS present in PD plasma samples the species found were the same as described in chapter 3 for normal controls. There was evidence of some citrate binding, but the majority of Ga was bound to an unidentified (possibly phosphate) species, which should merit further investigation.

Analysis of Fe, Tf, and ferritin results in the PD group and comparing them to controls revealed only Fe concentrations to be significantly different. Although Tf levels were within the normal range, there was a trend to observe lower values in the PD group. The lower Fe results are reflected in a lower mean Tf Fe saturation value. In the neonate, one may assume low Ga-Tf binding was due to high Tf Fe saturation. However, these results suggest lower Ga-Tf binding in PD is not due to such an observation. Evidence from treated PD patients also supports a hypothesis which would imply an alternative mechanism is responsible for reduced Ga-Tf binding. This will be discussed later.

The low Ga binding control group matched almost identically the PD group in respect to Ga-Tf binding. The only observable difference between the two groups was a significant increase in the mean age of the PD subjects. If an age matched group of low

Ga binding controls were selected no differences would exist. It is reasonable to assume the low Ga binding controls which also have an increase in 'mobile' Ga (and Al) could represent a group of people who are nonsymptomatic PD sufferers (i.e. they are in a pre Parkinsonian phase of the disease). With time and a reduction in dopamine concentration (indirectly due to the low Ga binding) in the basal ganglia, the subjects present in the low Ga binding group who may display the classical symptoms of PD. This hypothesis will be dealt with in greater detail in chapter 10. If the PD and low control group are combined, there is a significant correlation between Ga-Tf binding and age. The trend observed was to increase Ga binding with age. This implies the higher the Ga-TF binding the later a person would develop PD.

As defined, the cause of PD is unknown, but secondary Parkinsonism has many causes (see table 5.1). The group of idiopathic PD patients studied in this set of experiments may represent two subgroups which developed PD by alternative routes. The majority of patients had Ga-Tf binding in the range of 10-40%, and two with just slightly higher values. There was a group of five PD patients who had Ga-Tf binding in the normal control range and it is this small group of patients which possibly represents a route into PD which is not dependent upon the speciation of metal ions in the plasma. Alternatively, it is possible these patients may be anemic. Anemia could explain an increase in Ga-Tf binding (a reduction in plasma Fe would result in an increase in available binding sites for Ga). However, the consultant who supplied the plasma samples thought the possibility that the patients were anemic was small. Thus, the latter explanation does not seem feasible, although plasma Fe measurements would have given a definitive answer.

Permanganate (a powerful oxidising agent) inhibited Ga-Tf binding in vitro. Although in a physiological situation permanganate would not be present in the blood stream, it does show that Tf can undergo oxidative attack which causes less Ga to bind to the Tf molecules. The binding ligands within the protein molecules' binding sites are especially susceptible to attack because they are exposed to extracellular fluids. The most likely ligands to undergo oxidative attack are the tyrosine residues which, once damaged, Fe probably would not bind to. Thus, Fe would not bind to the lobe of Tf which had undergone oxidation. In support of this (Dr. R. Evans personal communication 1992), a case has been described where a subject had tyrosine residues absent from the lobe of the C-terminal of Tf and there was no Fe binding in that particular site. There are also two naturally occurring lactoferrins that have been identified with tyrosine residues missing from the binding site. Fe binding does not occur in these molecules. 'Activated' magnanese dioxide also showed the ability to detrimentally affect Ga-Tf binding by oxidative attack, but to a lesser degree and normal manganese dioxide showed little effect at all on the binding of Ga to Tf in the concentrations used in the experiments.

With the concentration of Mn found in the plasma of an unexposed individual (3.3 - 5.6 x 10^{-8} M) no effect on Ga-Tf binding would be demonstrated. However, in the presence of increased hydrogen peroxide production, Mn could participate in free radical formation via a Fenton type reaction. It has already been demonstrated in chapter 3 that Fe can take part in such a reaction in plasma which results in a decrease in Ga-Tf binding. Furthermore, an increase in Mn concentration in the blood stream (as appears in chronic Mn exposure with subsequent Parkinsonism) (3.35 x 10^{-6} M Suzuki cited by Manganese, Environmental Health Criteria 17) would increase a cascade system in which progressively less Mn, Al ,and possibly Fe are bound by Tf due to oxidative damage. This releases the metals into a potentially neurotoxic LMWS to accumulate in the brain and take part in further reactive oxygen species formation in the brain and blood stream.

Drug treatment for PD increased Ga-Tf binding. L-dopa was given for at least six weeks before Ga-Tf binding in treated PD patients was analysed. The mechanism behind the increase in Ga-Tf binding is believed to be the antioxidant effect of L-dopa. Farrar et al (1991b) has demonstrated the inhibition of oxidation of 5-methyltetrahydrofolate in the presence of L-dopa. The mechanism was suggested to be the ability of L-dopa to break the chain reaction initiated by hydroxyl radicals (by forming a relatively stable complex. The effect of L-dopa on increasing treated PD Ga-Tf binding suggests that the primary mechanism behind the original observations (reduced Ga-Tf binding) is due to oxidative damage.

CHAPTER SIX

DOWN'S SYNDROME

6.1 INTRODUCTION

Down's syndrome (DS) is one of the most common disorders which affect human intelligence. The condition (named after Langdon-Down) occurs in approximately 0.1-0.12% of live births in the developed countries of the world (Hook 1981) and has a prevalence of 15% in the mentally retarded population (Heller 1969). There is an increase in incidence of DS births with an increase in age of the mother over the age of forty. Baraitser (1985) proposed that it is due to a fetus with DS aborting less frequently with an increase in the age of the mother. It has also been reported (Stene and Stene 1977, Matsunaga et al 1978 and Stene et al 1981) that an increase in the age of the father can increase the risk of DS in the child. Therefore, the ages of both parents should be taken into account when evaluating the risk of chromosomal abnormality in the fetus.

The origins of the DS are genetic, with 95% of all cases resulting from meiotic non-disjunction of chromosome 21 (Williams and Matthysse 1986), more commonly known as trisomy 21. At conception, the zygote receives half the genetic material from each of the reproductive cells of the parents. In trisomy 21, one of the reproductive cells (usually the ovum) carries a duplicate of chromosome 21, thereby giving the child an extra chromosome in each cell. Another cause of DS are translocation errors (partial trisomy) where part of chromosome 21 is broken off and joined to another (usually chromosome 14). Finally, DS can arise through some, but not all cells in the body having an extra chromosome 21. When this occurs it is known as mosaic DS.

Life expectancy at birth of DS patients was only nine years in the early part of the twentieth century (Penrose 1932), but has risen steadily over the last five decades. Improvements in medical and surgical techniques (especially in the treatment of congenital heart disease, a major cause of death in young DS patients (Owens et al 1971)) and the increased availability of antibiotics (to overcome infections due to a relatively poor immune response) have been the most significant contributors to this. Recent survival rates of DS patients reaching sixty years old has been calculated to be 44.4% compared to 86.4% in the normal population. 13.6% of all DS live births reach 68 years old compared to 78.4% in the normal population (Baird and Sadovnick 1988). Steffelaar and Evenhuis (1989) have estimated the number of DS who live to or beyond 40 years will increase by 75% in the next two decades and during the same time the number of DS patients surviving beyond 50 years will increase by 200%.

The advances in medicine which increased the life expectancy of DS patients has also created new problems for the individual and for the carers. With a greater number of DS patients surviving longer, there is an increased responsibility placed upon the carers within the community and on the National Health Service (many older DS need to be hospitalised). There is also the added burden of increasing numbers of demented

patients within this population. Dementia (typical early features are apathy, loss of skills, and development of seizures (Holden et al 1991)) in the DS population occurs at a much younger age and with increased frequency compared to the general population. Neuropathologically (Williams and Matthysse 1986) and neurochemically (Yates et al 1980, Godridge et al 1987) older DS brains are very similar to AD brains post mortem.

Fraser and Mitchell (1876) described an association between DS and dementia and Strewe (1929) (cited by Yatham et al 1988) reported the characteristic senile plaques of AD in the brains of DS patients. However, it was Jervis (1948) and Verhaart and Jelgersma (1952) who were the first to describe DS patients who showed clinical deterioration and died after development of dementia. Characteristic neuropathological changes in DS brains was confirmed post mortem to be identical to SP and NFT of AD. It is now well established that neuropathological features of AD are present in the majority ($\approx$ 90%) of DS patients over the age of forty (Olson and Shaw 1969, Burger and Vogel 1973, Ellis et al 1974, Sylvester 1984, Hyman and Mann 1991) and in all DS patients over sixty (Mann 1988). Furthermore, Parkinsonism has been noted in 20% of a demented DS study group and epileptic seizures was noted to be in 84% of the patients in the same group (Lai and Williams 1989).

6.1.1 AIMS OF THE CHAPTER

DS subjects have shown to accumulate Al in the brain in senile plaques (Edwardson et al 1991) and to display neurochemical (Godridge et al 1987) and neuropathological (Mann 1988) changes identical to those in the brains of individuals with post mortem confirmed AD. It was therefore decided to study DS patients because they represent a suitable model for the occurrence and development of AD in the general population. The use of DS patients has an advantage over using AD patients diagnosed ante mortem because of the possibility of misdiagnosis. It could also offer a possible insight into the mechanisms of progression into dementia of the Alzheimer type.

6.2 METHODS

6.2.1 PATIENT DETAILS

DS plasma samples (prepared from blood collected, separated and stored as described in section 2.3.1) were obtained from: Prof. J. Lejuene, Hospital des Enfants Malades, Paris, France; Dr S. Sturman, Lecturer in Neurology, Smethwick Neurology and Neurosurgical Hospital, Smethwick and Dr V. Prasher, Lecturer in Psychiatry, Monyhull Hospital, Kings Norton, Birmingham. The subjects were hospitalised individuals or people living in the community (with relatives or in small group homes). All patients in the study group were trisomy 21 (except for one individual with translocation DS) which was confirmed by standard cytogenetic analysis.

6.2.2 GEL FILTRATION CHROMATOGRAPHY

see section 2.3.2

6.3 RESULTS

The control group used in this chapter was defined in chapter 5. Age and sex matching the DS subjects was performed as previously described (section 5.3)

The elution profile (fig 6.1) from a G75 column containing 25 mM bicarbonate demonstrates the Ga distribution in DS plasma is bimodal. There were two observable peaks in all experiments which eluted in similar positions and recovery of radioactivity was always above 85%. The positions of the two peaks was identical to those seen in previous chapters; Ga was bound to Tf in the first peak at fraction ten and at fraction twenty two to twenty four, Ga was bound to a LMWS.

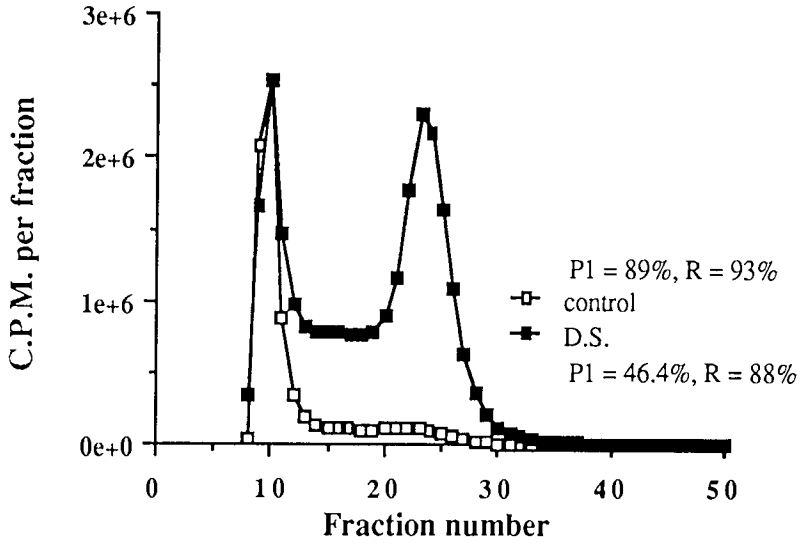


Fig 6.1 Comparison of Ga-Tf binding in DS and control plasma using a G75 column with a 25 mM bicarbonate buffer at pH 7.4

In the presence of 25 mM bicarbonate the mean Ga-Tf binding (as a percentage of recovered activity) in DS plasma was 54.9% ± 29.9 (N=32). This was significantly depressed ($p < 0.0001$) compared to age/sex matched controls (86.2% ± 8.8 (N=32)) and the whole control group (86.9% ± 8.2 (N=86)) but significantly increased ($p < 0.0001$) compared to the low Ga binding control group (23.9 ± 10.1 (N=21)). When bicarbonate was omitted, Ga-Tf binding in DS plasma was still significantly reduced ($p < 0.02$) compared to controls (9.1 ± 3.4 (N=23) c.f. 13.3 ± 6.1 (N=18)), but was not significantly different from the low Ga binding controls. This was in agreement with the results of Farrar et al (1990) (DS Ga-Tf binding 6.9% ± 2.4 (N=12) c.f. control Ga-Tf binding 17.1 ± 7.4 (N=22)). Fig 6.2 shows the Ga-Tf binding results in the DS

group have a large coefficience of variance about the mean which results from a large range of values for Ga-Tf binding within the group. In this respect they are not a well defined group.

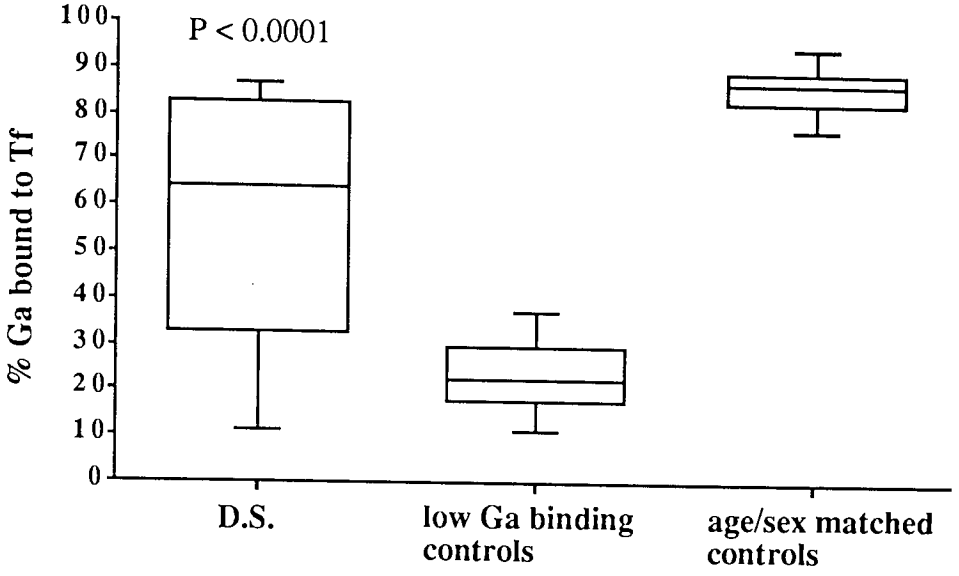


Fig 6.2 Percentile plots (10, 25, 50, 75, and 90th) of Ga-Tf binding in plasma from D.S. subjects (N=32), low Ga binding controls (N=21), and age/sex matched controls (N=32).

	DS (N=32)	age/sex matched controls (N=32)	high* controls (N=86)	low* controls (N=21)
M/F	21/11	21/11	42/44	12/9
age (years)	32.4 ± 17.9	32.7 ± 17.0	42.2 ± 18.6	42.0 ± 16.2
age range	7-70	5-70	5-82	15-67
% Ga-Tf (HCO_3^-)	54.9 ± 28.9	86.2 ± 8.8	86.9 ± 8.2	23.9 ± 10.1
% Ga-Tf (std)	9.1 ± 3.4 (N=23)	-	13.3 ± 6.1 (N=18)	6.9 ± 3.5 (N=5)

* refers to high or low Ga binding

Table 6.1 Ga-Tf binding with and without 25 mM bicarbonate in DS and controls groups.

When all the DS data was examined there was a significant correlation between Ga-Tf binding in the presence of 25 mM bicarbonate and the age of a subject (R = 0.48, p = 0.006) (see fig 6.3). No such correlation existed when Ga-Tf binding occurred without bicarbonate.

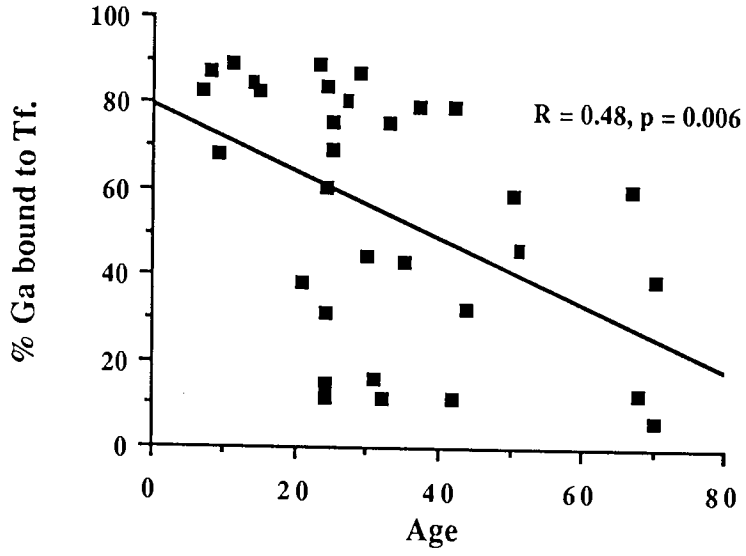


Fig 6.3 The relationship between Ga-Tf binding and age in DS patients.

When the DS group was split by sex no difference in Ga-Tf binding was evident between males and females (54.8 ± 30.2 (N=21) c.f. 54.9 ± 27.6 (N=11) respectively). As shown in Fig 6.3, Ga-Tf binding decreased with age. Splitting the DS subjects into two groups by age (forty was taken as a cut off point because it is generally accepted that the majority of DS by this age have characteristic AD neuropathological hallmarks in the brain) revealed a significant decrease ($p < 0.05$) in Ga-Tf binding in the older (40 +) group (see table 6.2). Further division of the DS group into decades was not completed due to lack of subjects at the two ends of the age range. Fig 6.4 shows the similarity between the high Ga binding DS group and the young DS group, correspondingly the old DS group match well the low Ga Tf binding group.

	all DS	young DS (< 40)	high Ga binders	old DS (>40)	low Ga binders
% Ga bound to Tf	54.9 ± 28.9 (N=32)	61.2 ± 28.2 (N=9)	77.6 ± 9.7 (N=18)	38.7 ± 25.3 (N=23)	25.6 ± 14.8 (N=14)

Table 6.2 Ga-Tf binding in DS subjects split by age and Ga binding.

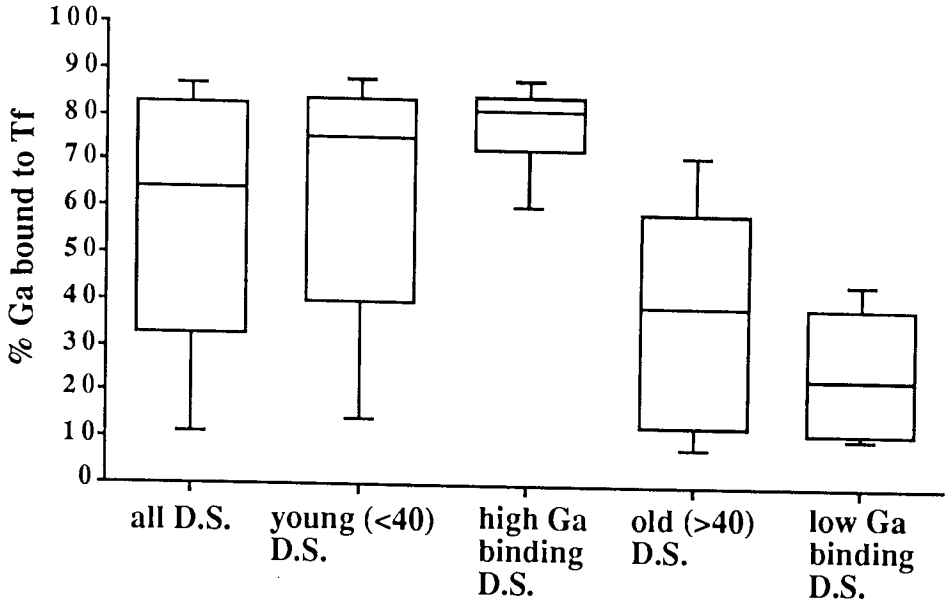


Fig 6.4 Percentile plots (10, 25, 50, 75, and 90th) of Ga-Tf binding in DS subjects split by age and Ga binding.

Sixteen DS patients were tested for dementia (clinical and adaptive behaviour scale (ABS)) by Dr V. Prasher (see appendix 7). There was no correlation between Ga-Tf binding, age, and dementia. This was not unexpected because there are certain problems in the ante mortem diagnosis of AD in the DS population. Therefore any potential correlation with dementia has significant problems to overcome.

If one examined Ga-Tf binding in those DS who were demented (ABS and clinically diagnosed dementia, including those who were possibly demented), it was of interest to note that demented DS patients had significantly lower Ga-Tf binding than the control group as a whole (23.68% $\pm$ 21.6 (N=6) c.f 86.9% $\pm$ 8.2 (N=86) respectively, $p < 0.0001$), but also had significantly lower Ga-Tf binding than those DS patients who were diagnosed as non-demented (23.68% $\pm$ 21.6 (N=6) c.f 44.4% $\pm$ 22.7 (N=10) respectively, $p < 0.05$). This latter statistical analysis was only significant if one hypothesised that Ga-Tf binding in demented DS patients was reduced compared to non-demented DS patients, this enabled a one tailed t-test to be performed.

The composition of the DS demented group included ABS and clinically defined patients as well as any possible case of either dementia. The decision to include possible cases of dementia was to increase the sample size because the groups were so small.

6.4 DISCUSSION

Ga-Tf binding in DS plasma (using both a bicarbonate containing and a bicarbonate free buffer) was significantly reduced compared to age/sex matched controls. The

consequences of reduced Ga-Tf binding have been discussed in previous chapters.

There was a large range of values within the DS group, ranging from normal controls results to below 10% of Ga bound to Tf. Examination of the results showed that younger DS patients generally had high Ga-Tf binding and older DS patients had low Ga-Tf binding. It has been demonstrated by others that in young DS patients functions are normal, with an increase in age these parameters move from healthy to diseased values. For example, Kish et al (1989) demonstrated the activity of choline acetyltransferase and acetylcholinesterase to be normal in all areas of the brain from DS babies less than one year old compared to age matched controls (except the putamen where it was above average), while Godridge et al (1987) has reported a reduction in the same enzyme systems in the adult DS brain compared to age matched controls.

Mann (1988) examined 392 DS brains post mortem and noted the percentage of patients within a given age range that showed senile plaques and neurofibrillary tangles (see table 6.3). Using the data of Mann and superimposing the Ga bound to a LMWS in DS patients (in the same age range) (fig 6 .5) it can be seen that a sharp increase in the LMWS of Ga (or conversely a sharp decrease in the amount of Ga bound to Tf) in the second, third, and forth decade of life is matched by an increase in plaques and tangles observed in the brains of DS patients some ten years later. This observation reinforces the importance of the equilibrium between Ga bound by Tf and a LMWS has on the possible development of neurodegenerative diseases such as AD or PD. It also demonstrates that it may take many years for the development of such a disease to develop into a clinically definable condition.

Possible mechanisms for the development of AD in DS will be discussed in detail in chapter 10.

