THE DEVELOPMENT AND APPLICATION OF TRACE METAL

ANALYSIS IN CLINICAL CHEMISTRY

by

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The author graduated with Honours in Chemistry at the
University of Manchester in 1983. Since October 1983 the
author has been engaged in full time research under the
supervision of Dr. J.P. Day.

No portion of the work referred to in this thesis has been
submitted in support of an application for another degree or
qualification of this or any other university or other
institute of learning.

ACKNOWLEDGEMENTS

To My

Parents

Plate 1. Fast Protein Liquid Chromatography System. (Pharmacia Ltd.). See Chapter 8.
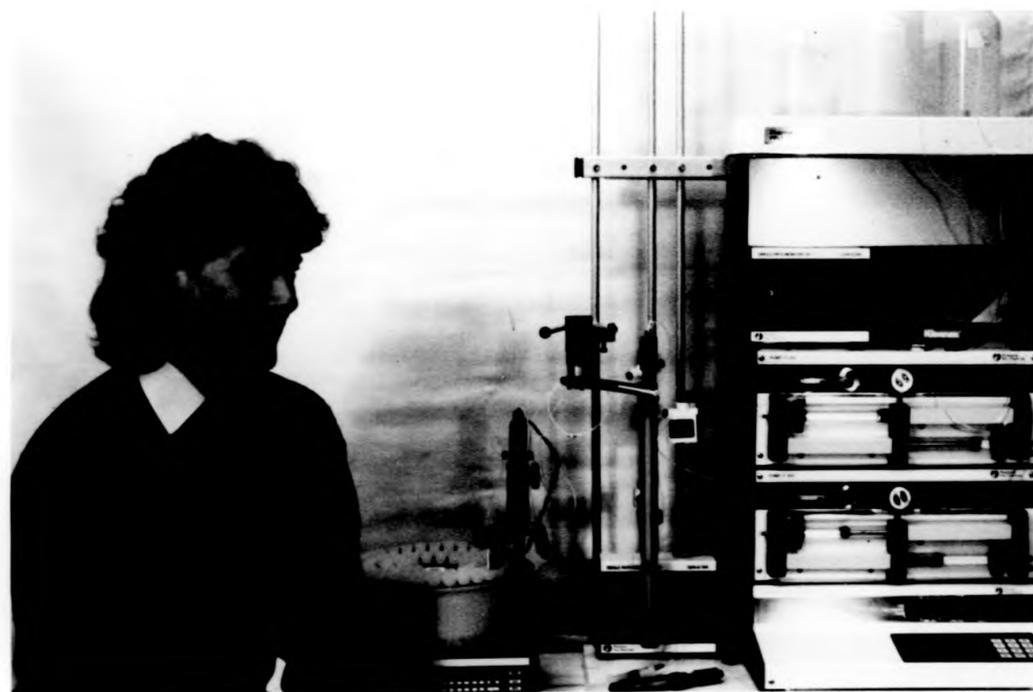
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We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time.
                    T.S. Eliot 1888-1965.

ABSTRACT.

This thesis reports studies related to the toxicology of
aluminium in renal dialysis patients and preterm infants.
Four areas have been investigated experimentally.

1.   Analytical methodology for patient aluminium status. -
Patient monitoring for aluminium exposure is traditionally
carried out by serum/plasma aluminium determination.
Aluminium accumulation can be estimated by the analysis of
bone tissue, obtained by biopsy. Both analyses have
advantages and disadvantages, and these are reviewed. The
potential for useful analysis of hair is then investigated
experimentally. A straightforward and reproducible method is
developed and described for hair collection, pretreatment and
analysis, although no correlation was found between hair and
serum aluminium or copper levels in renal patients or
controls.

A methodology was also developed for the determination of Al
and Cu in erythrocytes, which is particularly suitable for
the small samples obtained in paediatric work.

2.   Mobilisation of Al, Fe and Cu by desferrioxamine therapy.
Aluminium removal from patients is universally effected by
desferrioxamine (DFO) chelation therapy. Three clinical
studies are reported in which the mobilisation and removal of
aluminium (in vivo) during DFO therapy is characterised,
through the analysis of patients' serum, red cells, bone,

urine, faeces and (where appropriate) dialysis solutions from
the kidney machines. Iron and copper are measured in the same
substrates to determine their removal in patients treated
with DFO, in cases both with and without iron overload. The
effect of DFO therapy on aluminium induced anaemia and the
probable interaction with iron and copper biochemistry,is
also reported.

3.   Aluminium in preterm infants. - Intravenous solutions for
total parenteral nutrition (TPN), and milk formula feeds
given to perterm infants, contain aluminium. Infants on TPN,
who are exposed to higher levels of aluminium than infants
fed on milk formula. The former have raised serum aluminium
levels and calculations suggest that this intake from TPN is
potentially much more serious than from oral feeds. Two
infants who had received prolonged TPN were found to have
high tissue aluminium levels at post mortem.

4.   Serum protein separation. - A method is described for the
separation of serum plasma proteins by high performance
liquid chromatography, using both molecular exclusion and ion
exchange. Separation by anion exchange chromatography enabled
direct metal analysis of protein fractions and allowed
elucidation of the probable identity of the metal-carrier
proteins. Transferrin is the major (probably the only) serum
protein which binds aluminium.

The thesis includes an extensive review of the toxicology of
aluminium, and ends with a detailed discussion of the
interaction between the biochemistry of aluminium, iron and

copper particularly in relation to the metabolism of red cells and the problems of microcytic anaemia in renal patients.

CONTENTS.

NORMAL RANGES OF METALS IN HUMAN TISSUE.

		ALUMINIUM	COPPER	IRON
BLOOD (μg/l)	Total	-	1010 640-1280	-
	Erythrocytes	2-10	890 720-1140	-
	Plasma	2-10	1100 790-1410	750-1750
BONE (μg/g)	Whole	-	1-25	110-170
	Marrow	< 20	-	-
	Trabecula	< 20	-	-

OTHER NORMAL VALUES.

		MEAN	RANGE
HAEMOGLOBIN	g/dl	15.9	13.4-17.3
CAERULOPLASMIN	g/l	0.38($\pm$0.08)	
UREA	nmoles/l		2.5-7.5
CREATININE	μmoles/l		30-80
GFR	ml/min/1.73m^2		50-85

Principal Abbreviations Used in the Text.

AAS	Atomic Absorption Spectroscopy.
CAPD	Continuous Ambulatory Peritoneal Dialysis.
Cp	Caeruloplasmin.
CRF	Chronic Renal Failure.
CSF	Cerebral Spinal Fluid.
DE	Dialysis Encephalopathy.
DFO	Desferrioxamine.
Dx	Dialysis.
EEG	Electroencephalogram.
EDTA	Ethylenediaminetetraacetic acid.
Fn	Ferritin.
FO	Ferrioxamine.
FPLC	Fast Protein Liquid Chromatography.
FRO	Fracturing Renal Osteodystrophy.
GFR	Glomerular Filtration Rate.
GFAAS	Graphite Furnace Atomic Absorption Spectrophotometry.
IAEA	International Atomic Energy Association.
Hb	Haemoglobin.
Hct	Haemocrit.
LS	Liquid Scintillation Counting.
MA	Microcytic Anaemia.
M.Wt.	Molecular Weight.
OM	Osteomalacia.
PTH	Parathyroid Hormone.
iPTH	Active Parathyroid Hormone.
RBC	Red Blood Cell (Erythrocyte).
RE	Reticuloendothelial Cell.
Tf	Transferrin.
TMAH	Tetramethylammonium hydroxide.
TPN	Total Parenteral Nutrition.

PREFACE.

Three diseases of renal failure, namely dialysis
encephalopathy, fracturing renal osteodystraphy, and
hypochromic microcytic anaemia, are known to be associated
with the accumulation of toxic quantities of aluminium. Since
1978, the research group at the Department of Chemistry has
collaborated with the Renal Unit at Withington Hospital, and
other Greater Manchester Hospitals, in research to understand
the cause of aluminium toxicity, the routes of aluminium
exposure, and techniques for aluminium detoxification.