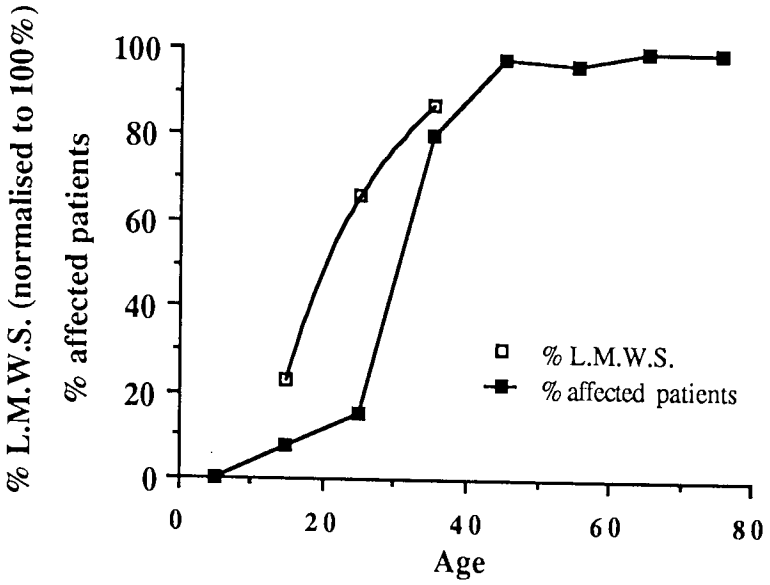


Fig 6.5 The incidence of senile plaques and neurofibrillary tangles in DS subjects related to an increase in LMWS and age.

age range (years)	number of patients	% of affected patients	Ga bound to LMWS
0-9	37	0	-
10-19	79	7.6	14.5%± 3.3 (N=3)
20-29	58	15.5	41.6% ± 29 (N=11)
30-39	35	80.0	54.9% ± 28.9 (N=6)
40-49	50	98.0	-
50-59	87	97.7	-
60-69	43	100.0	-
70-79	3	100.0	-

Table 6.3 Prevalence of senile plaques and neurofibrillary tangles in the brains of DS patients with an increase in the LMWS of Ga in the plasma.

CHAPTER SEVEN

HAEMODIALYSIS, MND, AND AFFECTIVE DISORDER STUDIES

7.1 INTRODUCTION

The relationship between Al, dementia, and chronic renal failure has been discussed in chapter 1 and it is therefore not appropriate to consider haemodialysis patients further.

Motor neuron disease (MND) results from the death of a specific type of nerve cell (motor neurons) which is present in the brain stem and innervates a group of skeletal muscle fibres to control movement. Damaged or dead motor neurons which fail to stimulate muscle fibres cause paralysis and atrophy of fibres. This results in the affected parts of the body becoming increasingly weak and uncoordinated. The disease can attack most motor neurons which can affect virtually all physical functions. Treatment is only palliative and life expectancy at the onset of MND is 5 - 10 years.

Affective disorders are characterised by a disturbance of mood associated with alterations in behaviour, energy, appetite, sleep, and weight (Neal 1987). From intense excitement, elation, and delusions of grandeur (mania) to a depressed mood, morbid thought content, and some suicidal tendencies (depression). These are the two extremes in affective disorders. Depression (unipolar depression) is the most common affective disorder and may occur from an extreme emotional blow such as bereavement (reactive depression) or from an endogenous source such as the result of hormonal changes after childbirth (endogenous depression). Patients categorised as unipolar must have at least three episodes of depression separated by periods of complete remission. A diagnosis of bipolar depression (manic depressive illness) is made if a period of mania follows on from a period of depression (Leonard 1963 cited by Jones 1988).

Depression can be readily treated. A combination of drugs and psychotherapy usually returns the patient to normality, although further episodes of depression may occur later in life. Tricyclic antidepressants (so called due to the ring structure of the drug) or related drugs are the usual choice of medication and at least 70% of patients respond satisfactorily to them. Monoamine oxidase inhibitors (MAOIs) are effective antidepressants but rarely used because of the severe adverse side effects. With patients who fail to respond to a single drug, a combination of drugs and/or electroconvulsive therapy (ECT) can be used. ECT involves the passage of an electric current between electrodes applied to the scalp. This generates a large amount of activity in the brain which disappears quickly. The mechanism behind an improvement of depression post ECT is not understood. The most effective treatment of mania is by lithium carbonate administration and/or a neuroleptic drug.

Depression in demented patients has been recorded in approximately half the patients observed by Ernst et al (1977) and in a slightly lower proportion (30-40%) of AD patients (Wragg and Jeste 1989). Parkinsonism is also strongly associated with depression (Sano et al 1989). Furthermore, Agbayewa (1986) and Rovner et al (1989) noted that ≈ 20% of AD patients had a previous history of psychiatric illness (most

likely to be unipolar depression). However, Murphy (1983) reported that the incidence of dementia in depressed elderly patients was comparable to that of the general population. Alexopoulos (1991) reported the high comorbidity between depression and dementia results from depressives being vulnerable to develop dementing disorders.

7.1.1 AIMS OF THE CHAPTER

Reduced Ga-Tf binding has been observed in diseased groups where neurological dysfunction was present. Similarly, this has been discovered in a small number of healthy and hospitalised controls. It was therefore decided to examine other groups of people which are susceptible to, or suffering from neurological dysfunction (long term haemodialysis patients and individuals with an affective disorder) and a group who retrospectively, show an increased incidence of reduced Ga-Tf (MND patients).

7.2 METHODS

7.2.1 PATIENT DETAILS

Plasma from six haemodialysis patients (M/F - 2/4) were obtained from Dr. P. Altmann, Senior Registrar in Nephrology, The Royal London Hospital, London. Plasma Al concentrations were measured courtesy of Dr. Altmann using a Varian AA1275 atomic absorption spectrophotometer and GTA95 electrothermal atomiser.

Dr. H. Pall, Consultant Neurologist, Queen Elizabeth Hospital, Edgbaston, Birmingham, supplied seven plasma samples from MND patients. The study group consisted of three males and four female, the mean age was 54.4 years $\pm$ 9.3, with a range from 44 to 67 years. This group appeared in chapter 3 as part of the hospital control group. The inclusion criteria of the hospital control group was the absence of any known dementing disorder and so all MND patients were judged suitable control subjects. They do however, represent a sub-group of great interest because of the Ga-Tf results and so merit further analysis.

Dr. D. Anderson, Consultant Psychogeriatrician, Fazakerley Hospital, Liverpool, supplied plasma from twenty severely depressed patients (M/F - 6/14, mean age 68.6 years $\pm$ 15.3, age range 24 - 81) who received ECT as part of their treatment. Diagnosis of depression can be rather difficult and may vary from Clinician to Clinician. It was therefore decided to use only depressive patients who had undergone ECT, as it is generally accepted that any individual who has ECT is severely depressed. By using these patients any possible chance of misdiagnosis was removed. Plasma was obtained prior to and after a course of ECT in ten patients in the study group. Patients were classified as: unipolar or bipolar depressive, psychotic or not, and whether they responded to ECT (the Hamilton depression score (HDS) was used pre and post ECT to determine the degree of depression. A response to ECT was defined as a 50% or

more reduction in the HDS). Medication of the patients was noted, all patients (except five who received a 5-HT specific antidepressant) received tricyclic antidepressants and one patient also received lithium carbonate (see appendix 8 for details).

7.2.2 Ga SPECIATION

see section 2.3.3 and 2.3.5

7.3 RESULTS

The control group used in this chapter was defined in chapter 5. Plasma Al concentrations were measured in thirteen subjects from the control group by Dr. Altmann using the same technique employed to measure the haemodialysis patients. Control plasma Al concentrations were within the range 2-3 µg/l and Ga-Tf binding was between 90-100%.

Ga speciation in haemodialysis, MND, and affective disorder patients was identical to all other plasma samples analysed in respect to being bimodal and in the position of the two peaks in the elution profile. Recovery of radioactivity in the study groups was greater than 85%.

7.3.1 HAEMODIALYSIS PATIENTS

Ga-Tf binding in haemodialysis patients was significantly depressed ($p < 0.05$) compared to normal (high Ga binding) controls (74.95 ± 14.4 (N=6) c.f. 86.9 ± 8.2 (N=86) respectively). Ga-Tf binding appeared to be a function of plasma Al concentration in the haemodialysis group, a significant correlation ($R = 0.93$, $p < 0.0001$) was found using the control and study group (see fig 7.1). If the one out lying point (600 µg/l Al, Ga-Tf binding 47.7%) was removed the correlation was still statistically significant ($R = 0.88$, $p = 0.0001$). Furthermore Ga-Tf binding in the haemodialysis group was very similar to that obtained from in vitro additions of Al to control plasma (see section 3.11).

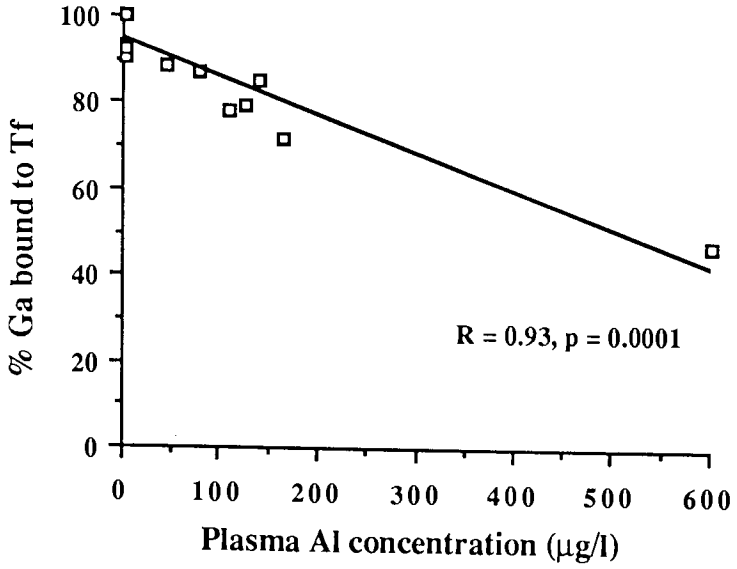


Fig 7.1 Ga-Tf binding as a function of Al plasma concentration in haemodialysis (N=7) and control (N=13) subjects.

I.D.	sex	plasma Al conc. (µg/l)	% Ga-Tf binding	% Ga bound to a LMWS	Al (µg/l) in LMWS
J.T.	M	80 (2.96 x 10^{-6} M)	87.0	13.0	10.4
E.S.	F	110 (4.07 x 10^{-6} M)	78.2	21.8	23.98
B.P.	F	125 (4.63 x 10^{-6} M)	79.5	20.5	25.63
D.R.	M	140 (5.19 x 10^{-6} M)	85.3	14.7	20.58
J.W.	F	165 (6.11 x 10^{-6} M)	72.0	28.0	46.2
M.B.	F	600 (2.22 x 10^{-5} M)	47.7	52.3	313.8
M.B.*	F	45 (1.67 x 10^{-6} M)	85.5	14.5	6.525

* denotes post DFO treatment

Table 7.1 Plasma Al concentrations and Ga-Tf binding in haemodialysis patients.

If one considers Ga-Tf binding and the total concentration of plasma Al in each of the haemodialysis patients (excluding M.B. pre DFO treatment), it is possible to calculate the concentration of Al in a LMWS (assuming the concentration of Ga added does not effect the equilibrium between the high and low molecular weight species) for each subject (see table 7.1 and fig 7.2). Fig 7.2 demonstrates that as Ga-Tf binding decreased, the concentration of Al in a LMWS increased. There was a significant correlation (R = 0.96, p = 0.0028) between the two.

It is not only the concentration of plasma Al that is important in subsequent toxic effects observed in vivo, but the species that Al is present in. For example, if the plasma Al concentration was 10 µg/l and Ga-Tf binding was 90% in a patient, 9 µg/l of Al would be bound by Tf (and therefore it would be non toxic), 1µg/l would be present as a

LMWS which could represent a neurotoxic threat. However, if the same concentration of plasma Al is taken but the Ga-Tf binding is reduced to 70%, 7 μg/l would be bound to Tf and 3 μg/l Al would be present in a LMWS which represents a much greater potential neurotoxic threat.

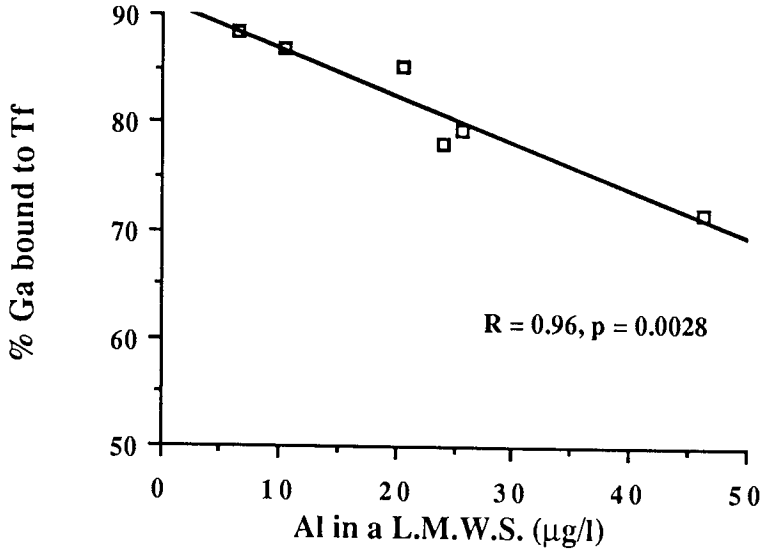


Fig 7.2 The concentration of non Tf bound Al in haemodialysis patients with respect to Ga-Tf binding.

In one haemodialysis patient, a reduced Al concentration in the plasma was observed after treatment with the chelating agent, desferrioxamine (DFO). The reduction in plasma Al concentration post treatment significantly increased Ga-Tf binding from 47.7% to 85.5% (see fig 7.3). The second peak from each of the elution profiles was run on a G15 column to try and identify the Ga binding species (fig 7.4). In both samples there was a small peak at fraction ten (3% pre DFO treatment and 19% post) and a major peak at fraction twenty five and twenty eight respectively. This indicated citrate was binding a small proportion of the Ga.

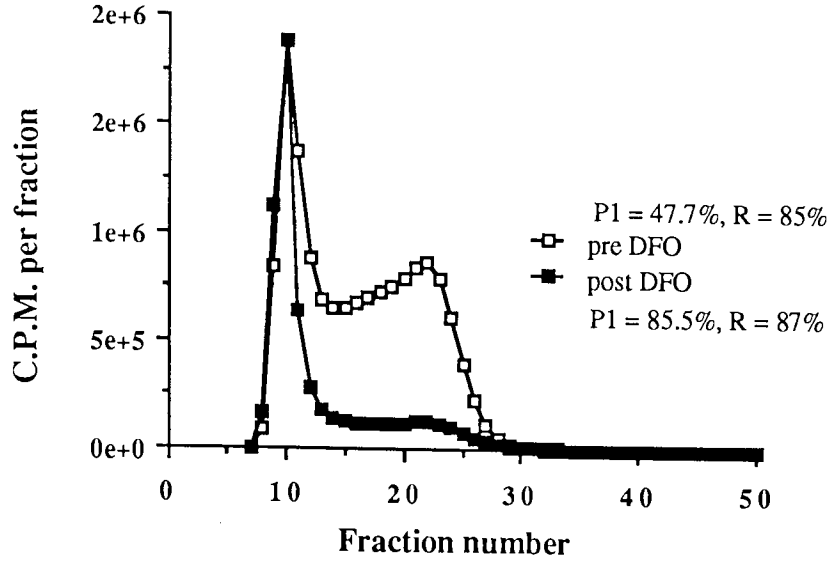


Fig 7.3 Ga-Tf binding in plasma pre and post DFO treatment in a haemodialysis patient.

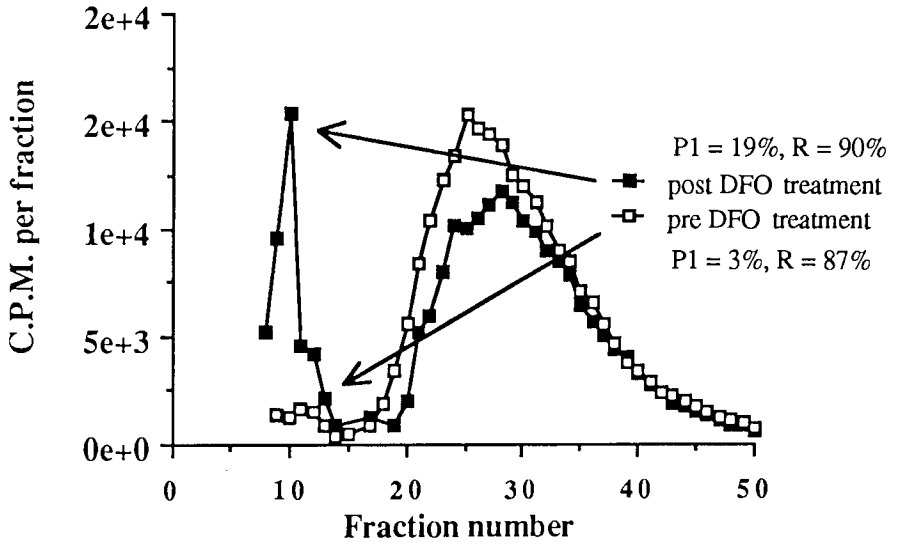


Fig 7.4 Ga-LMWS binding in plasma on a G15 column, pre and post DFO treatment in a haemodialysis patient.

7.3.2 MND PATIENTS

In MND patients, the mean Ga Tf binding was 59.7% ± 36.1 (N=7). Examination of the data showed a bimodal distribution of Ga-Tf binding. The high and low Ga binding MND groups had a mean percentage Ga-Tf binding of 88.6% ± 2.3 (N=4) and 22.1% ± 1.5 (N=3) respectively. This was very similar to that found in the large control group study of chapter 3 (86.9% ± 8.2 (N=86) and 23.9% ± 8.8 (N=21) for high and low Ga binders respectively). Without the MND patients in the control groups of chapter 3 the data changed negligibly (86.8% ± 8.4 (N=82) and 24.4 ± 10.8 (N=18)). Although the distribution of high and low Ga-Tf binding values in MND patients was very similar to controls, the ratio of low: high Ga-Tf binders was greatly increased. To test whether

this increased ratio was statistically significant, contingency table analysis was used with Yates correction for continuity. There was no statistical significance in the results (chi squared = 0.96), but this was probably due to the small sample size of the MND group. There were no apparent age or sex differences in Ga-Tf binding of the MND patients.

MND high Ga binders	88.6% ± 2.3 (N=4)
all control high binders	86.9% ± 8.2 (N=86)
control (excluding MND) high binders	86.8% ± 8.4 (N=82)
MND low binders	21.1% ± 1.5 (N=3)
all control low binders	23.9% ± 10.1 (N=21)
control (excluding MND) low binders	24.4% ± 10.8 (N=18)

Table 7.2 Ga-Tf binding in MND patients and control volunteers.

I.D.	Sex	Age	% Ga bound to Tf
J.Cl	F	44	88.0
C.J.	F	64	91.4
C.H.	F	46	85.9
M.T.	F	49	22.9
B.M.	M	50	89.0
J.C.	M	67	20.2
W.R.	M	61	20.3
		mean % Ga-Tf binding	59.7 ± 36.1 (N=7)

Table 7.3 Ga-Tf binding in MND patients.

7.3.3 AFFECTIVE DISORDER PATIENTS

Plasma from two bipolar depressives was analysed for Ga-Tf binding (see table 7.4). As this was such a small sample size very little could be deduced from the results, so they will not be discussed further although the results are available in table 7.4.

Ga-Tf binding in unipolar depressives prior to ECT was significantly reduced ($p < 0.0001$) compared to normal controls (57.8% ± 20.5 (N=8) c.f 86.9% ± 8.2 (N=86) respectively). After a course of ECT (mean number of treatments: 8 ± 1.4) Ga-Tf binding rose to 66.6% ± 17.1(N=8) . Although this was not a significant increase, there was a general trend for Ga-Tf binding to either remain constant or rise by approximately 30% points. Including all unipolar post ECT samples, Ga-Tf binding increased further to 73.4% ± 15 (N=18), this was not a statistically significant rise

compared to the pre ECT group but was significantly depressed compared to controls ($p < 0.0001$).

There was no statistical difference in the Ga-Tf binding in male and female patients post ECT although the Ga-Tf binding in females was slightly higher and medication of the patients did not appear to influence the binding results. There was no correlation found between the degree of depression of patients pre or post ECT (as calculated using HDS) and Ga-Tf binding or between age and Ga-Tf binding.

	I.D.	% Ga-Tf (pre ECT)	% Ga-Tf (post ECT)		I.D.	% Ga-Tf (pre ECT)	% Ga-Tf (post ECT)
1)	A. K.	-	73.0	3)	T.G.	-	82.6
	J. L.	68.8	93.8		V.H.	-	90.3
	J. W.	64.0	59.7		E.I.	-	83.4
	I. T.	49.5	48.1		D.F.	-	91.9
	E. S.	38.4	75.4		M.B.	79.0	76.5
	S. P.	49.1	46.9		H.K.	87.0	79.1
	B. C.	26.8	53.0	4)	E.W.	-	80.6
2)	R. H.	-	82.5		J.S.	-	58.5
	D. H.	-	62.0		J.D.	-	84.3
				5)	F. A.	88.4	75.4
mean % Ga-Tf pre ECT: 57.8 ± 20.5 (N=8)				6)	A. D.	22.4	84.8
mean % Ga-Tf post ECT: 73.4 ± 15.0 (N=18) (bipolar depressive results excluded)							

1) Unipolar, non psychotic responders to ECT
2) Unipolar, psychotic responders to ECT
3) Unipolar, non psychotic, non responders to ECT
4) Unipolar, psychotic, non responders to ECT
5) Bipolar, psychotic responder to ECT
6) Bipolar, non psychotic, non responder to ECT

Table 7.4 Ga-Tf binding pre and post ECT in unipolar and bipolar depressives.

7.4 DISCUSSION

The results from the haemodialysis group indicate that an increase in the LMWS of Al in the plasma would normally occur only when the capacity of Tf to bind Al was exceeded. The series of experiments which examined Ga-Tf binding with differing plasma Al concentrations (section 3.11) also points towards this. These findings could explain why haemodialysis patients do not show a higher incidence of AD. The saturation point of Tf would be unique for all individuals and therefore an increase in

the neurotoxic LMWS would differ in all individuals exposed to the same concentration of Al in the plasma. Normal control subjects are able to bind Al to Tf at concentrations usually seen in haemodialysis patients, only in such people as the low Ga binding control group would an increase in plasma Al represent a threat of Al associated diseases. Furthermore, recent measurements of silicon in haemodialysis patients has demonstrated elevated levels compared to normal controls (Dr. P. Altmann personal communication, 1992). This provides a further mechanisms of detoxifying Al because insoluble hydroxy-aluminosilicates could be formed in plasma, given a large enough concentration of silicon (100 μM/l) (Birchall 1991b).

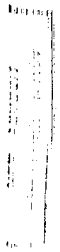
Treatment with DFO reduces both the Al content of the plasma and increases Ga-Tf binding, thus reducing the LMWS of Al which is potentially neurotoxic. In a recent DFO drug trial in AD patients, McLachlan et al (1991) reported a decreased rate of deterioration in the daily living skills associated with AD in patients given intramuscular injections of DFO compared to patients who did not receive this treatment. The same group have also shown the death rate of patients in the DFO group to be considerably less than that of the control group (McLachlan 1991). It is possible the decrease in decline of living skills and reduction of the death rate reported is due to the removal or reduction in the LMWS of Al as seen in fig 7.2.

The apparent bimodal split between high and low Ga-Tf binders in MND is of interest because of the increased incidence of the low binders. Neary (1990) has described a rapidly progressive dementia (dementia of the frontal-lobe type (DFT) which was clinically and pathologically distinct from AD) in association with clinical symptoms of MND. An association between dementia in MND and an increase in LMWS of Al in some patients is possible. However, one should be very cautious about extrapolating too much from these results because of the small sample size involved. The data does indicate MND patients are a group of interest and further studies into the mechanisms and consequences of reduced Ga-Tf binding should be performed.

Reduced Ga-Tf binding in plasma from unipolar depressives pre ECT indicates this group of people are more susceptible to metal ion uptake in the brain. The presence of Al in the brain could affect enzymes which would result in functional defects of neurotransmitter systems (such as the noradrenergic system) as seen in depression. Urinary methoxyhydroxyphenylglycol (MHPG) is the main central metabolite of noradrenaline and its excretion is low in depression (Neal 1987). This indicates noradrenaline metabolism is also decreased. Al has been shown to inhibit the biosynthesis of tetrahydrobiopterin (BH_4) in brain homogenates from controls (Cowburn and Blair 1989). BH_4 is the natural co-factor for the conversion of phenylalanine to tyrosine via phenylalanine hydroxylase in the synthetic pathway of the neurotransmitters noradrenaline and dopamine, so any factors affecting BH_4 synthesis

would result in a decrease in neurotransmitter production. It is possible the increase in the Al-LMWS associated with decreased Tf binding in unipolar depression is responsible or at least contributes towards the decrease in noradrenaline metabolism in depression.

These studies do not show whether reduced Ga-Tf binding in unipolar depressives is a cause or consequence of the disease. However, it does offer a possible explanation of why people suffering from depression develop AD or PD more frequently than the general population.



CHAPTER EIGHT

ALZHEIMER'S DISEASE

8.1 INTRODUCTION

Dementia is a clinical term which refers to a progressive loss of cognitive functions commonly involving memory, orientation, and the ability to learn new tasks. A disturbance of higher brain functions which involves language, perceptual impairment, and a change in personality are also common features of dementia. They may be expressed either alone or in combination with others.

In 1907, Alois Alzheimer first described neurofibrillary tangles and senile plaques during a post mortem examination of a brain from a fifty one year old woman whose memory deteriorated to such an extent that she was admitted to hospital. She was found to be disorientated, paranoid, and dysphasic. The illness was rapidly progressive and within four years the patient died. The coupling of NFT and SP in brain tissue to clinical dementia by Alzheimer, led to the establishment of the condition known as AD.

A review of AD is given in chapter 1 and it is therefore not appropriate to consider it further here.

8.1.1 AIMS OF THE CHAPTER

The etiology of AD remains unresolved and a role for Al neurotoxicity as the primary cause of AD remains controversial. However, there is mounting evidence that implicates Al in the pathogenesis of the disease. It is therefore the aim of this chapter to investigate Al speciation (using ^{67}Ga as a marker) in AD plasma to identify a possible mechanism of accumulation.

8.2 METHODS

8.2.1 PATIENT DETAILS

Twenty four AD patients (M/F 12/12) were selected from three different geographical locations throughout the U.K. The mean age of the patients was 72.8 years $\pm$ 9.8 with an age range of 56 - 90 . The following clinicians supplied blood or plasma from AD patients: Dr. D. Anderson, Consultant Psychogeriatrician, Fazakerley Hospital, Liverpool; Dr. O. Wychrij, Consultant Psychiatrist for the elderly, Barnsley Hall Hospital, Bromsgrove and Dr H. Pall, Consultant Neurologist, Queen Elizabeth Hospital, Edgbaston, Birmingham. If blood was received, plasma was prepared and stored as described in section 2.3.1. AD was diagnosed by standard neuropsychiatric techniques (including: computerised tomography (CT) scans, exclusion of other dementias and examination of the clinical history).

8.2.2 Ga SPECIATION

see section 2.3.2

8.3 RESULTS

The control group for the AD patients in which Ga-Tf binding was examined in the absence of 25 mM bicarbonate was made up of volunteers from Aston University and Wolverhampton Polytechnic. The control group used for all other studies was defined in chapter 5. Individual age/sex matching for AD patients was not possible. Therefore, any control subject over the age of 60 was used to form an age/sex matched control group.

After gel-filtration chromatography, the distribution of Ga in AD plasma was observed to be bimodal. The position of the two peaks (a Ga-Tf (HMWS) and Ga-LMWS) was constant in all experiments and similar to that found in other groups studied. Recovery of radioactivity was always between 85 - 95%.

Using the standard buffer, Ga-Tf binding was significantly depressed ($p < 0.002$ and $p < 0.001$) in the AD group compared to the young and old controls respectively (see table 8.1). These observations were in agreement with those of Farrar et al (1990) (Ga-Tf binding in AD patients; 7.9% $\pm$ 2.5 (N=13)) and Brammer et al (1990) whose AD patients were selected according to NINCDS-ADRDA criteria (Ga-Tf binding in AD patients; 10.6% $\pm$ 2.3 (N=10)). Ga-Tf binding differences in AD patients with respect to age and/or sex were not evident using the standard buffer.

	AD patients	young controls	old controls
M/F	7/5	13/5	3/3
age (years)	74.6 $\pm$ 8.9	24.8 $\pm$ 36	66.7 $\pm$ 4.3
age range	64 - 90	20 - 32	62 - 73
% Ga-Tf	6.25 $\pm$ 5.3	13.8 $\pm$ 6.1	15.2 $\pm$ 2.4

old controls were courtesy of Dr. G. Farrar

Table 8.1 Ga-Tf binding in a standard (bicarbonate free) buffer using AD patients and young and old controls.

In the presence of 25 mM bicarbonate, Ga-Tf binding in AD patients was still significantly depressed compared to age/sex matched controls and the normal control group ($p < 0.02$ and $p < 0.01$ respectively) (see table 8.2 and fig 8.1). The mean Ga-Tf in AD plasma was 68.9% $\pm$ 27.6 (N=20) compared to 85.9% $\pm$ 8.3 (N=20) in the age/sex matched control group and 86.9% $\pm$ 8.2 (N=86) in the normal control group. Again, no Ga-Tf binding differences existed between male and females and there was no correlation with age.

	AD patients	age/sex matched controls	high Ga binding controls
M/F	11/9	10/10	42/44
age (years)	71.8 ± 9.1	67.7 ± 7.3	42.2 ± 18.6
age range	56 - 86	58 - 82	5 - 82
% Ga-Tf	68.9 ± 27.6	85.9 ± 8.3	86.9 ± 8.2

Table 8.2 Ga-Tf binding in the presence of 25 mM bicarbonate using AD patients, age/sex matched controls, and normal controls.

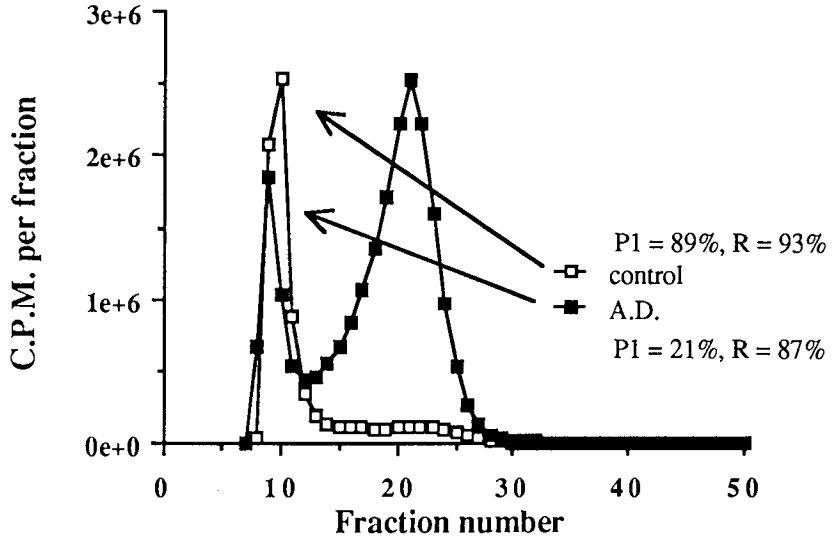


Fig 8.1 Comparison of Ga-Tf binding in AD and control plasma in the presence of a 25 mM bicarbonate buffer at pH 7.4

The Ga-Tf binding in AD plasma showed a large coefficience of variance (see fig 8.2) which resulted from a large range of Ga-Tf binding results (range 21 - 100%). This was in contrast to other disease groups studied with the exception of DS which was due to age and MND.

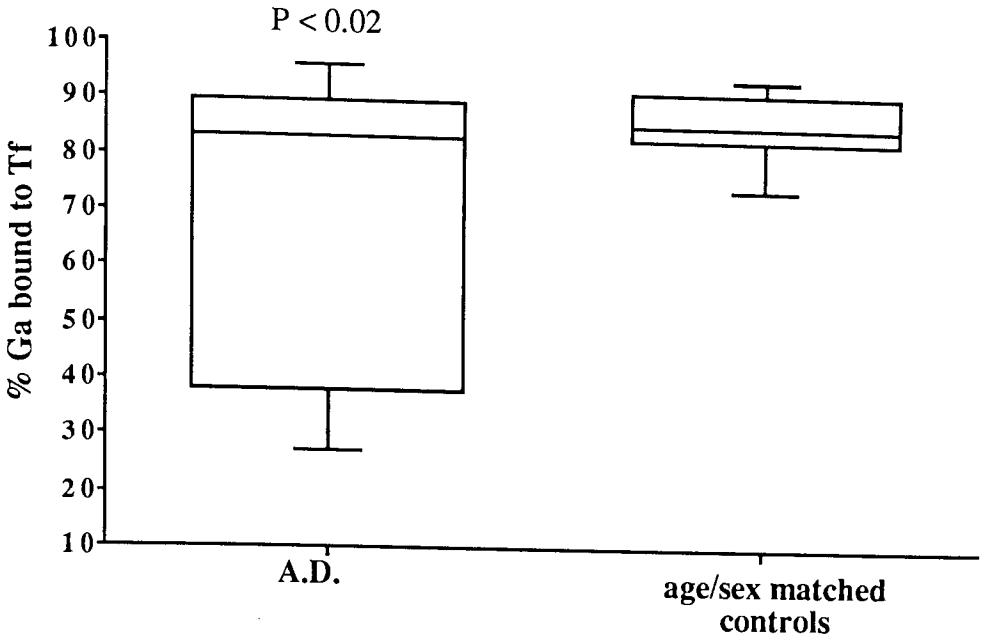


Fig 8.2 Percentile plots (10, 25, 50, 75, and 90th percentiles) of Ga-Tf binding in plasma from A.D. patients (N=20) and age/sex matched controls (N=20).

Fig 8.3 shows the distribution of Ga-Tf results with respect to age in AD.patients and normal controls. It is evident that there are some AD patients with normal Ga-Tf binding and some of the control group which (although by definition are normal Ga binders) show reduced Ga-Tf binding.

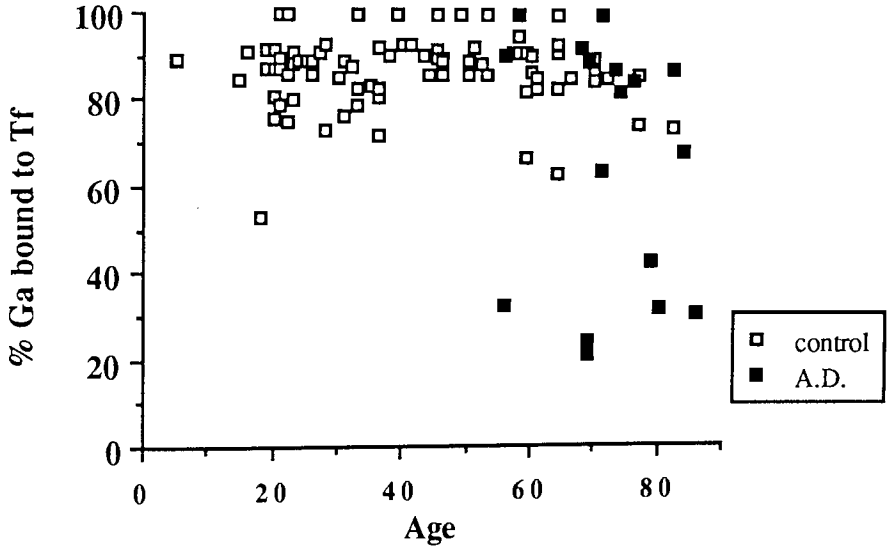


Fig 8.3 The relationship between Ga-Tf binding and age in AD and controls.

8.4 DISCUSSION

The results show that in AD, significantly less Ga (and by implication Al) was bound to Tf compared to both age/sex matched controls and the control group as a whole. Consequently, in AD, an increase in Al bound to LMWS would serve to increase the entry of Al into the brain. Once in the brain, Al would be neurotoxic and accumulate in

the core of SP and in NFT within neurons. As a detailed discussion of Al neurotoxicity is given in chapter ten, it will suffice to have given a very brief account in this section.

Ga-Tf binding in AD was shown to have a large coefficients of variance within the group. Many of the AD patients had normal binding, the reasons for this may be two fold. Diagnosis of AD is only confirmed at post mortem examination. Ante mortem diagnosis by even the most experienced clinicians rarely exceeds 80% accuracy. Homer et al (1988) who followed the clinical diagnosis of AD by neuropathological examination found an accuracy rate of only 46%. By strictly adopting the criteria of NINCDS-ADRDA, Burns et al (1990) achieved an accuracy rate of 88%. Therefore, it is probable that between 4 and 9 of the AD group were in fact misdiagnosed and could account for at least some of the AD patients being in the control range for Ga-Tf binding. The second reason why AD patients are seen in the control range could be due to the nature of the disease. Entry into AD is probably by more than one mechanism and subtle subtypes of AD may well exist. Certainly recent genetic studies would support this idea (St George-Hyslop et al 1990).

CHAPTER NINE

AL-27 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

9.1 INTRODUCTION

NMR is a particularly good method of examining liquids because it is a direct, non invasive technique which does not disrupt or separate the solution under analysis. It is a proven method in the study of small molecules isolates from biological systems (Moore et al 1983) and can be used to examine intact body fluids such as plasma, urine, sweat, seminal and eye fluid (Bell et al 1988). Furthermore, NMR is a powerful tool in the investigation of metal ion speciation (Fatemi et al 1991b).

Spectroscopy is the study of the interaction of electromagnetic radiation with matter and depends upon the absorption and emission of energy associated with the transitions between different energy levels. Measurements made with this technique are as a function of the frequency and the amount of radiation applied to the sample (Moore et al 1983, Fatemi 1990). The spectrum obtained is made up of a series of discrete lines each with an energy, an intensity, and a line width. In general, a spectrum is individual for a given sample and data such as atomic, electronic, or molecular information is available for interpretation from the spectrum. The nucleus of an atom has magnetic properties and this fact is exploited in NMR studies. By placing samples in a strong magnetic field, the degeneracy of various energy levels is removed and the transition between the energy levels is induced by a suitable radio-frequency field. The size and frequency of emissions or absorptions depends upon the splitting of the energy levels and this is in turn is dependent on the nucleus under examination, the applied field strength, and its chemical environment.

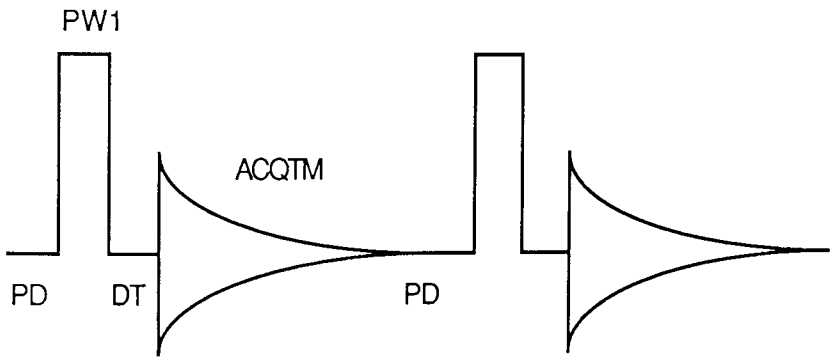


Fig 9.1 Simplified diagram of the build up of a spectrum.

PW1: Pulse Width. Time taken in which a sample is subjected to radiation (radio waves) thereby altering the energy levels within the sample.

DT: Delay Time. Time before measurement of the absorption or emission of a sample takes place.

ACQTM: Acquisition Time. Time of measurement of absorption or emission from sample.

PD: Pulse Delay. Time taken before another pulse of radiation is given to the sample.

Time of one scan is calculated by : $\frac{(PD + PW1 + DT + ACQTM)}{3600}$ = Hours

in practise PW1 and DT are ignored as they are only a few micro seconds.

A spectrum for a sample is built up from the accumulation of individual scans. As the number of scans increases, background noise is reduced (noise is random and therefore the positive and negative elements cancel each other out) and a signal for a particular species increases in intensity.

^{27}Al NMR spectroscopy has been used to characterise the aqueous chemistry and the small ligand complexes of Al^{3+} (Akitt 1989). Its major spectroscopic draw back is that the ^{27}Al nucleus, though 1.17 x 10 ^{3} times more receptive than naturally abundant ^{13}C, has a nuclear spin quantum number of 5/2 and hence a quadrupole moment. The consequence of this is that generally its lines are broad (Akitt 1989). Spectra obtained from NMR will not identify an Al-Tf complex because the bound Al^{3+} would exist in a low site of symmetry. This enhances the quadrupole induced relaxation of the Al-Tf complex and hence gives a broader signal (Fatemi et al 1991b). A signal for Al-Tf would also be masked by the general background noise in the spectrum due to the complex make up of plasma. However, Al-citrate complexes have distinctive NMR signals that are easily detected (Jackson 1982, Karlik et al 1983).

9.1.1 AIMS OF THE CHAPTER

The use of gel-filtration chromatography has identified Tf as the major Ga and Al binding constituent in plasma of normal adults (Cochran et al 1983, Trapp 1983, Rahman et al 1985). However, the identity of the LMWS in plasma has still not been fully resolved (see section 3.10), although citrate remains the most likely candidate both from analysis of theoretical stability constants (Martin 1986, Martin 1987) and from experimental work (Fatemi 1990, van Ginkel et al 1990, Fatemi et al 1991a). In an attempt to confirm Al-citrate is the LMWS present in plasma, NMR was employed. Studies of normal and AD plasma have shown an increased proportion of Ga (and by implication Al) bound to a LMWS in AD (Farrar et al 1990 and Hodgkins et al 1991). Therefore, it was decided to further characterise Al speciation in both groups with the aim of demonstrating additional differences between AD and normal control plasma.

9.2 METHOD

9.2.1 PATIENT DETAILS

Plasma samples from seven control subjects were obtained from volunteers at Aston University, Birmingham; Dr. O. Wychrij, Consultant Psychiatrist to the elderly,

Barnsley Hall, Hospital, Bromsgrove supplied six samples from AD patients and Dr. P. Altmann, Senior Registrar in Nephrology, Royal London Hospital, Whitechapel, London, supplied a plasma sample from a haemodialysis patient with a plasma Al concentration of 600 μg/l. For the diagnostic criteria of AD patients see section 8.2.1

9.2.2 PROCEDURE

see section 2.5

9.3 RESULTS

I am grateful to Dr. G. Farrar for allowing me to use some of her NMR data (pH curve, figs 9.7a - 9.7c inclusive) in this chapter.

A solution of 10^{-4} M $Al(NO_3)_3$ in distilled water at pH 7.3 and 0.9 gave rise to spectra after 10,000 scans (fig 9.2), as did equimolar solutions of 10^{-2} M and 10^{-4} M $AlCl_3$ and citric acid after 512 scans at pH 7.4 and 4096 scans at pH 2.2 respectively (fig 9.3). These spectra have been previously described by Jackson (1982), Karlik et al (1983), Bertsch et al (1986) and Akitt (1989).

Plasma samples were measured by ^{27} Al NMR and spectra obtained (spectra not shown). As expected, the endogenous concentration of Al was too small to be detected, so samples were doped with Al to a final concentration of 10^{-4} M, pH 7.4. At this concentration all available Tf binding sites would be occupied with approximately 4 - 6 x 10^{-5} M Al left to bind to other ligands (Williamson et al 1992). The samples were incubated for one hour at 37°C and analysed over an eighteen hour time period (450,000 scans) by NMR spectroscopy. Fig 9.4a represents a typical spectra from the subjects studied. In all cases with freshly prepared plasma at pH 7.4, no Al signal was seen.

The plasma used in these experiments was prepared from blood which was collected using lithium heparin sulphate as an anti-coagulant. To ensure heparin sulphate did not alter the binding equilibrium between Al and citrate, a simple control experiment was performed. A 5 x 10^{-5} M Al-citrate solution was prepared in a blood collection tube and its subsequent NMR spectra was compared to a spectra of Al-citrate which was prepared in a conventional way. There was no difference in the two spectra, showing heparin sulphate does not interfere with the formation of Al-citrate.

With the inability to obtain an Al signal at a concentration of 10^{-4} M, a plasma sample which had been previously analysed was doped with more Al to a final concentration of 5 x 10^{-4} M. Again no signal was observable after eighteen hours (fig 9.4b). Finally a plasma sample was obtained from a haemodialysis patient who had an elevated Al level in the blood (approximately 3 x 10^{-5} M), the spectra (fig 9.4c) was very similar to that

of all the other controls. This sample was important because the plasma had a naturally high level of Al, as opposed to the other samples which were artificially high.

Al spectra were not being produced in the experimental conditions described, so it was decided to force the Al into a low molecular weight pool (LMWP) by blocking the majority of the Tf binding sites with Fe (as described in section 3.11). Ferric chloride was added to a plasma sample to give a final concentration of 5×10^{-5} M. At this concentration only a minority of Al (approximately 2%) should be bound in the high molecular weight pool (HMWP) (see section 3.11). The Fe was left to incubate at 37°C, pH 7.4 for two hours and then Al was added to a final concentration of 10^{-4} M. The sample was incubated for a further hour at pH 7.4 and then an NMR spectra obtained. After an eighteen hour accumulation time, no observable Al signal was seen.

As no Al signal was seen from plasma samples, it was necessary to confirm a detectable concentration of Al was in fact added to the plasma. Precipitation of the proteins from whole plasma by perchloric acid identified an Al signal at $\partial = 0$ ppm (the $[Al(H_2O)_6]^{3+}$ ion) which proved that Al was in the plasma at a relatively high concentration and it was easily detectable (fig 9.5a). The spectra was obtained at a low pH, upon bringing the pH back to a physiological level the $Al(OH_4)^-$ signal was lost and no new signals observed (fig 9.5b).

In one control subject, a spectra produced a clearly defined peak of $Al(OH)_4^-$ at $\partial = 80$ ppm, no citrate peak was seen (spectra not shown). It was found that the same signal could be reproduced in plasma which had been incubated for 48 - 96 hours at 37°C (fig 9.6). During the incubation, the pH of the sample rose from 7.4 to 7.9. It was thought this pH change caused the formation of the hydroxide peak, because when the pH was returned to 7.4 the peak disappeared. The $Al(OH)_4^-$ signal was also reproducible by directly altering the pH of a plasma sample. Fig 9.7 illustrates a pH curve of a plasma sample from a control subject. It can be seen that at an acid pH no observable signal was apparent, however at a slightly alkaline pH (pH 7.98 in fig 9.7b) the peak at $\partial =$ 80 ppm was present and this was even more evident at pH 8.19 (fig 9.7c).

With the absence of an Al signal in plasma, it was decided to separate the high and low molecular weight components of the plasma and examine the Al speciation of each solution. An arbitrary cut off point between each fraction was 30 KD. Plasma separation was carried out using a 30 KD Centricon ultrafiltration device (centrifugation was at 4,000 r.p.m. at 5°C for five hours). 55% of the plasma went into the HMWP, 45% into the LMWP. The LMWP was checked for protein content by SDS gel-electrophoresis and found to be substantially protein free and no proteins were identified above the cut off point of 30 KD. Subsequently, the LMWP was freeze dried (Edwards Modulyo freeze drier) for three hours to concentrate the potential binding

species. Freeze drying (samples were frozen in liquid nitrogen and the water vapourized) was used to remove water from proteins containing solutions which were heat sensitive.

Subsequent ^{27}Al NMR spectra of both the high and low molecular weight fractions failed to produce any observable Al signal. Upon addition of Al to give a final concentration of 1×10^{-4} M in the LMWP, pH 7.4, the spectra still showed no Al signal. Raising the concentration to $\approx 2 \times 10^{-4}$ M, only the $Al(OH)_4^-$ signal at $\partial = 80$ ppm was seen (fig 9.8). When Al was added to the HMWP to give a concentration of $\approx 1.6 \times 10^{-3}$ M, a small broad peak appeared at $\partial = -7$ ppm. This peak increased in intensity with larger concentrations of Al up to $\approx 5.5 \times 10^{-3}$ M. An $Al(OH)_4^-$ signal at $\partial = 80$ ppm also began to develop with increasing concentrations of Al (fig 9.9). The NMR signal for Al bound to albumin has been observed by Fatemi et al (1991a, 1991b) with a chemical shift of -7 ppm at pH 7.4 and 37°C. Repetition of Fatemi's work produced similar results (fig 9.10) and both the peaks obtained by Fatemi and by the work presented here at -7 ppm closely resembled that of the peak in the HMWP sample.