As a result of this collaborative work, aluminium exposure,
through haemodialysis, medication, and other routes, has been
progressively reduced, to the extent that the aluminium-
related diseases are now relatively rare in Manchester Renal
Units. Additionally, there was no effective treatment for the
diseases associated with aluminium overload until 1979, when
patients with dialysis encephalopathy at the Artifical Kidney
Unit, Withington Hospital, successfully began treatment with
desferrioxamine, a chelating agent usually used in the
treatment of iron overload. This drug is now used worldwide.

The objective of the present research was to study the
transport and storage of aluminium in patients in aluminium
overload in vivo, including during desferrioxamine treatment.
A similarity between aluminium and iron biochemistry is
apparent, and this research examines the interaction between

aluminium, iron and copper in the body, particularly the effect of chelation therapy on these metals. The results of this work are of particular relevance to the causes of aluminium induced microcytic anaemia.

This thesis is written so that each of the chapters is self-contained and can be read independently of one another. This has therefore necessitated a certain amount of repetition, particularly in the reporting of the clinical case studies. The background to the work is discused in Chapter 1, and many of the experimental details are presented in Chapter 2. Chapters 3 to 8 cover various specific topics, some of which are clinically related case studies. Chapter 9 examines the underlying biochemistry of aluminium, copper and iron revealed by the preceeding experimental work.

CHAPTER 1.

INTRODUCTION AND SURVEY OF LITERATURE.

ALUMINUM TOXICOLOGY.

1. INTRODUCTION.

Aluminium is the third most abundant element in the earth's crust, occurring naturally in aluminosilicates, oxides and hydroxides in rocks and soils. Aluminium is a member of group III of the periodic table, and in aqueous, environmental and biological systems is stable only in the oxidation state +3 (1).

The concentration of aluminium in most natural fresh water is below 20 µg/l. When the pH of run-off water is consistantly below 5 concentrations of aluminium may increase to > 100 µg/l. These conditions may result from industrial waste water, mine drainage or natural phenomenan such as acid spring waters in volcanic regions (2). However, because aluminium sulphate is widely used as a flocculant in the treatment of drinking water supplies, the level of aluminium in domestic water may range up to several 1000 µg/l (3).

The first documented case of aluminium poisoning was reported in 1921 (4). The patient, a bauxite worker, had loss of memory, jerking movements, incoordination and marked tremor. Another case of progressive encephalopathy ascribed to aluminium toxicity was reported in 1962. The patient had been exposed to heavily aluminium-contaminated dust for a long period of time while working in an aluminium-powder factory (5). However, the potential toxicity of aluminium was

dismissed in early reviews in 1957 and 1974 (6, 2), and it was concluded that industrial exposure through inhalation of heavily aluminium-contaminated dust particles was the only potential source which might present a health hazard.

In health, aluminium is poorly absorbed through the gastrointestinal tract, resulting in a low body-burden of the metal. Furthermore, the amount of aluminium absorbed depends on the other dietary components, for example, the presence of fluoride in the diet increases the faecal and urinary excretion of aluminium and decreases its retention in the body (7, 8).

The kidneys are the main excretory organ for aluminium (10-13). Kovalchik (13) reported that the kidneys comprise the major route for excretion of aluminium, whilst biliary excretion plays only a minor role. He observed that biliary excretion increases in cases of aluminium overload, but even so accounts for only 0.1% of the total flux.

Whereas aluminium may not be toxic in relation to normal environmental exposure and renal function, toxicity may result in patients with chronic renal failure (CRF) (14-16). Uraemic patients with reduced or non-existant renal function may also be exposed to increased levels of aluminium as a result of medication, and of the use of kidney machines for dialysis.

Increased serum aluminium levels in CRF were first observed

by Berlyne et al in 1970 (10). One year later Parsons et al (17) reported raised bone aluminium levels in uraemic patients. Bone aluminium concentrations tended to be higher in patients who had been in renal failure or on dialysis the longest.

Clarkson et al (18) carried out an aluminium balance study in patients with CRF receiving aluminium-containing phosphate-binding gels. A positive aluminium balance was observed in uraemic patients which was not observed in the control study.