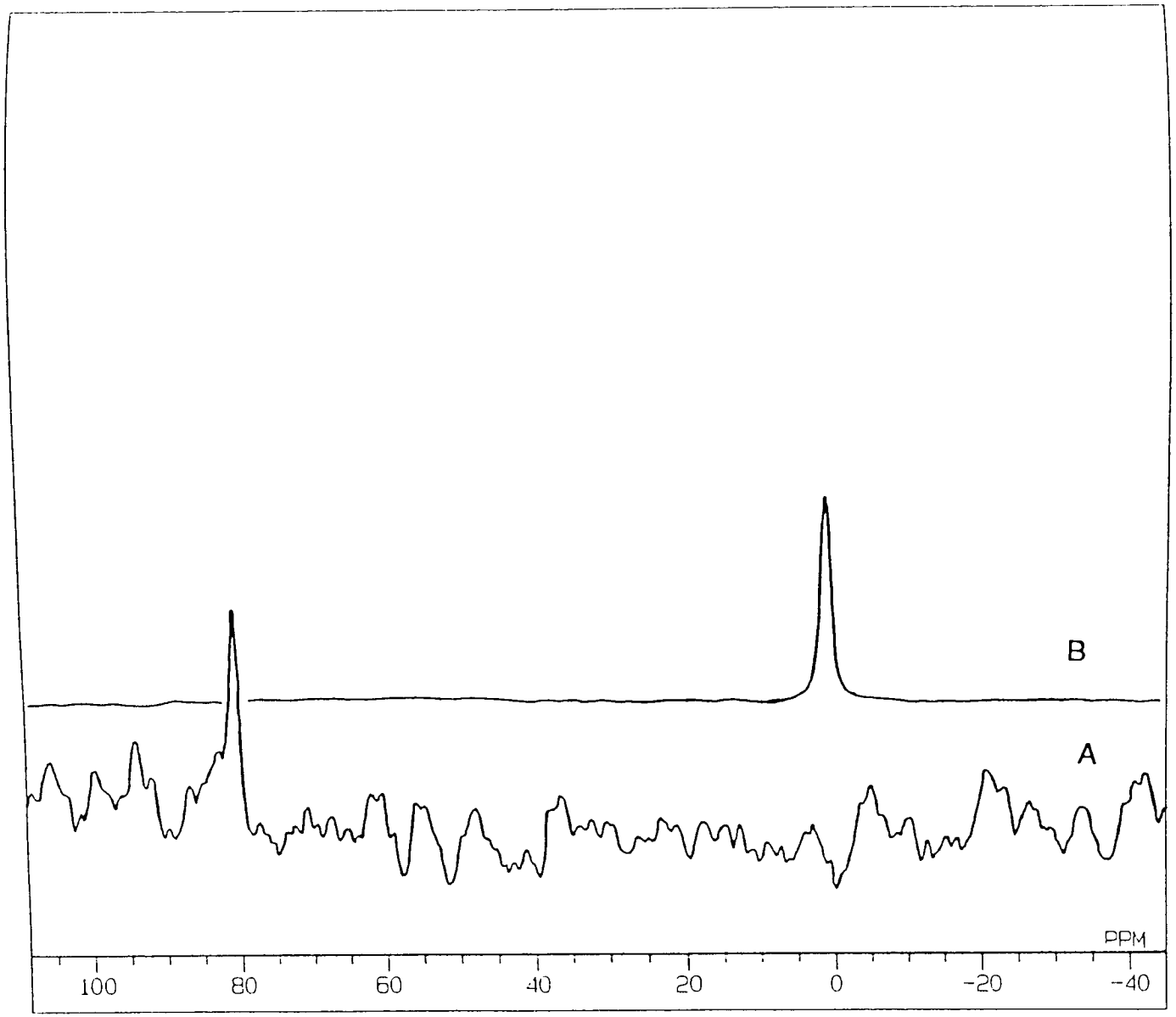


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Fig 9.2a Al nitrate (10^{-4} M); pH 0.9
Fig 9.2b Al nitrate (10^{-4} M), pH 7.3

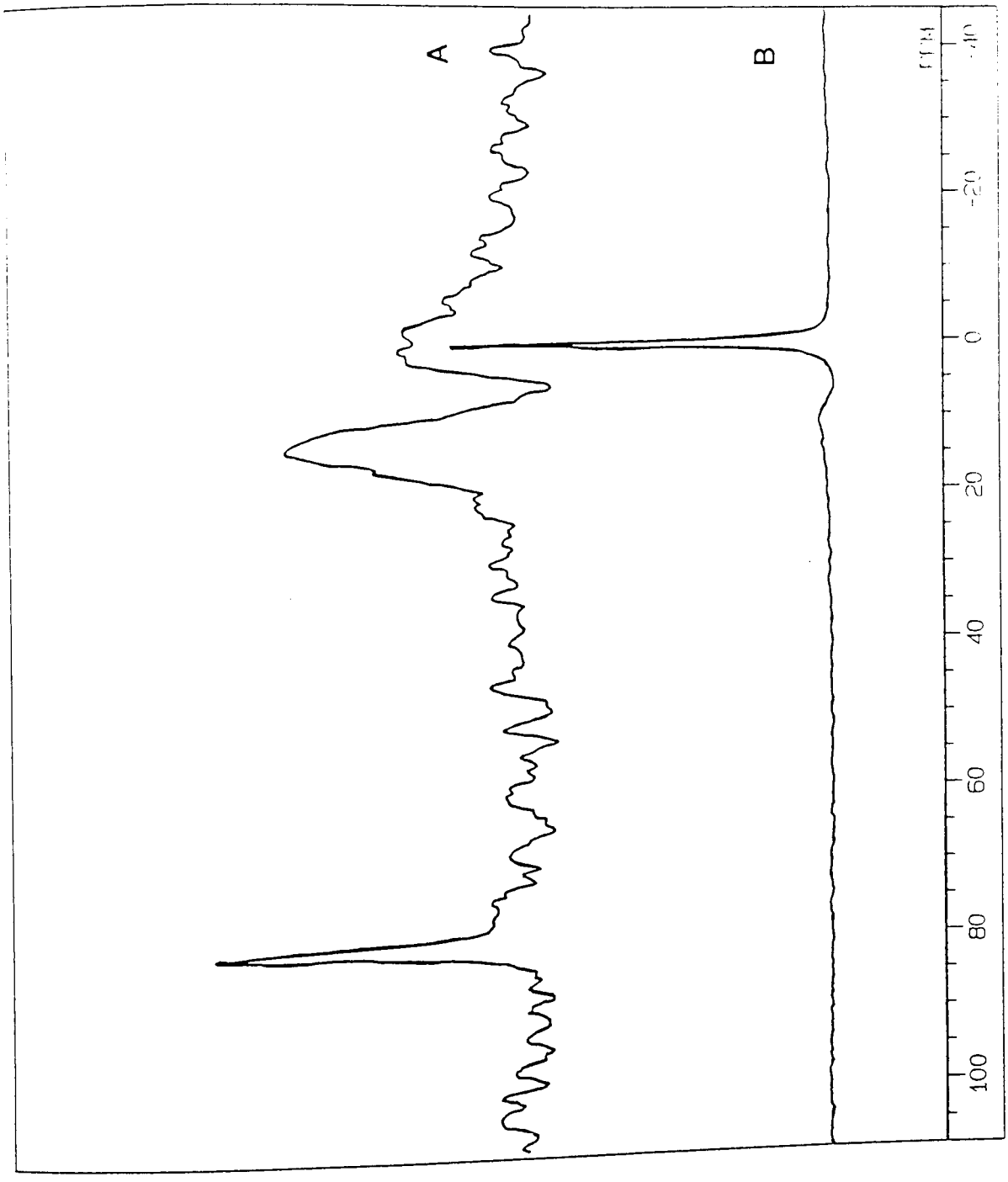

Fig 9.3a Equimolar Al chloride and citric acid solutions (10^{-2} M), pH 7.4
Fig 9.3b Equimolar Al chloride and citric acid solutions (10^{-4} M), pH 2.2

Fig 9.4a Control plasma sample doped with Al to a final concentration of 10^{-4} M

Fig 9.4b Sample 9.4a doped with Al to a final concentration of 5×10^{-4} M

Fig 9.4c Haemodialysis patient with an endogenous Al concentration of 3×10^{-5} M

Fig 9.5a Plasma sample after protein precipitation (pH 0.5)

Fig 9.5b Sample 9.5a at pH 7.4

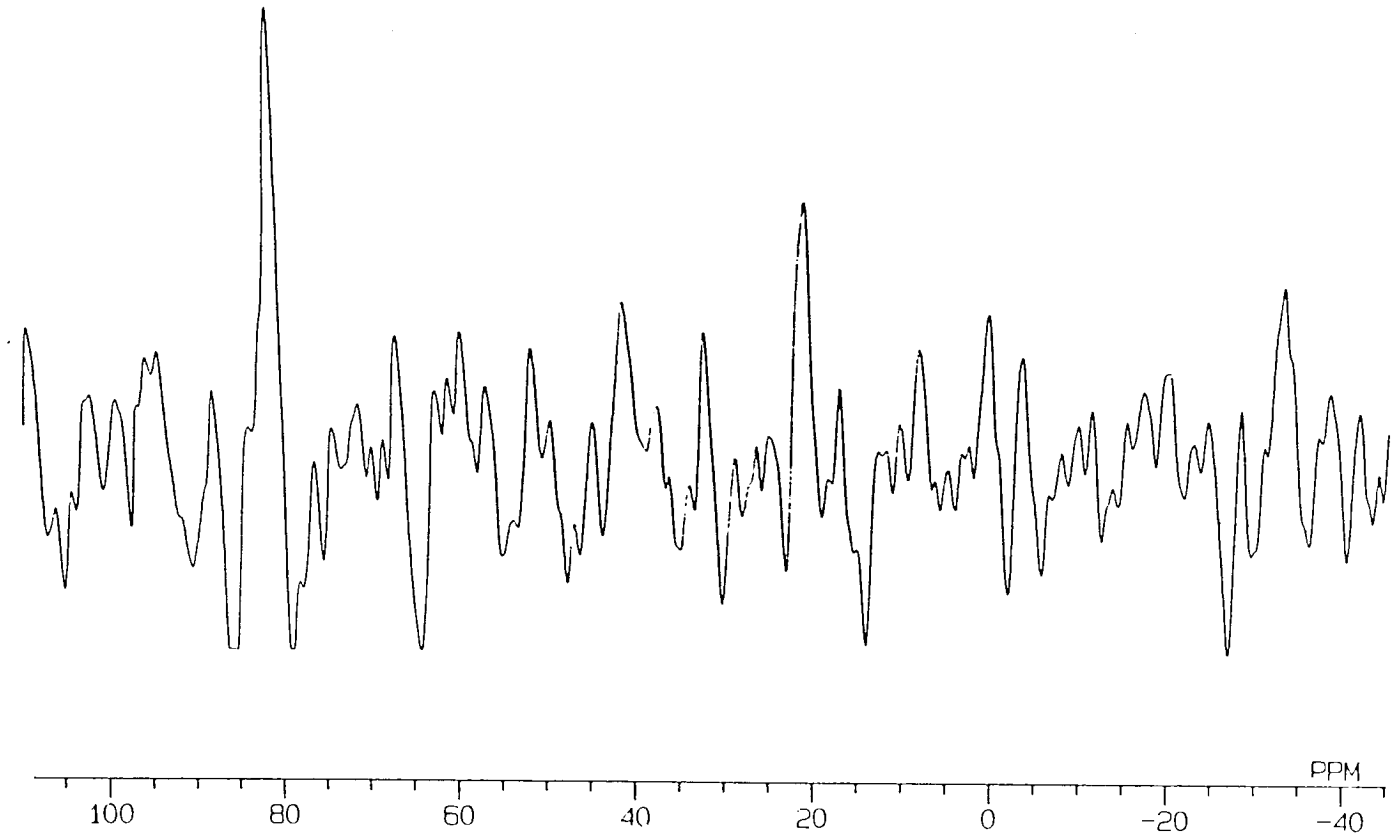

Fig 9.6 Aged plasma sample

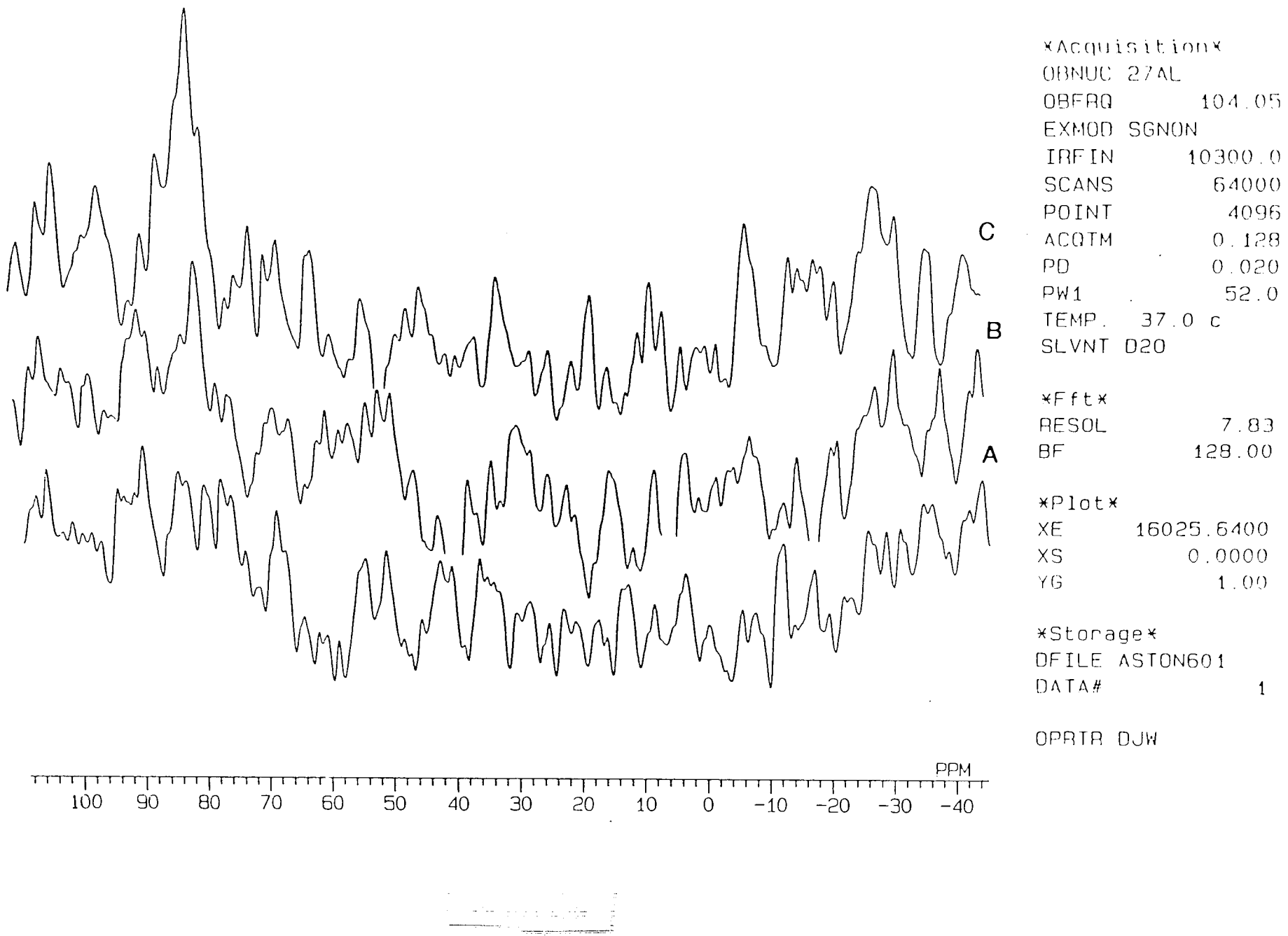

Fig 9.7a, b, c Control plasma at pH 7.3, 7.98, and 8.19 respectively

Fig 9.8 Low molecular weight plasma fraction with 2 x 10^{-4} M Al added

Fig 9.9a, b, c High molecular weight plasma fraction with 0, 3.2 x 10^{-3} M, and 5.3 x 10^{-3} M Al

Fig 9.10a, b, c Albumin solution (1.6×10^{-3} M) with 1.6×10^{-3} M, 3.2×10^{-3} M, and 5.9×10^{-3} M Al

9.4 DISCUSSION

From initial studies of Al containing solutions (fig 9.2 and 9.3) it was obvious NMR spectroscopy could identify Al complexes at a given pH and concentration. The variation in chemical shifts and the difference in resonance line width within the spectra were readily apparent by peaks of different height and width, arising at specific points along the spectra. For example, at ∂ = 80 ppm the $Al(OH)_4^-$ peak was seen in fig 9.2b and 9.3a, while at ∂ = 0 ppm a peak of $[Al(H_20)_6]^{3+}$ was observed in fig 9.2a and 9.3a. It should be noted that it is the integrated peak area that is proportional to concentration and not peak height. The difference in chemical shift is a salient feature of NMR and is exploited in speciation studies.

From fig 9.2 and 9.3 it was important to note that the resonances of the Al-citrate complexes had quite different chemical shifts from that of the $Al(NO_3)_3$ solution, which had a relatively sharp signal. With an equimolar (10^{-2} M) Al-citrate solution the spectra gave rise to four signals. This indicates there are three different complexes of Al-citrate given the experimental conditions. The Al-citrate region of the spectra (∂ = 0 - 20 ppm) does not vary greatly with increased pH, except that the broad background signal increases in intensity at the expense of a sharper signal for Al-citrate. The small amount of $Al(OH)_4^-$ that was present with a chemical shift of ∂ = 80 ppm at pH 7.4 indicates the OH^- anion can compete effectively for Al with citrate (Williamson et al 1992).

With no observable signal obtained from plasma doped with 10^{-4} M $Al(NO_3)_3$, one can assume that in both groups that were studied, Al was bound to high molecular weight species including Tf, albumin and other proteins which give broad signals (Bertholf et al 1984, Fatemi et al 1991a) or to polymeric solvated species with a lower symmetry than those seen in fig 9.2. The polymeric solvated species would also give broad signals but were not expected to be observed in plasma at the concentration of Al used in the experiments (Baes and Mesmer 1976). No Al cations were free (unbound) in the plasma even at a concentration which is known that the major metal binding protein would be fully bound with Al. An Al signal which is not detected in plasma suggests that Al which was not bound to Tf (or other high molecular weight species) was not bound to a low molecular weight ligand in a discrete and relatively symmetrical complex at a concentration of 2 x 10^{-5} M. These spectra also clearly indicate that Al citrate or any other solvated Al species (from fig 9.2 and 9.3) were not found in detectable concentrations in freshly prepared plasma, given the experimental conditions.

After an addition of perchloric acid, the resulting protein free plasma solution gave a sharp signal for $[Al(H_20_6)]^{3+}$ at a low pH. Upon returning to a physiological pH, the signal was lost and no new ones observed. This was not pursued further because a

perchlorate rich, protein free medium was far removed from that of plasma. These observations do show that any Al bound to macromolecules was released with a reduction in pH. Lestas (1976) showed that at pH 6.7 diferric Tf readily loses Fe (and by implication Al) and Hodgkins et al (1991) have provided chromatographic evidence to show reduced binding to high molecular weight species with a deviation from a physiological pH. With a high ionic strength and a pH of 7.4, Al in a protein free plasma was not bound to citrate in a detectable concentration.

An Al signal ($[Al(OH)_4]^-$ with a chemical shift of ∂ = 80 ppm) could be detected in a plasma sample which prior to incubation had a pH of 7.4 and a pH of 7.9 after an hour incubation. The Al signal could be reproduced if the pH of the sample was altered directly or by incubating the plasma over extended periods of time. The reproduction of the $Al(OH)_4^-$ peak at ∂ = 80 ppm in the plasma sample was probably due to a pH rise during the incubation of the sample. The pH increase may be associated with the loss of bicarbonate as CO_2. The increase in pH is known to reduce Tf binding as would a decrease in the bicarbonate concentration of the plasma (Hodgkins et al 1991) and an increase in pH to $\approx$ 8 would generate hydroxide anions to compete with existing Al ligands.

The binding of Al to albumin is known to occur (Glick 1990, Glick 1991, Fatemi et al 1991a) and the stoichiometry has also been elucidated (Fatemi 1991b). Approximately three Al ions are bound to each albumin molecule. Normal plasma albumin concentration is 6×10^{-4} M (Peters 1985), so there are enough vacant binding sites on the albumin molecules to be able to bind any Al which is not bound by Tf. Even though a signal for Al bound to albumin was observed in the HMWP, this does not seem plausible in vivo because of the large number of observations which refer to a low molecular weight fraction of Al, unless analytical methods to measure Al in plasma disrupted the equilibrium of Al bound to albumin. Alternatively, there could be two other explanations.

Physiological levels of bicarbonate (approximately 2.5×10^{-4} M) could assist in the partial solubilisation of Al which was not bound to Tf. An Al carbonate complex may well have been present in the plasma given the fact there was a 250 fold excess of bicarbonate over Al at the start of the experiments. Al carbonate complexes in solution have been identified (Chappell and Birchall 1988) although the stability constants which would be relevant to plasma are not known. In minerals, a mixed hydroxy-carbonate complex exists and is thought to be present in aqueous solutions at a high ionic strength. It has been proposed the complex would be the $[Al_2(H_2O)_6(OH)_2(CO_3)]^{2+}$ ion (Ohman and Forsling 1981). This would have a relatively large electrical field gradient for Al and would therefore have signals which were broadened by quadrupolar relaxation. The second possibility is the Al forms asymmetrical polymeric hydroxide

complexes, which would produce broad Al signals.

Although Al-citrate species are detectable by ^{27}Al NMR and are fairly stable, no observable signal was seen in freshly prepared plasma at pH 7.4 given the conditions of the experiment. Furthermore, there were no observable differences between AD and normal control plasma using NMR.

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CHAPTER TEN

GENERAL DISCUSSION AND FUTURE WORK

10.1 INTRODUCTION

It has only been during the last two decades that the potent neurotoxic effects of Al have become recognised. Prior to this, it was generally accepted as an innocuous element (Sorenson et al 1974). Scientific interest and public concern over Al has grown rapidly over the years and this has been reflected in the number of research papers produced on the subject. In 1970, less than 200 papers were published on Al. This figure rose to over 1 300 by 1988 and has no doubt increased since.

The cause of sporadic AD remains unknown, although the pathogenic mechanisms behind familial AD are beginning to be understood. Environmental factors (as exemplified in the population of Guam) are most probably the key to some cases of sporadic AD, Al possibly being the major risk factor involved. Similarly, the majority of cases of PD are idiopathic. The development of this disease may be derived from a genetic susceptibility coupled to exposure to an environmental toxin (Marsden 1990).

10.1.1 AIMS OF THE CHAPTER

This chapter will bring together the results from previous sections with the aim of identifying a possible mechanism for the pathological changes observed in both AD and PD. An initial description of familial AD will be reviewed (this can be used to develop arguments for sporadic AD) followed by a discussion of sporadic AD in the general population and AD in the DS population. An argument for a role for oxidation (and enhancement by Al) in the development of PD will be considered and finally, the neurodegenerative implications of decreased Ga-Tf binding will be discussed.

10.2 REPLICATION OF WORK BY FARRAR *et al* (1990)

The original observations by Farrar et al (1990) described an increased low molecular weight species of Ga in plasma samples from AD and DS patients compared to suitable control groups. They suggested this species could account for the increased Al concentration observed in AD brains. The paper received a mixed response from the scientific community. Criticisms and possible methodological flaws were soon to follow (Spear 1990, McGregor et al 1991, Taylor et al 1991). Brammer et al (1990) confirmed the findings of Farrar and in repeating the work, answered some of the criticisms directed at the original article. The results obtained in this thesis also fully corroborate the observations of Farrar and furthermore, provide additional, supporting evidence to strengthen their hypothesis for Al accumulation in AD brains.

Despite the disconcerting comments made about the methodology used, the elution system which best discriminates between AD and controls is the one used by Farrar. Using a bicarbonate buffer, the relative differences between the two groups is not so clear: many AD patients having binding figures which were comparable with controls. The same samples in the original buffer would indicate a binding figure appropriate to

the AD group.

10.3 Ga SPECIATION IN HUMAN PLASMA

G75 gel-filtration chromatography has established the species of ^{67}Ga present in human plasma after an in vitro incubation. The results can be interpreted in relation to Al because it is generally accepted that ^{67}Ga is a suitable marker for Al. Furthermore, it does not involve any of the technical difficulties of working with Al compounds.

In normal healthy people and those from diseased groups (AD, PD, DS, haemodialysis, and affective disorder patients), Ga was bound to a high molecular weight species and to a second, low molecular weight species. The differentiation between normal and diseased subjects (also between normal and 'at risk' controls and between individual diseased groups to a limited degree) was the altered ratio of Ga bound to the two species identified on a G75 column.

The first peak to elute off the G75 column was taken to be Tf (Farrar et al 1990), observations made during this investigation support this. Further characterisation of the high molecular weight peak using a G150 column revealed another Ga binding species. Apart from noting the molecular weight of the species to be over 80 KD, further identification was not pursued.

The identity of the low molecular weight species (second peak) was attempted using a G15 gel-filtration chromatography column and radio-labelled citrate (section 3.10) and by NMR spectroscopy (chapter 9). Citrate was considered to be the most likely ligand to bind Ga. Accordingly, work was directed to the positive identification of this species. Gel-filtration chromatography did reveal Ga-citrate binding in plasma samples, but the major low molecular weight species of Ga was not identified, although the elution profile was similar to that of Ga phosphate. NMR failed to show an Al-citrate signal in plasma samples given the experimental conditions and detection limit of the technique. It was possible Al-citrate was present in plasma at a concentration lower than 2×10^{-5} M and therefore, a signal for the species would not observed. The results did not differ between controls and other diseased groups studied.

Work described above used the original method of Farrar et al (1990). One of the criticisms of Farrar's work was the lack of a physiologically suitable environment in which binding to Tf occurred. This problem was addressed in chapter 3 and suitably discussed. Therefore, it is adequate to only briefly discuss the results in this chapter.

The primary missing constituent of the original buffering system was a physiological concentration of the bicarbonate anion ((bi)carbonate in vivo is known to bind concomitantly with Fe to Tf and is a synergistic anion). Additions of known concentrations of bicarbonate to the elution buffer drastically increased Ga-Tf binding

up to a concentration of 25 mM bicarbonate. Further additions of bicarbonate negligibly decreased the binding, thus maximal Ga-Tf binding was observed in vitro at an approximately physiological concentration of bicarbonate.

Initial results suggested all normal controls displayed a high level of Ga-Tf binding (85% or greater), with only a small percentage of Ga bound to a low molecular weight species. However, upon increasing the sample size of the control group to include hospitalised patients (to eliminate a hospital environment as a cause of the low Ga binding results observed in the diseased group in this study) and normal people from different geographical locations throughout the UK, it became apparent that a few people had low Ga-Tf binding (in the order of 20%). These subjects were found in each control group, the frequency of appearance and binding results was similar in all groups. The implication of these findings can be interpreted as the identification of a group of otherwise healthy people who are at a greater risk than the general population from the toxicity of metals not bound by Tf.

It was interesting to note in the Second Report of the Lowermoor Incident Health Advisory Group (1991), in the appraisal of Farrar's paper, the original work was dismissed partially on the basis that no persons in the control group were identified as having defective (low) Ga-Tf binding. The advisory group commented if defective binding was a factor in AD, "It would be expected to be present in a significant proportion of the population." By extending the sample size of the control group, such people have been identified.

The ratio of Al in plasma bound to high and low molecular weight species can be influenced by physiological factors such as pH and plasma bicarbonate concentration (section 3.4 and 3.6 respectively). It has already been mentioned that in extreme cases, the concentration of bicarbonate in plasma can be reduced to 5 mM. Although large variations in pH are not evident in the circulatory system, by reducing both the pH and the concentration of bicarbonate a subsequent reduction in Al-Tf binding may be observed. Similarly, the presence of other metal ions in the blood stream which can bind to Tf will (depending on relative stability constants) force more Al to bind to a low molecular weight ligand. Probably the metal to exert the most detrimental effect on Al-Tf binding would be Fe. Fig 3.9 demonstrates the rapid shift of Ga into a low molecular weight pool with only a small concentration change of Fe in the plasma.

There are a variety of Fe metabolism disorders which these can serve to illustrate the effects of altering the equilibrium which exists between the high and low molecular weight binding species. Atransferrinaemia is a condition in which the patient has little or no endogenous Tf and in haemochromatosis there is an acute plasma Fe overload. Both conditions result in the deposition of Fe in tissue such as the spleen, kidney and heart. By increasing plasma Fe levels or decreasing Tf levels, the Tf Fe saturation will

increase. This will affect Al-Tf binding at any given concentration of either Fe or Tf, the significant correlations observed between Tf Fe saturation and Ga-Tf binding in chapter 4 support this. Further examples of altered Fe and Tf values in physiological situations are given in Huebers and Finch (1987).

10.4 Fe SPECIATION IN HUMAN PLASMA

If one considers the stability constants for Tf-Fe, Ga, and Al in the presence of 27 mM bicarbonate at pH 7.4, Martin et al (1987) have calculated an approximately 10^9 greater affinity for Fe compared to Al. Similarly, Ga was shown to have a 10^7 greater affinity than Al. The implication of this in terms of the relative amount of Tf bound metal is apparent if one considers Ga and Fe plasma speciation.

Preliminary experiments from our laboratory (J. Howson) have indicated the species of Fe present in plasma following an identical procedure to the one used for Ga. Fe was bound only to a high molecular weight peak in all four samples studied, there was no suggestion of a low molecular weight species. Tf is known to be the major Fe binding protein and the elution position of the Fe peak was identical to that of a Ga-Tf peak. Therefore, one can assume the Fe binding species present in the peak was Tf. The presence of bicarbonate did not effect the elution profile (i.e. Fe-Tf binding was 100% in the presence or absence of bicarbonate) which has not been described previously. Therefore, it is suggestive that if a 10^2 decrease in the stability constant observed in a Ga-Tf complex compared to an Fe-Tf complex results in lower Ga-Tf in vitro, it is possible that Ga-Tf does not reflect Al-Tf binding, but over estimates it. If this be the case, the results presented in this thesis are still valid because differences noted between control and diseased patients are comparative and a toxic Al species would still exist. It also suggests the low Ga-Tf binding subjects (who are suggested to be at risk from a neurotoxic low molecular weight Al species) are also underrepresented in the control group.

10.5 REACTIVE OXYGEN SPECIES AND CELL MEMBRANE DAMAGE

In the following sections, the mechanisms behind the pathological events in AD and PD relate in part, to free radical attack on cell membranes. Accordingly, it is appropriate to introduce the concept of free radical production and subsequent damage to biological tissues.

Free radicals are species which are unstable and react almost instantaneously with neighbouring molecules to become more stable. They may be defined as, "Any species capable of independent existence that contains one or more unpaired electrons" (Halliwell 1989). The hydrogen atom, most transition metals, and dioxygen are all by definition free radicals. Others include: superoxide (O_2^-), peroxyl ($\cdot O_2$), and hydroxyl

(·OH) radicals. Hydrogen peroxide (H_2O_2) is not a free radical (it has no unpaired electrons) so the term reactive oxygen species has been introduced to describe not only the superoxide and hydroxyl radicals, but hydrogen peroxide as well (Halliwell 1989).

Transition elements have variable valencies, a change in valency state involves the addition or removal of an electron (reduction and oxidation respectively). This means transition elements are very effective at promoting free radical formation. Fe, Cu, and Mn are three such metal ions which are readily found in the body. During the conversion of oxygen to the superoxide radical, which in turn can be converted to hydrogen peroxide and then to the hydroxyl radical the three metals are able to participate in all stages of the reactions (equations 1, 2, 3, and 4).

1) $O_2 + e^- \text{——} O_2^{\bullet -}$

2) $O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \text{———} H_2O_2 + O_2$

3) $H_2O_2 + M^x \text{———} {\bullet}OH + OH^- + M^{x+1}$ (where M^x is a metal in its reduced form and M^{x+1} is the oxidised form of the metal)

4) $H_2O_2 + O_2^- \text{———} O_2 + OH^- + {\bullet}OH$

Fe is known to participate in a Fenton reaction to form the hydroxyl radical (equation 3). In this reaction, hydrogen peroxide is converted to the hydroxyl radical by the addition of an electron from ferrous Fe. Hydroxyl radicals can also be produced by a Haber-Weiss reaction where the superoxide radical donates an electron to hydrogen peroxide to form the hydroxyl radical (equation 4).

The hydroxyl radical is the most reactive species known and in biology the damaged caused by this species is demonstrated well by lipid peroxidation. When a hydroxyl radical is produced near a membrane, it will remove a hydrogen atom from a carbon atom of a fatty acid side chain to form water. Under physiological conditions, the removal of the hydroxyl radical will result in the formation of a carbon centred radical which will react with oxygen to produce the peroxyl radical. This new radical will remove another hydrogen atom from an adjacent fatty acid side chain to form the carbon centred radical again. The production of one hydroxyl radical sets off a chain reaction which will convert the fatty acid side chains into lipid hydroperoxides. An accumulation of the lipid hydroperoxidases will disrupt the membrane causing it to dysfunction and possibly collapse.

Organisms use superoxide dismutase (SOD), catalase, and glutathione peroxidase to protect against the generation of damaging reactive oxygen species (although hydrogen peroxide is formed as a result of the reaction using SOD which can then form the even more damaging hydroxyl radical).

Catalase and glutathione peroxidase are used to remove hydrogen peroxide and stop the production of hydroxyl radicals. Free radical scavengers such as vitamins C (ascorbic acid) and E (alpha-tocopherol) interact with them once they are formed to protect cell membranes. Ascorbic acid functions as a hydrophilic chain-breaking antioxidant and alpha-tocopherol as a lipophilic chain-breaking antioxidant (Niki et al (1988), the latter being the most important in humans (Halliwell 1989). When peroxyl radicals are formed during lipid peroxidation, they combine preferentially to a hydroxyl radical attached to alpha-tocopherol rather than to forming a carbon centred radical in the plasma membrane. A new tocopherol-O• radical is formed which is unable to react with the fatty acid side chains and so the chain-reaction is broken.

10.6 A BIOCHEMICAL MECHANISM FOR ALZHEIMER'S DISEASE

10.6.1 INTRODUCTION

The formation of senile plaques is central to the pathology of AD and it is possible the deposition of amyloid A4 protein (the primary proteinaceous component of the plaque) is a key event in the initiation of the other characteristic changes observed in the AD brain (Kosik 1991, Hardy and Allsop 1991, Hardy and Higgins 1992). Therefore, although neurofibrillary tangle formation is another important pathological marker, it will not be discussed further.

The transmembrane amyloid precursor protein (APP) contains the A4 protein, which itself is situated partially in the membrane and partially extracellularly (Anderson et al 1991). Proteolysis (via a secretory pathway) of APP normally occurs to produce three isoforms of the protein which, if containing an inserted exon, are functionally homologous to the kunitz-type serine protease inhibitors (Tanzi et al 1988). More specifically to protease nexin-II (Oltersdorf et al 1989, van Nostrand et al 1989). The isoforms of APP are derived from alternative pre-mRNA splicing of the APP gene which is located on chromosome 21. The major type of APP found in the brain lacks the inserted exon which is found in the other two isoforms (Kang and Muller-Hill 1990), although the significance of this was not determined. The cleavage site of APP has been identified in the region of the A4 sequence (Sisodia et al 1990, Anderson et al 1991). Therefore, under normal circumstances, intact A4 is not produced by secretase and cannot lead to A4 deposition in the senile plaque (Estus et al 1992, Hardy and Higgins 1992). A study by Golde et al (1992) has described an alternative endosomal-lysosomal pathway for the processing of the precursor protein to form C-terminal derivatives of APP, including the A4 protein. Support for this hypothesis has been provided by the work of Cole et al (1990, 1991) and Chen et al (1990) (cited by Golde et al 1992).

10.6.2 GENETIC MUTATIONS AND A4 DEPOSITION IN FAMILIAL ALZHEIMER'S DISEASE

Goate et al (1991) have described a mutation in the APP gene on the long arm of chromosome 21 in a family with post mortem confirmed early-onset AD. An amino acid substitution (valine is replaced by isoleucine) close to the end of the C-terminus (within the transmembrane domain) of the A4 protein was reported by the group. The same mutation was noted in Japanese families by Naruse et al (1991) and they claim this supports the argument that the mutation was pathogenic because it occurred in patients of varying ethnic origin. Other mutations at the same locus have been identified by Murrell et al (1991) (valine replaced with phenylalanine) and by Chartier-Harlin et al (1991) (valine replaced with glycine).

van Broeckhoven et al (1990) postulated that a single mutation in the APP gene causes human hereditary cerebral haemorrhage with amyloidosis of the Dutch type (HCHWA-D), an autosomal dominant form of cerebral amyloid angiopathy. This has been corroborated by Levy et al (1990), who have identified a single amino acid substitution on the exon encoding the APP (glutamic acid replaced by glutamine). Apart from cerebral angiopathy, deposition of A4 protein occurs in senile plaque like structures in patients with HCHWA-D (van Duinen et al 1987).

It appears therefore, that AD can, in a small number of cases be attributed to an altered protein sequence of APP. The normal processing pathway of APP seems to be blocked/inhibited and an alternative pathological route taken. It has yet to be determined the exact mechanism behind the switch in the pathways.

10.6.3 SPORADIC ALZHEIMER'S DISEASE

In sporadic AD, it is probable that A4 protein production is due to an alteration in the normal secretase pathway of proteolysis of APP. Thus, it is proposed that free radical attack of membrane sections containing the APP occurs. This would result in the withdrawal of the damaged membrane section into a lysosome where the A4 protein could be cleaved (as described above). Cell death (with subsequent lysis of the plasma membrane) or exocytosis could deposit the A4 protein into the extracellular medium. Plaque formation would follow.

Lipid peroxidation is known to increase with age (Yagi and Komura 1990) and it has been demonstrated that Al can increase membrane hydroxyl radical attack (Quinlan et al 1988). It is suggested that these factors are important in the development of an increased production of A4 protein from APP, rather than APP following the secretase pathway to form protease nexin-II. Dyrks et al (1988) suggest membrane damage may result in A4 production, but do not specify any possible mechanism.

The presence of Al in the area of peroxidation can be explained by reference to Ga-Tf binding. It has been demonstrated in chapter 8, that patients with AD have significantly lower Ga-Tf binding than age/sex matched controls. Consequently the low molecular weight species of Al will be enhanced which can lead to accumulation of Al in the brains of AD patients. Furthermore, Fleming and Joshi (1987) demonstrated the increased presence of Al (≈ 6 times higher) in ferritin extracted from AD patients brains compared to age matched control brains. They suggest this was in response to a general elevation of brain Al levels and could possibly lead to the release of Fe in its ferrous state. This would facilitate the production of free radicals possibly via a Fenton type reaction. Edwardson (1991) has also suggested that Al accumulation in neurons may lead to altered biosynthesis or processing of APP.

Al has been identified as an aluminosilicate in the central core of senile plaques from the brains of clinically and neuropathologically confirmed AD patients (Candy et al 1986), increased staining for APP in cortical pyramidal neurons has also been demonstrated in patients with chronic renal failure and it is those neurons which selectively accumulate Al (Candy et al 1992). These findings have lead Crapper Mclachlan et al (1991) to suggest that elevated levels of Al in the brain and serum can induce some of the cellular responses contributing to amyloid deposition.

Alzheimer like changes are demonstrated in dementia pugilistica (Roberts 1988) and other forms of head trauma are correlated with a risk of developing AD (Mortimer et al 1985). Roberts et al (1991) have demonstrated the formation of A4 containing plaques within days of head trauma and Braugher et al (1985) have shown in cells which are subjected to trauma, the normal xanthine dehydrogenase enzyme is swithed to a xanthine oxidase enzyme which produces hydrogen peroxide. Therefore, it is possible that A4 protein production can be initiated after one of a few 'insults' to the brain. These 'insults' may be in the form of a genetic predisposition (as in familial AD and DS) or increased oxidative attack resulting from Al or a head injury.

10.7 ALZHEIMER'S DISEASE IN DOWN'S SYNDROME

10.7.1 INTRODUCTION

In the majority of cases, the phenotype of DS is expressed as a result of an extra copy of chromosome 21 being present in all cells and it has been suggested that this is the cause of AD in DS. Neuropathological changes of the Alzheimer type are not associated with the mentally handicap population in general, but is restricted to DS. Therefore, these changes cannot be primarily due to mental retardation in DS, but can be attributed to the extra copy of chromosome 21.

Approximately 90% of DS subjects over the age of forty have neuropathological features of AD. (Sylvester 1984) and found in all DS over the age of sixty (Mann

1988). Generally, senile plaque and neurofibrillary tangle development occurs twenty to thirty years earlier in DS compared to the non-DS population (Wisniewski et al 1985). Neurochemically, DS patients show identical changes to those found in AD brains confirmed post mortem (Godridge et al 1987).

10.7.2 A BIOCHEMICAL MECHANISM FOR ALZHEIMER'S DISEASE IN DOWN'S SYNDROME

The additional chromosome 21 in DS indicates there would be an increase in APP production (due to an extra APP gene) which could possibly explain the deposition of A4 protein in senile plaques of all DS patients. If one assumes the rate limiting step in the secretase pathway of APP proteolysis is the concentration of available enzyme, then the overproduction of APP would result in the processing of the protein via alternative (endosomal-lysosomal) pathways which could result in excessive A4 production.

The gene for superoxide dismutase (SOD) is also found on chromosome 21 and a 50% increase in SOD activity has been reported in a variety of cell types including: red blood cells, platelets, leucocytes, and fibroblasts in DS patients (Sinet et al 1978 and references therein). An increase in SOD activity has also been reported in fibroblast cell lines derived from DS patients compared to controls (Zemlan et al 1989).

The function of SOD is to remove the potent superoxide free radical, the superoxide radical being reduced to hydrogen peroxide and oxygen (equation given in section 10.5). Hydrogen peroxide itself is potentially toxic and can cause membrane disruption (Halliwell 1989). In the presence of ferrous iron, hydrogen peroxide can be reduced to the more damaging (than both hydrogen peroxide and the superoxide free radical) hydroxyl free radical via a Fenton reaction (equation given in section 10.5).

The relevance of free radicals in DS is thus: with an increase in SOD activity in the DS brain, there would be a subsequent increase in hydrogen peroxide. Glutathione peroxidase is the primary system for the removal of hydrogen peroxide in the brain (Olanow 1990): De Marchena et al (1974) measured the activity of glutathione peroxidase in brain tissue of various animals and concluded that there was an insufficient concentration of the enzyme to provide protection from peroxidative damage, this was not taking into account the elevated levels of hydrogen peroxide seen in DS. Accordingly, the build up of hydrogen peroxide could increase cell membrane damage and produce hydroxyl free radicals which, again would damage cell membranes (containing APP) via lipid peroxidation. A4 production would occur as described in sporadic AD.

Ascorbic acid (a scavenger of hydroxyl free radicals once they are formed) concentrations are reduced in DS (Abalan et al 1990). Halliwell and Gutteridge (1985) have noted two effects of a reduced ascorbate concentration: a decrease in the

antioxidant capabilities and at low ascorbate concentrations, it acts as a pro-oxidant. This implies that free radicals could exist for longer periods of time before being removed by other appropriate mechanisms.

Increased oxidation in the DS brain has been discussed above, but the increased oxidation of blood constituents is also probable. Sinet et al (1978) have suggested accelerated oxidative processes are occurring in red blood cells which may be reflected by an increase in peroxide production. Niki et al (1988) have demonstrated that ascorbate is the first line of defence (by acting as a hydrophilic chain-breaker) against peroxy radicals formed in plasma. The decrease in ascorbate known to occur in DS implies increased lipid peroxidation would occur. Furthermore, enhanced SOD activity would not be restricted to the brain, but would occur throughout the body.

The mean Ga-Tf binding in DS patients was decreased compared to age/sex matched controls. Young DS patients (below 20 years) generally displayed normal Ga-Tf binding. Subsequently, Ga-Tf binding became increasingly varied with age. Both high and low results were observed, with a significant decrease in mean binding occurring with age (and a tendency to observe high binding less frequently). It is proposed that Ga-Tf binding reflects the development of characteristic neuropathological lesions in the brain by approximately a decade (see fig 6.5). Reduced Ga-Tf binding could increase both A4 production and free radical production by allowing an increase in low molecular weight Al species accumulate in the brain areas affected by plaques. Edwardson et al (1991) have observed Al and Si in senile plaques from DS brains, the presence in situ confirmed by: energy and wave length dispersive electron probe micro analysis, scanning proton microprobe X-ray analysis, and imaging secondary ion-mass spectrometry. This supports the idea that Al is present in the general area of A4 production

The actual reason for the reduced binding seen in DS may be two-fold: Farrar et al (1990) have measured Tf Fe saturation in a group of DS patients (mean age 38.4 years) and noted it was over twice that observed in controls. Fe loaded Tf has been shown to catalyse hydroxyl radical formation via superoxide production by neutrophil NADPH oxidase in the presence of hydrogen peroxide (Bannister et al 1982) and via a hypoxanthine-xanthine oxidase system (Motohashi and Mori 1983). Therefore, in young DS with a high Tf Fe saturation, the production of free radicals may be enhanced (certainly increased hydrogen peroxide should increase the rate of production by the first system mentioned). Secondly, the increase in free radical production would result in raised protein membrane lipid peroxidation in the plasma. Work detailed in chapter 3 has shown reduced Tf binding as a result of hydroxyl formation via a Fenton type reaction. Therefore, as the DS patient ages, reduced binding to Tf could shift from a result of high saturation to a result of damaged Tf molecules due to oxidative attack. In either circumstance, increased Al transport to the brain would occur to enhance A4

production.

In a review by Sinet et al (1978), many features of DS are claimed to be consistent with increased oxidative damage, these include: a rapid aging with decreasing I.Q., histological changes in the brain similar to that seen in neuronal degeneration, an accumulation of lipofuscine, and a shortened life span of cells in culture.

In summary, the presence of senile plaques in all DS patients could therefore be in response to the overproduction of APP to give increased amounts of A4. This would be coupled to increased membrane oxidation enhanced by SOD activity and the local presence of Al to further enhance oxidation, which was delivered to the brain as a result of defective Tf binding.

10.8 A BIOCHEMICAL MECHANISM FOR PARKINSON'S DISEASE

10.8.1 INTRODUCTION

PD can be the end result of various 'insults' to the body in general, or to the brain specifically (table 5.1). However, in the majority of patients who develop PD, a causative agent can not be identified. These people are therefore referred to as having idiopathic PD and there is great interest and research into the pathogenic mechanisms which underlie this particular form of PD. A hypothesis which accounts for the main neurological changes in the Parkinsonian brain and has much supporting evidence is that of oxidation reactions.

10.8.2 IDIOPATHIC PARKINSON'S DISEASE

Free radical attack on the nigrostriatal dopaminergic system in PD may represent a mechanism which results in the symptoms of the disease (Graham et al 1978, Dexter et al 1986, Spina and Cohen 1989, Olanow 1990). The basis for this hypothesis is that for each mole of dopamine oxidised specifically by MAO B, a mole of hydrogen peroxide is generated. Both dopamine and MAO B are highly concentrated in the substantia nigra (Langston 1989 and Konradi et al 1989 respectively). Graham et al (1978) suggest that a life long injury to the dopaminergic neurons may occur from the by products of dopamine oxidation.

Catalase, although the most effective enzyme responsible for removal of hydrogen peroxide is almost absent from the brain (Riederer et al 1989). Therefore, the most important defence mechanism in the brain is glutathione peroxidase. This enzyme system is highly dependent upon the concentration of reduced glutathione and was found not to be in sufficient concentration to provide protection from peroxidative attack (De Marchena et al 1974). A decreased glutathione and glutathione peroxidase

concentration in the substantia nigra (Perry et al 1982, Perry and Yong 1986, Riederer et al 1989), an increase in activity of superoxide dismutase (Saggu et al 1989), and a decrease in the concentration of ascorbate (Subramanian 1977, Tolbert et al 1979) suggests excess reactive oxygen species are produced in PD. The reduced ascorbate concentration has two effects (mentioned previously): the antioxidant capabilities would be decreased with a decrease in ascorbate concentration, and at low concentrations it has been shown ascorbate is a pro-oxidant (Halliwell and Gutteridge 1985). MAO B (which specifically promotes dopamine oxidation) activity has been shown to increase with age (Fowler et al 1980) and possibly increase in neurons that are not dead (Agid 1991). This again suggests that reactive oxygen species could increase in the Parkinsonian brain.