In 1972 (19) Alfrey reported a syndrome of dyspraxia and seizures associated with chronic haemodialysis. The 5 patients studied developed a progressive encephalopathy that had a characteristic course. Typically the patients' speech became slow and deliberate with stuttering. During the first few months the speech problem was intermittent and was usually more apparent during dialysis or immediately post dialysis. Subsequently dyspraxia of speech developed, which was associated with an abnormal electroencephalogram. After 3 months the encephalopathy progressed, characterised by marked tremour, myoclonus, and dyspraxia of movement which appeared to be aggravated by dialysis. The patients developed severe loss of memory (both recent and long term), and were unable to concentrate. The patients were unable to walk or feed themselves. Four of the five patients died, and the fifth patient had a renal transplant. The cause of this syndrome was not determined, enhanced aluminium was not observed in

brain or other tissue. Tin was suggested as a possible
causative agent, but subsequent research suggests that this
was the first fully documented case of aluminium
encephalopathy.

People became more conscious of the potential toxic effects
of aluminium in 1976. Alfrey et al (20) reported high brain
aluminium levels at autopsy of haemodialysis patients with
dialysis encephalopathy (DE). Ward et al (21) also observed a
characteristic neurological syndrome in 14 patients,
undergoing haemodialysis. Thirteen patients developed
associated renal osteomalacia. All patients had ingested
aluminium hydroxide and were dialysed in an area where the
level of aluminium in domestic water was high. Aluminium in
brain grey matter was found to be significantly elevated.

2.   ALUMINIUM EXPOSURE IN CHRONIC RENAL FAILURE.

Renal patients are exposed to increased amounts of aluminium
from a number of different sources; the major sources of
exposure are aluminium-containing phosphate binding gels and
aluminium transfer from the dialysate to the patient during
dialysis.

2.1.   Aluminium from Phosphate-binding Gels.

Aluminium-containing phosphate binding gels (antacids) are
administered to uraemic patients to control the absorption of

phosphate·from the stomach, and thus prevent uraemic hyperphophataemia, hypercalcaemia and consequent secondary hyperparathyroidism.

These materials are made of aluminium hydroxide ("Aludrox" or "Alucaps") or trisilicate and taken in tablet or liquid form. This medication is prescribed from the early stages of chronic renal failure, prior to any dialysis treatment. The dose given varies, patients take between 4-12 g/day Al(OH)₃ which is equivalent to 1-3 g/day of aluminium.

Berlyne et al (10) reported raised serum aluminium levels in dialysis patients receiving 3.6g Al(OH)₃ a day and suggested that the use of aluminium salts and resins should be avoided until the possible toxic effects of hyperaluminaemia was understood.

Clarkson et al (18) carried out balance studies on eight patients with chronic renal failure who received between 1.5 and 3.4g aluminium daily. Patients absorbed between 100-568mg of aluminium per day, and plasma aluminium concentrations increased in eight patients.

The amount of aluminium excreted in urine and faeces was measured by Gorsky (22) in people receiving aluminium from Al(OH)₃ tablets. Gorsky found approximately 99% faecal excretion in both normal and positive aluminium loading.

There have been several cases reported (21, 23-26), where

encephalopathy has developed and Al(OH)$_3$ tablets have been the only source of exposure. Withdrawal of the antacids resulted in symtomatic improvement.

Masselof et al (23) reported the disappearance of neurological symptoms of DE in a patient who stopped taking Aludrox. The patient had ingested between 1-3 g/day aluminium for 43 months while on dialysis, a cumulative dose of ca. 2800g of aluminium. After withdrawal of Aludrox, the serum aluminium level fell from 230 to 121 µg/l in one month.

In 1980 (24) a fatal case of DE was reported in a non-dialysed uraemic boy treated with Al(OH)$_3$ for four years. The boy had received an estimated 1200g of aluminium. Post mortem analysis of brain showed high levels of aluminium in grey matter and white matter of 80 µg/g and 47 µg/g, respectively.

Another case (27) in 1983 reported encephalopathy and vitamin D resistant osteomalacia in a nondialysed uraemic child, treated with Al(OH)$_3$. At 31 months the child had received an estimated 545g aluminium and had a blood aluminium level of 334 µg/l.

In a recent study, Brahm (28) compared serum aluminium in non-dialyzed chronic uraemic patients before and during treatment with aluminium-containing phosphate-binding gels. The study showed that serum aluminium levels increased rapidly after the start of Al(OH)$_3$ treatment, and that serum

aluminium varies with the daily dose of Al(OH)₃.