Another mechanism to increase the production of hydrogen peroxide which yields the hydroxyl radical is by either the presence of a transition element (Fenton reaction), or the presence of the superoxide radical (Haber-Weiss reaction) (Olanow 1990). There is evidence of increased lipid peroxidation in the substantia nigra of patients with PD (Dexter et al 1989) and this could be due to any of the possible mechanisms described above.

Fe concentrations have been found to be elevated in the substantia nigra of patients with PD (Riederer et al 1989, Jellinger et al 1990, Sofic et al 1991, Hirsch et al 1991). Fe bound to Tf or Ferritin is thought not to participate in free radical formation (Olanow 1990), although Bannister et al (1982) and Motohashi and Mori (1983) have demonstrated the production of hydroxyl radicals (from different substrates) which were catalysed by Fe saturated Tf in vitro. Fe in a species which is present in a low molecular weight pool would take part in free radical formation (Halliwell and Gutteridge 1984) and in certain circumstances low molecular weight ferrous Fe has been detected in plasma (Halliwell et al (1988) and cerebrospinal fluid (Gutteridge 1992). The increase in Fe in the brain is not associated with the density of Tf receptors (Hill et al 1985, Youdmin 1989) and a decrease in the ferritin concentration of the substantia nigra (Riederer et al 1989) and the brain generally (Dexter et al 1991) suggests the species of Fe available would be that which could participate in free radical production (Riederer et al 1989, Sofic et al 1991). Aluminium has been found in increased concentrations in the substantia nigra of patients with PD (Hirsch et al 1991). It has been demonstrated that SOD activity is inhibited by Al (at least in vitro) (Shainklin-Kestenbaum et al 1989) and Al increases Fe mediated lipid peroxidation (Quinlan et al 1988).

Support for a primary role for oxidative damage in idiopathic PD is given by examining secondary Parkinsonism (in particular MPTP and Mn induced PD) and Ga-Tf binding in treated and untreated PD plasma.

MPTP is a by product from the synthesis of 1-methyl-4-propion-oxypiperidine (MPPP), an analogue of meperidine (or 'new heroin'). Injection of MPTP in humans produces a clinically indistinguishable (Langston et al 1983) and neuropathological very similar (but not identical) lesions (Davie et al 1979) to that of idiopathic PD. Marker et al (1984) have shown MPTP is oxidised to MPP^+ (by MAO B (Chiba et al 1984)) and it this species which is believed accumulate in dopaminergic neurons and cause damage (Tanner 1989). Superoxide (Sinha et al 1986, Rossetti et al 1988) and hydroxyl (Poirer and Barbeau 1985, Sinha et al 1986) radical production have been demonstrated in vitro during the conversion of MPTP to MPP+ and this enhances dopamine autoxidation in the presence of transition metals (especially Fe^{2+} and Mn^{2+}) (Poirier et al 1985).

Mn is increased in a secondary type of Parkinsonism (section 5.1.5.3). The many valency states of this metal means it is an ideal candidate for participating in Fenton reactions to produce hydroxyl radicals, although Halliwell and Gutteridge (1984) have not been able to demonstrate Mn^{2+} dependent hydroxyl production. Donaldson (1981) have shown the possible enhancement of dopamine autoxidation resulting in free radical production by Mn. This would occur under terms of chronic exposure to the metal where the valency of Mn is raised above +2 (Donaldson et al 1982).

Ga-Tf binding in untreated PD plasma was much reduced compared to age matched controls. This would be one mechanism of increasing the concentration of Al in the substantia nigra that has been observed by Hirsch et al (1991) and to increase lipid peroxidation in the area. Tf Fe saturation in PD plasma was lower than observed in controls and in lower end of the normal range expected. In other diseased groups (AD and DS), controls, and in neonates, there is an inverse correlation between Tf Fe saturation and Ga-Tf binding: as Tf Fe saturation increased, Ga-Tf binding decreased. As there is no such relationship found in untreated PD, one most assume factors other than Tf saturation are responsible for the reduced binding. Accordingly, it is proposed that reduced Ga-Tf binding in unmedicated PD is due to increased oxidation reactions in the circulatory system.

Both Fenton type reactions (chapter 3) and oxidation reactions using manganous salts (chapter 5) has resulted in reduced Ga-Tf binding in control plasma. This is indicative of oxidative damage to the Tf protein molecule, leading to reduced binding. Baker et al (1987) have identified two tyrosine residues in the N-lobe of human lactoferrin which act as binding ligands for ferric Fe. They also argued for a homologous sequencing of binding ligands in human Tf. It is these ligands which are exposed to the extra cellular medium (see fig 1.1) that represent a primary target for oxidation (see section 5.4 for details). Once structurally altered, it is assumed they would not be able to participate in Fe binding and as a consequence, the damaged lobe of Tf would not be able to accommodate any Fe ions. Supporting this theory, there is one subject who has a variant type of Tf which results in reduced Fe binding. Analysis of the protein has

revealed that tyrosine residues are absent from the C-lobe of the molecule which does not allow Fe to bind in that particular lobe. There are also two naturally occurring lactoferrins which do not contain the tyrosine residues, they do not bind Fe (Dr. R. Evans personal communication, 1992).

Further evidence to strengthen an argument for increased oxidative activity in unmedicated PD plasma is given by the work of Farrar et al (1991b). Using an aliquot of plasma derived from the sample used for Ga-Tf binding studies, it was demonstrated that the concentration of 5methyltetrahydrofolate (5MeTHF) (as measured by HPLC) was significantly reduced compared to control plasma. A reduction in the concentrations of serum biopterins and cell fraction (erthyrocytes and other cells) biopterins (as measured by *Crithidia fasciculata*) was also noted. Post treatment, the concentration of serum biopterins increased to a similar value observed in controls. Oxidative mechanisms were believed to be responsible for the observed reduction in biopterins and 5MeTHF.

	5MeTHF (ng/ml)	serum biopterin (ng/ml)	cell fraction biopterin (ng/ml)
untreated PD	4.21 ± 3.44 (N=14)	0.96 ± 0.24 (N=42)	1.74 ± 0.68 (N=11)
treated PD	-	2.04 ± 0.85 (N=25)	-
control	8.18 ± 5.32 (N=16)	1.81 ± 0.64 (N=114)	3.57 ± 1.1 (N=20)

Table 10.1 Biopterin and folate reductions in PD.
Adapted from Farrar et al (1991b)

Already discussed (briefly in chapter 5) is the increased Ga-Tf binding observed in patients who have been treated with antiparkinsonian drugs for at least six weeks. It is possible the increase in binding observed after treatment is due to the administration and subsequent antioxidant effects of L-dopa. During lipid peroxidation, peroxyl radicals will be produced during the chain reaction. L-dopa can participate in this reaction by preferentially combining with the free radical rather than with a hydrogen atom from the plasma membrane (the mechanism of chain breaking antioxidant was describe in section 10.5). This halts the chain reaction and stops further oxidation occurring. Furthermore, Farrar and Blair (1991) have demonstrated that L-dopa will prevent oxidation of 5MeTHF, at least in vitro.

In summary, it is proposed that PD is a result of increased oxidation in key areas of the brain which selectively destroys dopaminergic neurons. These reactions are enhanced by Al accumulation in the brain which itself occurs as a result of increased oxidation reactions in the blood.

10.9 Ga-Tf BINDING AND NEURODEGENERATIVE DISEASES

The results of this thesis demonstrate reduced Ga-Tf binding in a variety of neurodegenerative disorders and in a small percentage of the control group used for comparative purposes (table 10.2).

Group	age range	% Ga-Tf binding	% Tf Fe saturation
normal controls	5 - 82	86.9 ± 8.2 (N=86)	* 0.39 ± 0.29 (N=15)
low controls	15 - 67	23.9 ± 10 (N=21)	NA
Neonate	-	8.3 ± 6.1 (N=11)	0.86 ± 0.38 (N=41)
untreated PD	38 - 79	31.5 ± 21.9 (N=32)	0.24 ± 0.13 (N=7)
DS	7 - 70	54.9 ± 28.9 (N=32)	* 0.81 ± 0.53 (N=14)
AD	56 - 86	68.9 ± 27.6 (N=20)	* 0.59 ± 0.64(N=10)

* taken from Farrar et al (1990)

NA not available

Table 10.2 Summary of Ga-Tf binding data in control and diseased groups.

It is highly unlikely that reduced Tf binding is the specific cause of any diseased state examined, but it is proposed this phenomenon potentiates the normal processes of aging in some but not all cases of AD and PD, if one assumes AD and PD are an acceleration of normal aging. To clarify this statement, it has been suggested that the normal rate of dopaminergic neuronal loss in the human brain would induce PD in all people over the age of 120 years (Nagatsu 1991). The prevalence rate of AD rises so steeply with age, one can assume the majority of people living to a similar age as calculated to develop PD would also have AD. In this context, it can be interpreted that both AD and PD are an accelerated aging of the brain above that of the rest of the body. The factor which enhances the accelerating degeneration of the brain (in some circumstances) is enhanced Al accumulation in the brain due to reduced Tf binding.

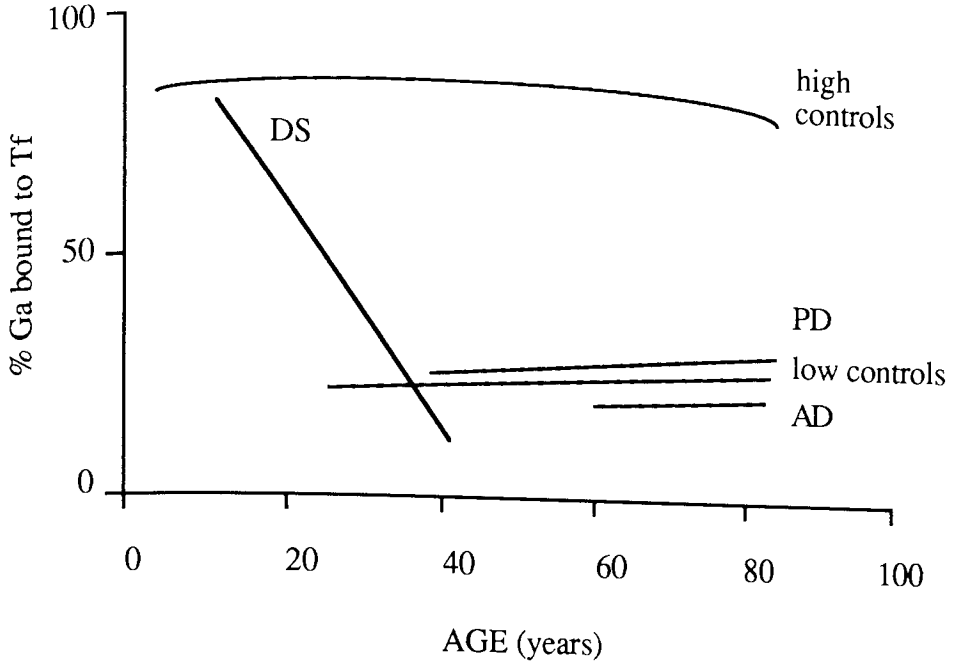

Fig 10.1 Simplified diagram demonstrating age related changes in plasma Ga-Tf binding in control and diseased groups.

Fig 10.1 demonstrates the Ga-Tf binding results observed in control, AD, and PD plasma samples. This simplified diagram can serve to illustrate the great differences in each group and the risk of development of a neurodegenerative disorder the low controls have.

Both AD and PD are a result of damage to specific areas of the brain: different areas are affected in each disease. If reduced Tf binding is a determining factor for both diseases, it raises the question why the cortex and hippocampus are affected in AD and the substantia nigra in PD ?

If one accepts the view there is a genetic susceptibility linked with exposure to an environmental toxin in PD (Marsden 1990) (the toxin being Al and susceptibility is defined as potential damage to the substantia nigra), it is feasible these people would develop PD if exposed to Al, because they are genetically determined. If however, exposure to Al is not accompanied to a genetic susceptibility, then AD may be the end result which shows a more generalised (yet still well defined) atrophy of the brain.

Alternatively, PD patients may have a disturbance in the Fe homeostasis mechanism in the body which results in the observed Fe accumulation in the substantia nigra. The increase in Fe would increase free radical production locally and result in atrophy in that given area of the brain. Increased peroxidation would also occur in the blood, damaging Tf molecules (amongst other components of the blood) which results in the inability to detoxify Al. Increased Al transport to the brain would follow to enhance peroxidative damage further. AD patients, without this defect would be relatively spared in the damaged seen at the substantia nigra in PD.

The presence of control people (apparently healthy individuals and those hospitalised for other medical conditions) with reduced Tf binding also poses the question: are these subjects at risk from developing AD or PD ? If so, which disease would they be more susceptible to ?

There is no firm evidence to either confirm or reject a hypothesis which states the low binding controls are at risk from developing a neurodegenerative disorder. However, the fact that the majority of all PD, DS patients over thirty, some AD and MND patients, and affective disorder patients all have reduced Ga-Tf binding is highly suggestive. One must remember, reduced Tf binding alone would probably not enhance the pathological changes involved in either AD or PD It would be a combination of reduced binding, exposure to Al (or another metal which Tf may usually bind to detoxify it), and increased oxidative damage.

The control group with reduced Ga-Tf binding match closely the pattern of binding observed with age in the PD group (fig 5.4). From this, one may assume that these people would develop PD. The expression of an individuals genetic susceptibility may be in the form of reduced Tf binding (three persons from a single family displayed reduced binding). However, the possibility of AD being developed can not be ignored. The low binding AD patients also match well to the low control group. The fact high binding AD patients are present in the experimental group does not help clarify the situation (although at least some of the results can be attributed to misdiagnosis).

It has been argued that AD and PD are not isolated diseases but are linked (van Duijn and Hofman 1991) and that both diseases have a common etiology (Calne et al 1986, Price et al 1986, Ditter and Mirra 1987). (Calne et al 1986 also suggest MND shares a common etiology with AD and PD, which is of interest in the light of Ga-Tf binding results in this group). Certainly, both disease have reduced Tf binding as a unifying factor and both diseases are becoming accepted as heterogeneous (clinically and neuropathologically) in nature. Idiopathic PD was found to develop at a rate seven times greater in AD patients compared to controls (36% c.f 5% respectively) (Morris et al 1989). Clinical Parkinsonism has also been detected in 20% of demented DS patients in a study group (Lai and Williams 1989). The frequency of AD in PD shows large variations between various reports and could reflect different diagnostic criteria. In a critical review by Mahler and Cummings (1990), the observed frequency of AD in PD was found to be 10 - 60%, PD in AD was found in 10 - 30% of all cases.

This thesis may be thought of as a retrospective study of diseased groups, but also as a prospective study of the control group. Individuals have been identified with defective Tf binding and by observing these people over time, it is an ideal opportunity to discover the exact consequences of low Ga-Tf binding.

10.10 SUMMARY

Normal control people have high Ga-Tf binding. This acts as a protective mechanism against brain Al accumulation. In a small percentage of controls, Tf binding is much reduced. It is believed these people are at greater risk of developing either AD or PD. This is a result of an increased Al low molecular weight species which can transport Al to the brain at a rate which is more rapid and uncontrolled than a Tf mediated mechanism.

In all subjects (healthy or diseased) the low molecular weight Al species was not identified as citrate by NMR and only traces of it were found using gel-filtration chromatography (with the exception of a few samples where citrate was the predominant species). A second species was present and this has tentatively been assigned to phosphate.

Neonates display reduced Tf binding and are known to be susceptible to Al toxicity. The mechanism behind reduced binding is believed to be the very high Tf Fe saturation observed. It is possible Al toxicity in the neonate is a direct reflection of the inability to incorporate Al into a relatively non-toxic Al-Tf species. Increased Tf Fe saturation in the neonate compared to adult values may be of benefit to the neonate in a number of ways, but is the determining factor in Al toxicity.

DS was found to be a suitable model for AD with respect to reduced Ga-Tf binding. Young DS patients generally have normal Tf binding, with an increase in age there is a significant decrease in binding. Abnormally high Tf Fe saturation in DS has been observed, which is one reason why reduced Tf binding is displayed. It has been proposed that as the DS patient ages, increased oxidation reactions (as a result of increased SOD) reduce Tf binding by attacking the tyrosine binding ligands within the Fe binding region of the lobes.

The enhanced entry of Al into the brains of AD patients can also be ascribed to reduced Ga-Tf binding. Al potentiates oxidation reactions which produces A4 protein that is deposited in senile plaques. Reduced Ga-Tf binding was probably due to high Tf saturation. Some AD patients displayed normal Tf binding. These people were either misdiagnosed or represent a subgroup of AD.patients whose dementia is not a result of oxidation damage enhanced by Al, AD being a heterogeneous disease.

In unmedicated PD, reduced Ga-Tf binding was a result of increased oxidative damage in the blood. Lower than normal saturation levels, L-dopa antioxidant observations in treated patients, and the work of Farrar et al (1991b) and Farrar and Blair (1991) support this theory. PD is believed to be in response to increased oxidative damage in the brain.

In haemodialysis patient, the concentration of Al in the plasma correlates well with the degree of Ga-Tf binding. However, it is more accurate to calculate the concentration of non-Tf bound Al (therefore in a low molecular weight species) in the plasma and draw conclusions from those figures. An example is given in 7.3.1. A disturbance in cerebral functioning in haemodialysis patients without clinical signs of Al toxicity could be attributed to reduced Tf binding.

MND as a group displayed normal and reduced Ga-Tf binding in approximately equal numbers. This was not a statistically significant compared to the ratio of high:low binders in the control group. It is possible this was due to the small size of the MND group. The MND patients represent a group who merit further investigation.

Affective disorder patients (pre ECT) display reduced Tf binding compared to controls. The binding generally increases post treatment. Mechanisms behind the benefits of ECT are poorly understood and it is hard to offer an explanation for the increase in binding post treatment. The response to ECT was positive in some patients and it may be a secondary consequence of the clinical symptoms of depression being removed that Tf binding is increased.

10.11 FUTURE WORK

10.11.1 INTRODUCTION

The work described in this thesis has answered many questions and the original aims have been fulfilled. However, it has also raised several other points, each of which deserve exploration.

10.11.2 PROPOSALS FOR FURTHER RESEARCH

To extend the initial Fe speciation studies with respect to Tf binding in diseased groups and controls. Similar experiments to those performed in this thesis would be most suitable. Factors which displace Fe from Tf would be of most interest with respect to the role Fe can play in oxidation reactions.

To initiate Al speciation studies using the radiolabel ^{26}Al. Ga-Tf binding has been shown to be less than that of Fe-Tf binding at identical concentrations of metals. Differing stability constants may be the reason for the observed differences. Therefore, the significantly reduced stability constant of Al-Tf may alter the equilibrium of Al bound to high and low molecular weight species.

To characterise the exact mechanism behind reduced Tf binding in each disease group examined. This could involve isolation of Tf followed by electrophoresis, or by genetically engineering human Tf without tyrosine residues in the binding lobes.

Sequencing of Tf from low binding patients could be attempted.

To clarify the identity of the low molecular weight species present in an elution profile from a G75 column when examining Ga speciation. Low molecular weight species identification from Fe and Al analysis would follow.

To observe the subjects in the control group who display reduced binding over time. Tf binding could be monitored in people with binding in the region of 60 - 70% to see if a further reduction in binding is seen.

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APPENDICES

APPENDIX A

ANIMAL STUDIES

INTRODUCTION

The use of animal studies enables scientists to model actions of drugs or biochemical reactions in man without exposing humans to the possible adverse side effects of the procedure or to the procedure itself. Theories derived from in vitro studies can be tested in vivo for accuracy. For the present study, the rat was chosen as a suitable model.

Previous chapters have shown that the speciation of Ga in plasma was bimodal, the majority of Ga being bound to Tf in the presence of physiological levels of bicarbonate. It was therefore the aim of this section to extend the in vitro examination of Ga-Tf binding to an in vivo situation. Furthermore, the accumulation of Ga in the major organs of the body could be determined by the use of the radiotracer ^{67}Ga.

ANIMAL DOSING REGIME

Fasted animals were dosed intra muscularly (left rear femur) with 300 µl gallium nitrate (10^{-3}, 10^{-5} or 10^{-6} M), a light anesthetic (ether) was used to restrain the animal during dosing. After the appropriate time (see individual results table for exact times), the animal was anaesthetised and 3-4 mls of blood was taken via cardiac puncture. The animal was sacrificed by stunning and cervical dislocation and the major internal organs and the femur muscle were counted for gamma emissions. A plasma sample was prepared and applied to a G75 gel-filtration column for Ga speciation analysis.

RESULTS AND DISCUSSION

Using G75 gel-filtration chromatography with and without the presence of 25 mM bicarbonate, a bimodal distribution of Ga was observed in all the elution profiles obtained. The two peaks eluted in similar positions in all experiments. Given various concentrations of Ga, the percentage of Ga bound to Tf remained similar in all experiments. However, this does not mean that the Ga-LMWS was present in the same concentration in each experiment.

There were many similarities between the rat in vivo and human in vitro studies. The elution profiles of plasma from both studies were virtually identical with the two peaks present at the same positions and both Ga-Tf peaks (peak 1) responded positively to the presence of bicarbonate in the elution buffer. It was also evident that Tf bound the majority of Ga in vivo and in vitro and this reproducibility in different systems was an important observation. The major difference between the rat and human studies was in the difference in the percentage of Ga bound to Tf in the absence of bicarbonate. In the rat, much more Ga was bound to Tf than in humans. Welsh (1990) has studied the

structural differences and properties of rat and human Tf, although there were no gross differences displayed between both Tfs, subtle changes in the amino acid sequence (97 substituted amino acids in all) were evident and this may offer an insight into the different Ga-Tf binding capabilities observed in the absence of bicarbonate.

Ga concentration *		3×10^{-7} M		3×10^{-9} M	3×10^{-10} M	
time (hrs)	0.5	4	24	4	4	24
% Ga-Tf	94.3%	94.5% ± 2.6	94.5% ± 1.5	86.7%	97.5%	97.4%
(HCO_3^- buffer)	(N=2)	(N=4)	(N=4)	(N=1)	(N=1)	(N=1)
% Ga-Tf	40.5%	25.6%	-	-	58.8%	74.5%
(std. buffer)	(N=1)	(N=1)			(N=1)	(N=1)

Ga concentration* refers to the actual concentration of Ga at the site of injection and not in the plasma

Table A1 Ga speciation in rat plasma with varying in vivo incubation times and concentrations of gallium nitrate.

dose (300 µl)	time (hours)	brain	heart	liver	kidney	spleen	muscle
1 M	4 *	0.058	0.74	0.34	0.61	0.25	1580
10^{-1} M	4 *	0.12	0.79	0.68	1.5	0.65	840
10^{-3} M	0.5 *	5.5	60	24	13	25	-
	4	1.3 ± 0.6	80 ± 25	34 ± 17	23 ± 2.0	53 ± 26	-
	24	2.6 ± 2.0	53 ± 28	45 ± 29	40 ± 8.0	84 ± 23	-
10^{-5} M	4	3.0 ± 1.0	45 ± 25	21 ± 12	26 ± 6.0	41 ± 27	-
10^{-6} M	4	6.0 ± 1.0	73 ± 16	37 ± 8.0	44 ± 0.8	68 ± 19	-
	24	2.0 ± 0.4	14 ± 1.0	70 ± 20	47 ± 0.8	111 ± 24	-

* = (N=2), others (N=5)

Results are expressed as a percentage of the TDA x 10^2 per gram wet weight of tissue

Table A2 Ga accumulation in the major organs of the rat.

Analysis of the Ga accumulation results demonstrated the localisation of Ga in all major organs of the body including the brain. The results are expressed without correction for blood within the tissue. Therefore, Ga accumulation in highly vascular tissue such as liver heart and spleen would be overestimated. However, because the tissues are highly vascular, Ga would preferentially accumulate there. At a Ga concentration of 10^0 and 10^{-1} M there was a large precipitation of Ga in the muscle (the site of injection) which

resulted in the reduced accumulation of Ga in other tissues. There were no observable trends with respect to Ga accumulation in specific tissues over time or at a given concentration of Ga. The data presented is a result of a few preliminary experiments, and as such little can be deduced. It is obvious though, Ga (and by implication Al) can be shown to accumulate in the brain and further in vivo studies would be appropriate.

APPENDIX B

POLYACRYLAMIDE GEL ELECTROPHORESIS

INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) is a technique which will separate proteins within a sample according to molecular weight and charge density. It is a quick and easy method which is suitable for screening blood plasma. The aim of this section was to evaluate AD plasma protein composition and compare the results with another dementia (multi infarct dementia (MID)), DS, and control plasma.

PLASMA PREPARATION

Plasma was diluted thirty times (using distilled water) to obtain a gel without distortion of protein bands. The dilution factor was decided upon after doing a series of preliminary experiments on plasma of varying dilutions.

When plasma was separated (by molecular weight), ultrafiltration was used to remove proteins of molecular weight > 10 KD or > 30 KD (MSE personal centrifuge, 3 250 x g for 15 minutes). Millipore ultra filtration membrane devices (Millipore (U.K.) Ltd.,Watford, U.K.) were used, 400 µl of plasma was loaded into the bucket and approximately 10% of the sample passed through the filter.

Molecular weight markers (Electran molecular weight markers, BDH Ltd.) were used (range 16.95 - 2.5 KD) at all times.

SOLUTIONS USED

A) 15% Separating gel

7.5 ml	acrylamide 44% w/v, bis-acrylamide 0.8% w/v
7.5 ml	1.5 M (Tris[hydroxymethyl]aminomethane) (TRIS), pH 8.8
8.0 ml	water
0.6 ml	sodium dodecyl sulphate (SDS) 10% w/v
56 ml	NNN'N'-tetramethylethylene diamine (TEMED)
80 ml	ammonium persulphate (AMPS) 10% w/v

B) Stacking gel

2 ml	acrylamide 30% w/v, bis-acrylamide 0.8% w/v
3 ml	0.5 M Tris pH 6.8
6.4 ml	water
120 ml	SDS 10% w/v
32 ml	TEMED
40 ml	AMPS 10% w/v

C) Sample buffer

5 ml	0.5 M Tris pH 6.8
10 ml	SDS 10% w/v
5 ml	glycerol
10 ml	water
0.5 ml	2-mercaptoethanol
0.4 ml	bromophenol blue 5%

D) Electrode buffer

6 g	Tris
28.8 g	glycine
20 ml	SDS 10% w/v
1980 ml	water

E) Stain solution

0.1%	Coomassie brilliant blue
50%	methanol
10%	glacial acetic acid
39.9%	water

F) Destain solution

10%	methanol
7.5%	glacial acetic acid
82.5%	water

PROCEDURE

The Mini-Protean gel electrophoresis package was used for PAGE (Bio-rad Ltd., Watford, UK). All items to be used were thoroughly cleaned in 100% methanol and then assembled. The separating gel was prepared and poured between the glass plates leaving a 2 cm space at the top. A small quantity of electrode buffer was sprayed on top of the gel to produce a level surface on which the stacking gel was to be placed. The gel was left for fifteen minutes to set. After removing the electrode buffer, the stacking gel was poured on top and the plastic comb inserted to form the wells. The gel was left to set for a further fifteen minutes. When the gel solidified the comb was removed and electrode buffer was put in the appropriate chambers. The diluted plasma samples (combined with the sample buffer in a ratio of 1:1) were injected into the wells using a Hamilton syringe: 10 µl of plasma/buffer solution and 5 µl of molecular weight marker were used.

The gel was run at two hundred volts for thirty five to forty minutes, or until the bromophenol blue marker was near the base of the gel. The gel was not run on constant current because there was a tendency for the gel to increase in temperature and this can adversely affected protein separation.

When the run was completed, the gel was removed from the apparatus and stained by Coomassie blue and/or silver staining. Coomassie blue can detect a band containing as little as 0.1 µg of protein, while the silver stain is at least one hundred times more sensitive and was comparable in sensitivity to autoradiography of radio-labelled proteins. Using the Coomassie stain, the gel was placed in solution E for thirty minutes and then in solution F overnight. Solution F was changed frequently to aid the destaining process. Silver staining was done by the Morrissey (1981) method due to its simplicity. At each stage of the procedure in both staining methods, the gels were slowly agitated. Once the gels were stained, they were vacuum dried (gel slab drier,

Bio-rad Ltd.) for storage purposes.

RESULTS AND DISCUSSION

Separation of plasma using ultrafiltration proved unsuccessful due to either blockage of the filter (thus no proteins were allowed through) or the filter ripped so rendering it useless. The idea behind using filters was to remove heavy molecular weight proteins so one could use undiluted plasma to screen for proteins: specifically the A4 protein which is known to be in senile plaques but unidentified in AD plasma as yet.

Plasma samples; three AD, four DS and five controls (see fig B1) revealed no differences in protein band composition of any sample studied unless haemolysed samples were examined. This was true when using either the Coomassie blue or the silver stain. Further studies would be appropriate using undiluted plasma as any differences in protein composition may be masked by the dilution process. Removal of high molecular weight proteins would be necessary to prevent distortion of protein bands within the gel.

When ten MID samples were examined, eight of the subjects showed a novel protein of molecular weight 13.5 KD which was not found in any other group studied (see fig B2). Initially, the protein was thought to be one or more of the iso-enzymes lactate dehydrogenase (LDH) as huge amounts of the protein are released into the blood stream when a stroke or cardiac arrest has occurred. Preliminary investigative studies using LDH markers proved unsuccessful and a literature review was unhelpful. This protein has remained unidentified, although it has been suggested the proteins are present in the plasma as a response to the individuals health status. All subjects were very ill when blood samples were taken, so it is possible they are acute phase proteins (Dr. P. Altmann, personal communication, 1991).

lane	fig B1	fig B2
1	molecular weight markers	molecular weight markers
2	control (male, 24)	control (female, 26)
3	control (female, 26)	M.I.D. (male, 84)
4	D.S.* (male)	M.I.D. (male, 81)
5	D.S.* (female)	M.I.D. (male, 89)
6	D.S. (male)	M.I.D. (male, 74)
7	D.S. (female)	M.I.D. (female, 77)
8	A.D. (female, 90)	molecular weight markers
9	molecular weight markers	

* haemolysed sample

Identification key for fig B1 and B2

Fig B1 Electrophoretic separation of plasma from AD, DS, and control subjects.

Fig B2 Electrophoretic separation of MID and control plasma.

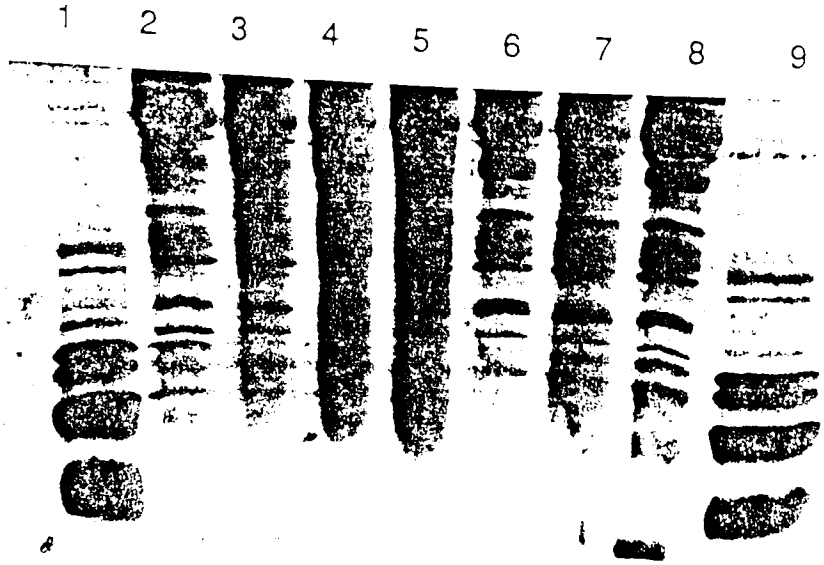

Fig B1 Electrophoretic separation of plasma from AD, DS, and control subjects.

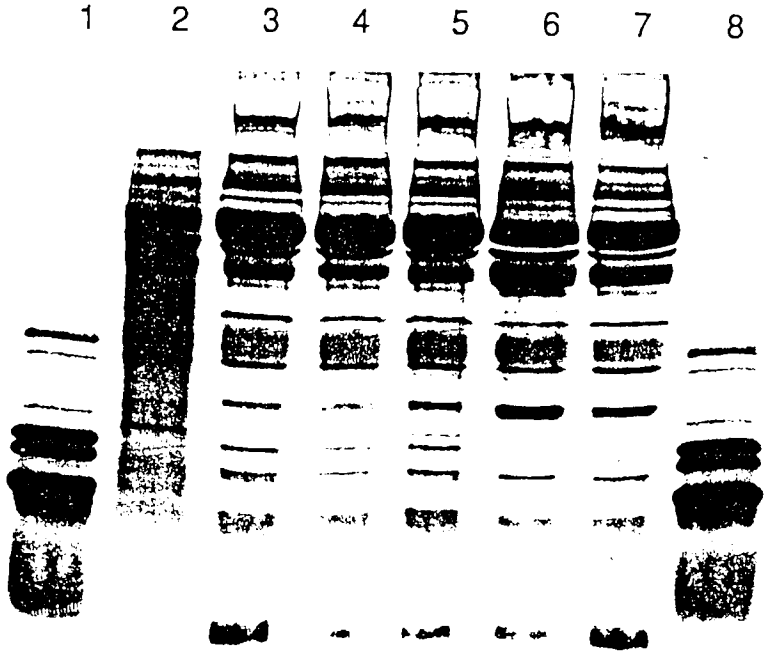

Fig B2 Electrophoretic separation of MID and control plasma.

APPENDIX C

IMMUNOPRECIPITATION OF Tf FROM PLASMA

INTRODUCTION

The use of antibodies to displace Tf from plasma is a good technique for precisely selecting the target protein. Gel-filtration results indicated a difference in the binding capabilities of Tf in control and AD groups, this may have been due to a structural difference on the Tf molecule. By using antibodies to Tf any gross differences in the tertiary structure of the protein could be detected by the inability of the antibodies to adhere to the protein. This would result in a lack of precipitate which could be measured using a radiotracer.

METHOD

To optimise the binding conditions for Tf, a physiological concentration of bicarbonate was added to the plasma and (if necessary) adjusted to pH 7.4 . Plasma (from control and AD subjects) was incubated with Ga to give a final concentration of 10 μg/l and 1-5 μCi ^{67}Ga tracer was added. The plasma was then incubated in a shaking water bath for one hour. 700 μl Tf antibody solution (pH adjusted to 7.4) (Sigma Chemical Company: Ig G fractionated antisera developed in the goat by immunisation with highly purified human Tf) was added to the plasma and further incubated for twenty minutes. After incubation, the plasma was spun down (20,000 R.P.M. x 20 minutes at 6^{o}C, MSE Superspeed 50 centrifuge (MSE Ltd.)) to force the precipitate to the bottom of the tube and another 300 μl Tf antibody solution were added to the plasma. Incubation, centrifugation and the addition of more Tf antibodies (300 μl) took place until all procedures in the protocol had been completed three times. Finally the precipitate (Ga-Tf/Tf antibody complex) was separated from the supernatant and both were counted for gamma emissions.

RESULTS AND DISCUSSION

The percentage of Ga bound to Tf antibodies was calculated as a ratio of the total activity within the plasma. In the control group (volunteers from Aston University) the percentage of Ga bound in the precipitate was 5.9 $\pm$ 2.9 (N=12). This was significantly different from a group of AD subjects at the 5% level (Student's unpaired t-test). The AD group had a percentage mean Ga binding to the Tf antibodies of 21.9 $\pm$ 19.0 (N=7). Within the AD group there was a large variation in the results: three subjects had Ga binding in the region 40% and the remainder of the group had similar values to the control group. If the high values were left out of any statistical analysis the significance of the results would disappear. This bimodal distribution of results was similar to that seen in the gel-filtration chromatography experiments with AD plasma, but there was no correlation between low Ga-Tf binding results and low or high

precipitation results.

Apart from the three AD values which were high, the percentage of Ga bound to the Tf antibodies (due to Ga-Tf binding) was small. Speciation analysis of the supernatant was performed on some of the samples. Peak one from the chromatograph eluted at fraction ten in all samples and the second peak was found at fractions twenty one to twenty three. Both these elution positions were identical to those found from normal plasma samples, therefore one can assume the first peak was Ga-Tf. The mean Ga-Tf binding was 44.6% ± 27.8 (N=6) for controls and 27.0% ± 17.1 (N=3) for the AD group. This observation shows that not all Tf was precipitated out during the experiment and the greater amount of precipitate produced lead to a greater proportion of Ga entering a LMWP. It is possible the reason for the incomplete removal of Tf from plasma was the Tf antibody was specific for a particular form of Tf, e.g. C-terminal mono ferric Tf.

	mean % precipitate	mean % Ga-Tf binding
control	5.9 ± 2.9 (N=12)	44.6 ± 27.8 (N=6)
AD	21.9 ± 19.0 (N=7)	27.0 ± 17.1 (N=3)

Mean % Ga-Tf binding was derived using the supernatant, not whole plasma.

Table C1. Comparison of Ga bound to precipitated Tf in control and AD plasma

APPENDIX D

U/V SPECTROPHOTOMETRY

INTRODUCTION

The aim of this series of experiments was to enable Al binding to Tf in plasma to be measured directly. The addition of Al to plasma of varying groups of subjects (AD, DS and controls) would enable a comparison of Al binding capabilities to be made.

Difference UV spectrophotometry was used to monitor Al binding to Tf. As Al^{3+} is a colourless ion, absorption was in the UV region only and therefore the reaction was recorded between the wavelength range 340 and 190 nm. The reaction occurred at 240 nm where a change in peak height would be indicative of Al-Tf binding (Cochran et al 1987). The binding of Al to Tf leads to a specific reaction in which the phenolic tyrosine of the polypeptide chain in the internal compartment of the lobe was converted to tyrosate. In the reaction a hydrogen ion from the phenol group was removed and replaced by an Al ion.

METHOD

Al binding to Tf (human and bovine) was measured using the UV-240 Shimadzu uv/visible recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and matching quartz cuvettes. The sample and reference compartments were jacketed and connected to an external water bath @ 37°C.

A sample solution was prepared to a final concentration of 10 mM Tf, 25 mM bicarbonate, pH 7.4. Equal volumes (3 mls) were placed in the cuvettes and a base line obtained. 5 µl aliquots of a 1 mM $Al(SO_4)_3$ solution was added to the the sample cuvette, while an equal volume of distilled water was added to the reference cuvette. After an equilibration time of five minutes, spectra were obtained until no further changes in the peak height were obvious. $Al(SO_4)_3$ and not $Al(NO_3)_3$ was used as the latter emits a confusing signal (Fatemi 1990).

Diluted human plasma was also used to observe Al-Tf binding. The plasma was diluted with distilled water by a factor of forty due to its high protein concentration: the sample must be sufficiently dilute to distinguish the general protein peak at 280 nm from that of the reaction at 240 nm which was to be monitored. The same methodology was used as above, except that the equilibrium time was lengthened to thirty minutes.

RESULTS AND DISCUSSION

Addition of Al to Tf was accompanied by an increase in absorbency at 235 and 285 nm

which has also been observed by Cochran et al (1984) and Fatemi (1990). This was indicative of Al /Tf interactions. Fig D1 and D2 illustrates the different spectra obtained upon the addition of increasing concentrations of Al to human and bovine Tf. From the spectra it is clear that the human Tf has a greater propensity to bind Al than does bovine Tf. However, this may be due to the Fe status of the Tf preparation which was used in the experiment. Future work should be done using apo Tf (either commercially prepared or dialysed).

The binding of Al in human blood plasma produced meaningless spectra which were neither consistent or reproducible. The major source of error was in the dilution of the plasma with distilled water which gave rise to a large background signal. Future work should be done using a Tris-HCL buffer which would eliminate the problems that were encountered at the earlier stages of this work (Dr G. Moore, personal communication, 1991).

Curve	Al^{3+} additions (µl)	Al^{3+} conc. (µM)	Curve	Al^{3+} additions (µl)	Al^{3+} conc. (µM)
1	5	1.66	5	25	8.2
2	10	3.32	6	30	9.9
3	15	4.97	7	35	11.53
4	20	6.62			

Conditions: pH = 7.4; T = 37°C; [bicarbonate] = 25 mM; [Tf] = 10 mM; sample volume = 3 ml.

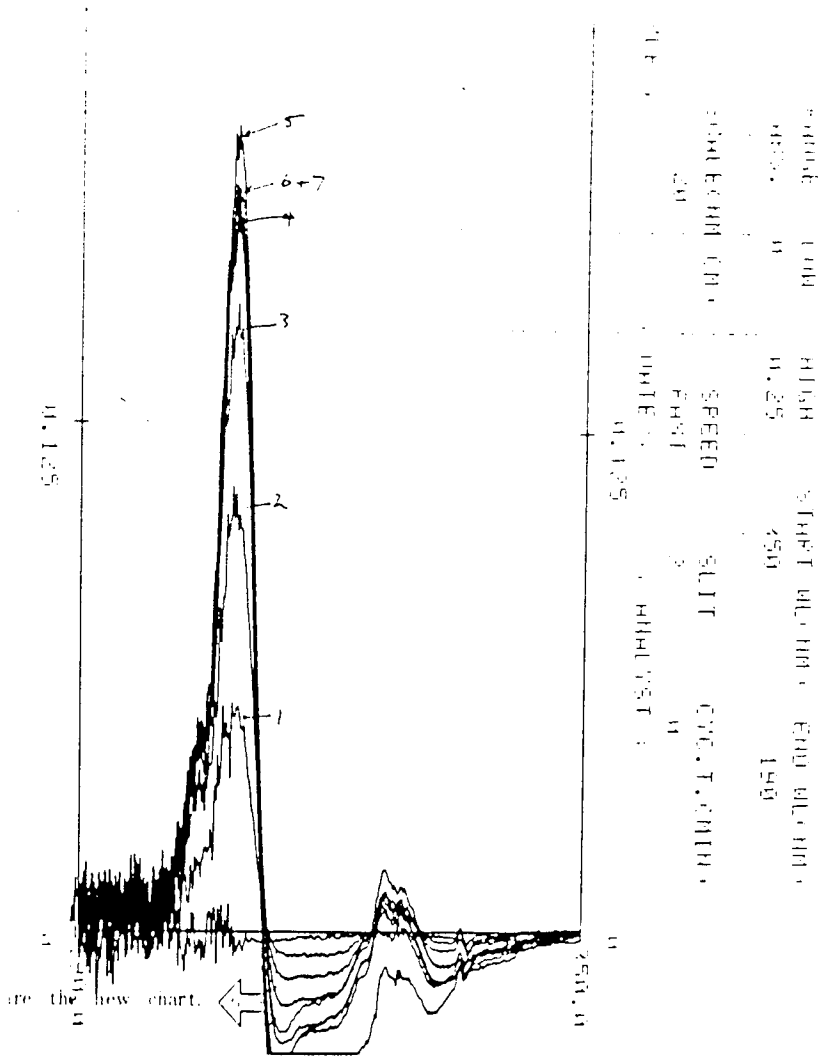

Fig. D1 Spectra of human Tf in an aqueous solution with the addition of 5µl aliquots of 1 mM Al Sulphate.

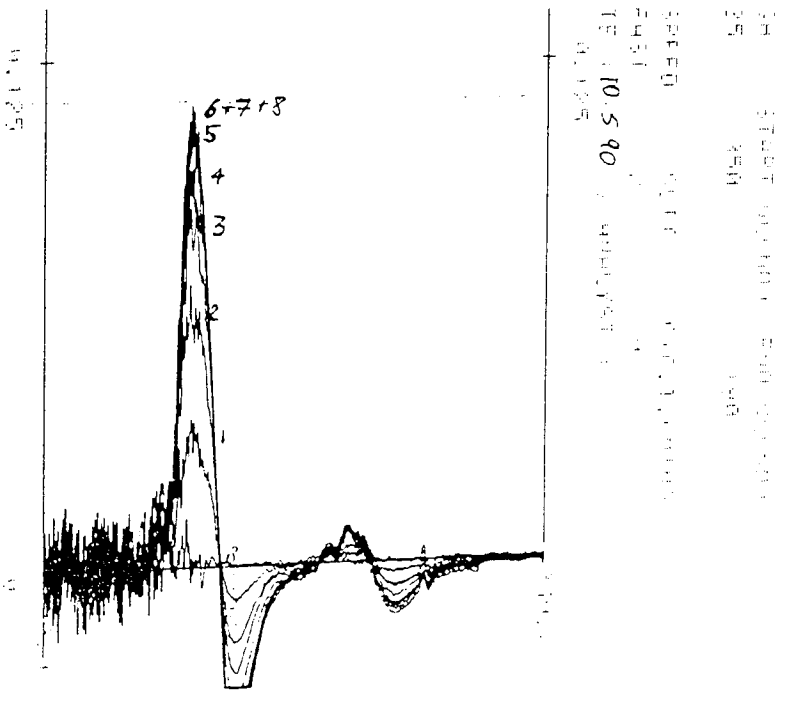

Fig D2 Spectra of bovine Tf in an aqueous solution with the addition of 5 µl aliquots of 1 mM Al Sulphate

APPENDIX 1

Statistical analysis

1) Statistical significance was taken at $p < 0.05$ (5%) or less

2) All results were expressed as: mean $\pm$ SD (N)
   SD = standard deviation
   N = number of observations

3) A two tailed unpaired Student's t-test was used to identify differences in two sets of means drawn from independent populations with normal distributions.

4) A two tailed paired Student's t-test was used to identify differences in two sets of means drawn from the same population which had a normal distribution.

5) The correlation coefficient (R) is a measure of strength of the relationship between two variables. Simple or polynomial regression was used appropriately.

6) All statistical analysis was performed using the statistical software 'Statview™ SE + Graphics' on an Apple Macintosh computer.

APPENDIX 2

Aston and Wolverhampton control results

I.D.	sex	age	% Ga-Tf (HCO_3^- buffer)	% Ga-Tf (std. buffer)
S.T	F	36	81.0	-
L.M.	M	36	72.0	-
A.R.	M	18	53.6	-
J.S.	M	28	37.3	-
M.P.	M	21	78.9	-
J.L.	F	20	87.2	-
H.B.	F	21	90.0	9.5
G.F.	F	27	91.0	20.0
M.Hi.	M	26	89.0	19.0
M.F.	M	22	75.0	11.0
K.H.	M	33	83.0	13.0
Q.W.	M	22	30.3	-
A.D.	M	28	22.4	8.5
R.Y.	F	20	87.0	19.3
R.J.	F	23	91.0	11.0
P.H.	M	24	89.0	25.0
S.E.	M	28	93.1	4.0
M.B.	M	20	11.2	-
B.P.	M	45	10.9	-
R.D.	M	31	88.9	14.0
S.B.	F	30	85.5	-
A.W.	M	29	9.0	-
E.B.	F	77	86.0	-
B.W.	M	82	74.0	-
D.I.	M	58	94.8	-
J.H.	F	23	88.6	-
S.D.	M	33	100.0	-
S.Ch.	M	26	89.0	-
S.E.	M	28	73.2	-
S.S.	F	20	-	12.0
M.H.	M	28	-	6.0
G.H.	M	24	-	11.0
C.D.	M	25	-	7.0
S.C.	M	23	-	10.0
D.J	M	23	-	15.8
C.B.	M	25	-	22.3

I.D.	repetition of samples in 25 mM bicarbonate buffer (std. buffer)
P.H.	83.0, 90.0, 88.8, 100 (25.0, 10.0, 15.1, 18.5, 25.5)
B.J.	89.0, 90.7, 100.0, 91.0, 85.0 (21.0, 11.0, 24.5, 27.8)
R.Y.	86.9, 92.4
M.F.	74.0, 64.5
G.F.	91.0, 100.0 (20.0, 21.0)
M.B.	85.5, 82.0
A.J.	14.9, 24.0
H.B.	(9.5, 8.5)
S.S.	(12.2, 24.4)

NB i) plasma samples collected at a single point in time have been repeated over time and ii) fresh plasma from the same subject has been collected and analysed at different times over the duration of this research project. Therefore, there is consistency in results with respect to sample, subject, and time.

APPENDIX 3

Community results

I.D.	sex	age	% Ga bound to peak 1 (Ga-Tf)	I.D.	sex	age	% Ga bound to peak 1 (Ga-Tf)
C1	F	40	93.0	C31	M	49	100.0
C2	M	19	92.0	C32	M	51	92.1
C3	F	42	18.0	C33	M	70	90.0
C4	M	15	13.0	C34	F	60	90.2
C5	F	18	16.0	C35	M	61	82.9
C6	M	40	25.0	C36	F	60	86.8
C7	F	42	30.0	C37	M	43	90.3
C8	M	49	27.0	C38	M	59	91.0
C9	F	46	28.0	C39	F	58	91.2
C10	F	46	100.0	C40	F	41	92.7
C11	F	64	100.0	C41	M	68	92.3
C12	F	39	100.0	C42	F	59	91.3
C13	F	22	100.0	C43	M	5	89.2
C14	M	21	100.0	C44	F	46	89.7
C15	M	64	93.0	C45	M	46	89.2
C16	M	45	92.0	C46	F	53	85.7
C17	M	38	91.0	C47	F	26	86.0
C18	F	36	93.0	C48	F	64	82.7
C19	M	53	100.0	C49	F	66	85.3
C20	F	45	100.0	C50	F	35	83.2
C21	-	-	-	C51	M	23	80.0
C22	F	32	88.2	C52	F	31	76.7
C23	F	21	87.2	C53	F	22	86.0
C24	-	-	-	C54	M	20	76.0
C25	M	16	90.9	C55	F	33	79.0
C26	M	45	89.7	C56	M	44	86.0
C27	F	46	88.8	C57	M	61	85.6
C28	F	19	87.2	C58	F	15	84.4
C29	M	20	80.6	C59	M	36	83.0
C30	F	52	88.3				

APPENDIX 4

Hospital control results

I.D.	sex	age	% Ga-Tf (HCO_3^-)	% Ga-Tf (std. buffer)	disease
W.H.	F	70	86.6	-	cervical myelopathy
H.S.	F	70	84.9	-	cervical myelopathy
R.T.	M	59	82.0	-	cervical spondylosis
E.P.	F	72	85.6	-	benign brain cyst
A.Mc.	F	50	85.7	-	frontal meningioma
I.D.	F	77	74.6	-	cerebrovascular cancer
R.C.	M	59	67.1	-	'turns' (prob. heart condition)
D.W.	M	64	63.3	-	multiple sclerosis
M.K.	F	55	38.0	-	benign brain tumour
S.M.	F	66	37.3	-	breast cancer
H.C.	M	62	20.8	7.5	epilepsy
A.R.	F	41	17.9	10.9	slipped disc
N.P.	M	57	90.8	-	multi system atrophy
E.G.	F	58	46.9	-	multi system atrophy
C.J.	F	64	91.4	-	motor neurone disease
B.M.	M	50	89.0	-	motor neurone disease
C.H.	F	46	85.9	-	motor neurone disease
J.C.	M	67	20.2	1.4	motor neurone disease
M.T.	F	49	22.9	-	motor neurone disease
W.R.	M	61	20.3	6.3	motor neurone disease

APPENDIX 5

Neonate results

I.D.	% Ga-Tf binding (std. buffer)	% Ga-Tf binding (HCO_3^- buffer)	% Fe Tf saturation	Fe (µg/l)	Tf (g/l)
C1	13.9	11.8	0.52	2100.0	2.86
I2	2.15	-	0.51	1462.4	2.05
K3	16.6	11.0	0.50	2052.8	2.95
C4	10.0	-	0.91	2881.8	2.25
N5	4.7	4.7	0.87	2601.6	2.13
B6	8.8	-	0.43	1355.2	-
B7	-	-	-	-	-
K8	-	-	2.02	6771.2	2.4
K9	-	-	-	-	-
G10	2.5	-	1.54	2929.6	1.36
B11	2.65	-	1.27	4411.2	2.49
C12	10.8	-	0.99	2476.0	1.79
S13	10.9	-	-	-	-
B14	6.4	-	0.77	2274.4	2.12
B15	8.9	-	1.20	3675.2	2.19
R16	0.84	1.95	0.79	2324.0	2.10
H17	19.1	-	0.60	2126.4	2.53
B18	2.24	-	0.80	2072.8	1.84
N19		-	0.32	1164.8	2.57
G20	-	-	1.14	2755.2	1.72
A21	7.9	-	1.93	4779.3	1.77
D22	7.6	-	0.44	1988.8	3.23
B23	-	-	0.89	3472.8	2.80
B24	4.2	-	0.72	1988.8	2.80
K25	4.8	-	1.07	2509.6	1.68
M26	9.5	-	-	-	-
B27	7.7	20.0	0.37	1164.8	2.27
B28	3.2	1.8	0.86	1652.0	2.27
O29	7.0	4.0	0.56	1556.8	2.00
B30	5.5	4.0	0.81	2286.0	2.01
P31	6.5	5.0	0.62	2144.8	2.47
C32	9.5	-	-	-	1.63
L33	6.8	-	0.68	1898.4	2.00
B34	13.0	16.4	0.55	2228.0	2.89
K35	13.4	10.6	0.80	2576.0	2.30
B36	6.0	-	0.86	1876.0	1.55
B37	10.9	-	0.92	2042.3	1.58
P38	2.0	-	1.20	1720.0	1.02
P39	2.5	-	1.56	3850.0	1.76
P40	5.0	-	0.80	1596.0	2.67
T41	1.0	-	0.73	1720.0	1.69
T42	5.0	-	0.82	1920.0	1.68
T43	3.0	-	0.92	1980.0	1.53
T44	8.0	-	-	-	1.50
T45	2.0	-	0.93	2150.0	1.66

APPENDIX 6

Parkinson's disease results

I.D.	sex	age	medication	% Ga-Tf (HCO_3^- buffer)	% Ga-Tf (std. buffer)
1) untreated P.D. patients					
W.W.	F	63	diclofenac	15.2	-
J.B.	M	63	insulin	15.2	-
M.H.	M	46	nil	14.2	-
M.U.	M	71	temazepam	17.4	-
D.M.	M	44	nil	21.6	-
M.R.	F	68	nil	25.9	19.8
J.G.	M	70	nil	17.9	-
E.S.	F	72	nil	22.1	-
F.S.	M	75	nil	30.3	-
E.P.	F	78	paracetamol	30.8	-
G.B.	M	46	nil	19.5	-
J.T.	M	49	adalat retard	17.0	-
R.A.	M	65	nil	9.1	-
L.H.	F	38	nil	28.0	10.6
B.S.	M	45	nil	17.3	20.4
B.H.	M	58	nil	30.6	14.0
H.B.	M	67	nil	30.7	-
C.T.	M	66	flucloxacillin	19.2	11.0
A.P.	M	68	beta blockers	9.0	-
D.T.	M	53		8.9	10.0
G.P.	M	63		21.6	6.0
W.H.	M	71	nitrazepam	25.3	-
J.W.	M	79		70.9	16.0
M.T.	F	72	nil	85.8	13.0
P.A.	M	58		46.0	16.0
P.B.	M	60		75.0	12.0
J.S.	M	75	nil	46.7	9.0
J.Sm.	M	61		65.0	6.0
E.C.	F	59	nil	82.0	15.0
J.Ta.	M	66		9.0	11.0
B.B.	M	51		40.0	10.0
E.M.	F	59	nil	39.2	11.0
2) treated P.D. patients					
J.P.	M	58	Madopar 125 ftd	76.0	-
J.C.	F	47	Selegiline 10 mg od	32.1	-
W.M.	F	52	Sinemet Plus	72.6	-
L.H.	F	38	Selegiline 10 mg od L-dopa	72.0	-
L.Ha.	F	56	Sinamet LS ttd	83.6	9.0
R.W.	M	56	Selegiline 5 mg od Sinemet Plus ttd	87.1	10.0
V.C.	F	58	Selegiline 5mg od Sinamet Plus	66.8	15.0
H.H.	F	65	Madopar	88.0	15.0
I.T.	F	72	Sinamet Plus ttd	76.8	12.0
R.C.	M	67	Selegiline 5mg td	73.9	14.0
M.M.	F	71	Sinamet LS ftd	73.0	9.0

od = once per day
td = twice daily
ttd = three times per day
ftd = five times per day

APPENDIX 7

Down's syndrome results

I.D.	sex	age	% Ga-Tf (HCO_3^- buffer)	% Ga-Tf (std. buffer)	dementia clinical	dementia A.B.S.*
F.R.	M	68	13.0	12.1	possible	yes
M.M.	M	24	11.2	7.6	no	possible
O.S.	F	70	6.6	8.0	possible	yes
R.B.	M	33	76.0	-	no	no
L.H.	F	44	32.3	11.0	no	no
A.J.	F	24	14.9	14.3	no	no
J.J.	M	21	37.9	11.5	no	no
P.B.	M	31	15.7	11.3	no	no
J.M.	M	42	11.3	6.0	possible	no
J.H.	F	67	60.7	10.6	possible	yes
P.D.	F	50	59.0	14.6	N/A	N/A
S.M.	F	35	43.0	8.0	N/A	N/A
M.C.	M	51	46.4	8.5	no	no
R.H.	M	70	39.3	12.7	yes	yes
M.S.	M	32	11.2	9.2	N/A	N/A
C.M.	F	24	60.3	9.0	N/A	N/A
J.C.	F	25	75.7	11.7	no	no
R.J.	M	25	69.4	10.8	no	no
R.T.	M	24	31.4	6.0	no	no
T.P.	M	30	44.6	4.0	no	no
A.L.	F	-	83.0	-	N/A	N/A
P.Bi.	F	-	84.0	-	N/A	N/A
R.S.	M	7	83.0	-	N/A	N/A
C.D.	M	29	87.5	-	N/A	N/A
J.Ma.	M	8	87.0	-	N/A	N/A
T.S.	M	9	68.0	-	N/A	N/A
C.B.	M	11	89.1	-	N/A	N/A
D.B.	M	24	84.2	-	N/A	N/A
B.D.	M	27	81.0	-	N/A	N/A
L.M.	M	14	85.0	-	N/A	N/A
G.D.	F	42	79.8	-	N/A	N/A
K.A.	F	15	82.5	-	N/A	N/A
B.K.	M	37	79.8	-	N/A	N/A
C.M.	F	23	89.0	-	N/A	N/A
S.Ma.	F	-	-	3.0	N/A	N/A
A.S.	M	-	-	5.3	N/A	N/A
A.W.	M	69	-	3.0	N/A	N/A
D.O.	M	49	-	11.0	N/A	N/A

ABS* = adaptive behaviour scale

APPENDIX 8

Affective disorder results

I.D.	sex	age	% Ga-Tf (pre)	% Ga-Tf (post)	No. ECT	pre HDS score	post HDS score	drugs
1) Unipolar, non psychotic responders to ECT								
A.K.	M	75	-	73.0	5	25	7	lofepramine
J.L.	F	72	68.8	93.8	9	28	9	lofepramine
J.W.	F	57	64.0	59.7	6	31	4	dothiepin
I.T.	F	80	49.5	48.1	9	51	3	lofepramine
E.S.	F	81	38.4	75.4	8	39	18	fluoxetine
S.P.	M	72	49.1	46.9	10	31	15	dothiepin
B.C.	F	72	26.8	53.0	8	32	3	lofepramine + lithium
2) Unipolar, psychotic responders to ECT								
R.H.	F	68	-	82.5	4	33	13	dothiepin + diazapam
D.H.	M	24	-	62.0	8	37	2	dothiepin
3) Unipolar, non psychotic, non responders to ECT								
T.G.	F	71	-	82.6	6	30	16	fluoxetine + diazapam
V.H.	F	71	-	90.3	8	34	22	fluoxetine + thioridazine, zimovane, and bendrofluazide
E.I.	F	75	-	83.4	4	31	21	fluoxetine
D.F.	F	37	-	91.9	12	37	21	imipramine
M.B.	F	80	79.0	76.5	8	33	31	fluoxetine
H.K.	F	65	87.0	79.1	6	38	23	lofepramine
4) Unipolar, psychotic, non responders to ECT								
E.W.	F	78	-	80.6	8	33	24	fluoxetine + thioridazine
J.S.	M	81	-	58.6	4	31	32	lofepramine
J.D.	M	76	-	84.3	12	46	26	lofepramine
5) Bipolar, psychotic responder to ECT								
F.A.	M	32	88.4	75.4	10	23	21	amitriptyline
6) Bipolar, non psychotic, non responder to ECT								
A.D.	F	83	22.4	84.8	4	43	10	lofepramine thioridazine and rifampicin

% Ga-Tf refers to plasma Ga-Tf binding in the presence of 25 mM bicarbonate
HDS: Hamilton depression score, response defined as > 50% reduction of HDS
pre and post refers to before and after ECT

lofepramine, dothiepin, amitriptyline, and imipramine are tricyclic antidepressants
fluoxetine: a 5-HT specific antidepressant
diazapam: a benzodiazepine tranquillizer
thioridazine: a (neuroleptic) major tranquillizer
rifampicin: an antibiotic used to treat tuberculosis
lithium: (administered as lithium carbonate)
bendrofluazide: a diuretic
zimovane: a hypnotic

APPENDIX 9

Alzheimer's disease results

I.D.	sex	age	% Ga-Tf (HCO_3^- buffer)	% Ga-Tf (std. buffer)
M.C.	F	84	68.0	21.0
C.D.	F	73	87.3	-
D.T.	F	71	64	-
R.T.	M	79	42.4	-
J.Lm.	M	86	30.5	2.0
A.K.	F	74	82.0	6.0
A.G.	M	69	24	10.5
G.H.	M	69	21	6.0
J.Le.	M	82	87	-
K.S.	F	76	85.0	5.2
F.C.	F	69	89.0	2.0
S.W.	M	-	75.0	1.2
I.D.	F	-	85	-
J.J	M	80	31.9	-
I.T.	F	68	92.1	-
G.L.	M	71	100	-
J.P.	M	56	90.6	-
G.I.	F	56	32.7	-
H.G.	M	-	91.4	-
C.E.	M	58	100.0	-
E.S.	F	90	-	4.0
J.S.	M	-	-	7.0
M.H.	F	-	-	5.0
M.F.	F	-	-	5.0

APPENDIX 10

Bicarbonate alteration

I.D.	bicarbonate concentration (M)						
	0	2.5×10^{-3}	0.01	0.025	0.05	0.1	0.25
G.F.	20.0	67.0	91.0	91.0	93.0	100.0	79.0
H.B.	9.5	66.0	91.0	90.0	91.0	100.0	78.0
P.H.	10.0	38.0	63.0	83.0	90.0	84.0	83.0
B.J.	11.0	58.0	86.0	85.0	91.0	87.0	78.0
M.Hi.	19.0	31.0	40.0	89.0	72.0	34.0	48.0
K.H.	13.0	22.0	74.0	83.0	71.0	83.0	63.0
mean	13.75	47.0	74.2	86.89	84.7	81.33	71.5
± S.D.	4.6	19.2	20.0	3.6	10.3	24.4	13.4

results are expressed as a percentage of Ga bound to Tf. Ga speciation was performed on a G75 gel-filtration chromatography column at pH 7.4 with varying concentration of bicarbonate.

APPENDIX 11

pH alterations

I.D.	pH of the elution buffer, column and plasma						
	6.0	6.5	7.0	7.4	7.7	8.0	8.3
P.H.	52.4	81.4	73.0	83.0	-	82.5	-
B.J.	63.1	82.5	88.1	85.0	88.3	72.0	73.2
S.T.	58.0	76.5	87.5	-	90.4	79.3	71.0
R.Y.	60.0	86.7	84.5	-	90.4	100.0	82.0
M.Hi.	36.0	-	-	89.0	-	51.0	-
C.D.	72.2	-	-	-	88.5	88.7	62.5
J.L.	-	86.0	-	-	-	-	-
J.M.	-	85.5	-	-	-	-	-
M.P.	-	82.0	-	-	-	-	-
K.H.	-	85.7	84.2	83.0	-	-	-
R.D.	-	-	-	-	87.0	-	-
G.F.	-	-	-	91.0	71.0	-	-
S.E.	-	-	-	93.1	-	-	33.0
M.F.	-	-	-	-	-	-	70.7
P.H.2	-	83.4	-	-	-	-	-
R.Y.2	-	88.3	-	-	-	80.8	-
J.L.2	-	85.5	-	-	-	-	-
S.T.2	-	-	-	-	-	92.0	-
C.D.2	-	-	-	-	-	64.4	-
B.J.2	-	-	-	-	-	83.5	-
mean	56.9	83.9	83.5	87.4	85.9	79.4	65.4
± SD	12.2	3.3	6.1	4.3	7.4	14.1	17.1

results are expressed as a percentage of Ga bound to Tf. Ga speciation was performed on a G75 gel-filtration chromatography column in the presence of 25 mM bicarbonate with varying pH of the column, buffer, and plasma.

APPENDIX 12

Fe chloride additions to plasma

I.D.	Fe chloride concentration (M) added to plasma			
	10^{-4}	8.3×10^{-5}	6.6×10^{-5}	5×10^{-5}
P.H.	1.9	3.2	1.6	2.1
S.T.	1.6	2.0	2.2	1.3
R.Y.	2.0	2.2	1.8	2.2
J.L.	-	3.8	1.3	1.8
J.M.	-	1.9	1.7	1.3
K.H.	-	2.9	1.8	1.4
B.J.	1.9	-	-	-
C.D.	1.7	-	-	-
G.F.	2.7	-	-	-
mean ± SD	2.0 ± 0.4	2.6 ± 0.8	1.7 ± 0.3	1.7 ± 0.4

I.D.	Fe chloride concentration (M) added to plasma			
	3.3×10^{-5}	1.6×10^{-5}	1.3×10^{-5}	8.3×10^{-6}
P.H.	3.1	6.8	-	80.9
S.T.	1.3	29.8	83.4	80.2
R.Y.	7.8	50.1	78.8	85.4
J.L.	1.5	9.5	74.2	75.9
J.M.	1.3	19.3	-	81.5
K.H.	6.7	27.2	56.0	80.3
B.J.	-	-	71.3	-
mean ± SD	3.6 ± 3.0	23.8 ± 15.8	71.7 ± 10.4	80.7 ± 3.0

results are expressed as a percentage of Ga bound to Tf. Ga speciation was performed on a G75 gel-filtration chromatography column in the presence of 25 mM bicarbonate at pH 7.4

APPENDIX 13

Al nitrate additions to plasma

I.D.	Al nitrate concentration (M) in plasma				
	10^{-4}	6.6×10^{-5}	3.3×10^{-5}	1.3×10^{-5}	8.3×10^{-6}
P.H.	11.1	16.9	22.9	37.3	67.9
R.Y.	10.6	25.7	48.0	77.5	75.4
J.L.	17.8	29.8	80.7	82.2	80.3
M.P.	6.2	5.8	17.6	34.7	62.5
S.S.	5.7	11.8	17.9	71.4	79.4
S.T.	14.0	26.9	43.8	70.8	77.4
mean ± S.D.	10.9 ± 4.6	19.5± 9.5	38.5 ± 24.5	62.3 ± 20.8	78.3 ± 16.0

results are expressed as a percentage of Ga bound to Tf. Ga speciation was performed on a G75 gel-filtration chromatography column in the presence of 25 mM bicarbonate with varying pH of the column, buffer, and plasma.

APPENDIX 14

Ga nitrate additions to plasma

I.D.	Ga nitrate concentration (M) in plasma				
	10^{-4}	6.6×10^{-5}	3.3×10^{-5}	1.3×10^{-5}	8.3×10^{-6}
P.H.	6.3	13.9	24.2	49.1	56.5
R.Y.	6.9	45.2	60.7	89.9	89.6
J.L.	9.5	21.4	49.2	77.6	61.7
M.P.	4.8	14.5	22.0	31.5	93.4
S.S.	5.0	21.2	31.3	70.6	91.6
S.T.	11.7	23.4	37.5	64.6	77.1
mean $\pm$ SD	7.4 ± 2.8	23.3 ± 11.4	37.5 ± 15.0	63.9 ± 20.8	78.3 ± 16.0

results are expressed as a percentage of Ga bound to Tf. Ga speciation was performed on a G75 gel-filtration chromatography column in the presence of 25 mM bicarbonate with varying pH of the column, buffer, and plasma.

APPENDIX 15

Mg chloride additions to plasma

I.D.	Mg chloride concentration (M) in plasma	
	10^{-4}	5×10^{-5}
P.H.	76.3	78.9
R.Y.	77.8	83.9
J.L.	82.0	86.8
M.P.	66.3	75.6
S.S.	64.8	81.4
S.T.	85.7	-
mean $\pm$ SD	75.5 ± 8.4	81.2 ± 4.3

results are expressed as a percentage of Ga bound to Tf. Ga speciation was performed on a G75 gel-filtration chromatography column in the presence of 25 mM bicarbonate with varying pH of the column, buffer, and plasma.

APPENDIX 16

Tf, Fe, AND FERRITIN RESULTS

I.D.	sex	age	disease	% Ga-Tf binding	Fe (µg/l)	Tf (g/l)	TIBC (µg/l)	Tf Fe sat.	ferritin (µg/l)
M.H.	M	46	PD	14.2	1243.779	2.19	3074.312	.405	167
D.M.	M	44	PD	21.6	568.553	1.27	1782.82	.319	74
E.P.	F	78	PD	30.8	409.939	3.706	5202.466	.079	16
B.S.	M	45	PD	17.3	1144.925	2.17	3046.236	.376	62
H.B.	M	58	PD	30.6	833.282	2.45	3439.299	.242	199
W.W.	F	63	PD	25.3	424.46	3.16	4435.993	.096	48
J.B.	M	63	PD	15.2	796.421	2.9	4071.007	.196	25
H.S.	F	70	con	84.9	1033.225	2.55	3579.678	.289	98
R.T.	M	59	con	82	1144.925	2.23	3130.464	.366	264
W.H.	F	70	con	86.6	346.27	2.84	3986.779	.087	39
A.Mc.	F	50	con	85.7	715.439	2.08	2919.894	.245	94
E.G.	F	59	con	46.9	729.401	1.16	1628.403	.448	176
C.H.	F	46	con	85.9	928.227	2.04	2863.743	.324	99
R.C.	M	59	con	67.1	1111.974	2.56	3593.716	.309	97
H.C.	M	62	con	20.8	822.112	1.36	1909.162	.431	79
M.K.	F	55	con	38	1162.239	2.47	3467.375	.335	115
A.R.	F	41	con	17.9	1105.271	1.65	2316.262	.477	19
B.M.	M	50	con	89	987.428	2.29	3214.691	.307	132
I.D.	F	77	con	74.6	528.341	1.55	2175.883	.243	> 300
M.T.	F	49	con	22.9	866.233	2.31	3242.767	.267	77
E.P.	F	72	con	85.6	351.855	2.07	2905.856	.121	248
S.M.	F	66	con	37.3	163.641	2.19	3074.312	.053	96
C1	F	40	con	92.8	•	•	•	.08	
C2	M	19	con	91.9	•	•	•	.24	
C3	F	42	con	18.3	•	•	•	.31	
C4	M	15	con	12.8	•	•	•	.49	
C5	F	18	con	16.2	•	•	•	.34	
C6	M	40	con	24.7	•	•	•	.19	
C7	F	42	con	29.6	•	•	•	.19	
C8	M	49	con	27.3	•	•	•	.2	
C9	F	46	con	27.9	•	•	•	.36	
C10	F	46	con	100	•	•	•	.19	
C11	F	64	con	100	•	•	•	.27	
C12	F	39	con	100	•	•	•	.31	
C13	F	22	con	100	•	•	•	.29	
C14	M	21	con	100	•	•	•	.36	
C15	M	64	con	93	•	•	•	.25	
C16	M	45	con	91.6	•	•	•	.33	
C17	M	38	con	90.5	•	•	•	.36	
C18	F	36	con	92.5	•	•	•	.12	
C19	M	53	con	100	•	•	•	.3	
C20	F	45	con	100	•	•	•	.25	