Fournier et al (29) reported a significant increase in plasma aluminium levels in patients on dialysis with low dialysate aluminium taking Al(OH)₃ while on l-alpha-hydroxycholecalciferol therapy. The increase in plasma aluminium was correlated with cumulative dose of Al(OH)₃.

A number of alternative phosphate-binders have been tested for use in chronic renal failure. Schneider et al (30) developed an aluminium-free phosphate-binder of natural polymers consisting of heteropolyuronic acid charged with different cations. In vitro experiments showed an efficiency two to three times greater than Aludrox, clinical studies showed no serious side effects, and serum phosphate was maintained at an acceptable level.

O'Donavan et al (31) substituted aluminium hydroxide with magnesium carbonate as a phosphate-binder. After 24 months pre dialysis serum aluminium concentration had fallen significantly, with no siginificant change in pre dialysis serum phosphate. This report suggests that aluminium containing antacids are unnecessary for the control of dialysis hyperphosphataemia and that magnesium carbonate may be an alternative, less toxic, binding gel. It seems unlikely that the only source of aluminium exposure in CRF is aluminium-containing antacids since not all dialysis centres observed cases of DE. In fact many patients who have taken

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antacids for years do not develop neurological symptoms (17, 32, 33).

2.2. Aluminium from Dialysate Fluid.

There have been number of reports concerning the transfer of aluminium from aluminium-contaminated dialysis fluid to the patient during haemodialysis (19, 20, 32-46) and continuous ambulatory peritoneal dialysis (CAPD), (47-50).

Alfrey et al (19), who first reported cases of DE, suggested that the sudden appearance of the syndrome indicated that the toxin came from the untreated tap water used for dialysis.

Platts et al (34) reported 3 fatal cases of DE of patients who had dialysed against untreated water in Sheffield, but no cases of DE in patients who used water treated by cation-exchange water softeners. This seems to support Alfrey's view of a water born toxin indicating that patients should not be dialysed against untreated tap water.

In 1976, four reports were published on dialysis dementia (20, 21, 35, 36). Platts and Hislop (35) reported 10 cases of encephalopathy in the Sheffield region, levels of aluminium in water ranged between 40-480 µg/l but the authors concluded that the role of aluminium in DE and patholgical fractures was not proven.

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Alfrey et al (20) reported high aluminium content of muscle,

bone and brain in dialysed CRF patients receiving oral aluminium. The aluminium content of water was found to be negligable with no net transfer of aluminium across the dialyser.

Flendrig et al (36), and Ward et al (21) reported high levels of aluminium in dialysate of patients with DE. In Flendrig's unit the dialysate levels were between 800-1000 $\mu g/l$. This high level of contamination resulted from two aluminium anodes present in a boiler. The anodes, which weighed 32.4kg, disappeared completely within about 2 years. In Newcastle (21) the aluminium content of untreated tap water was 180 $\mu g/l$, and three patients dialysed at home where aluminium content was <400 $\mu g/l$. They found that aluminium was not efficiently removed by water softeners, probably because aluminium was present as colloids or as fluorosilicates.

Platts et al (32) surveyed 202 patients undergoing home dialysis in the Trent region. In all cases of DE the tap water contained high levels of aluminium (<328 $\mu g/l$) compared to the unaffected patients (<24 $\mu g/l$).

Kaehny et al (11) showed that aluminium was readily transferred from dialysate to plasma during dialysis even in cases where plasma aluminium exceeded dialysate concentration, indicating that aluminium is strongly bound to a nondialysable plasma constituent, which precludes the removal of aluminium from the patient during dialysis.

Further papers were published in 1978 (33, 37-39) supporting the evidence for high levels of aluminium in dialysate causing DE in renal failure.

Parkinson et al published a survey of aluminium levels in water supplies to dialysis patients in the UK (40). The survey includes 1293 patients, of whom 71 had DE and 208 fracturing renal osteodystrophy. Parkinson found a significant correlation between mean aluminium content of water and the incidence of DE (r = 0.69) and fracturing renal osteodystrophy (r = 0.55) and recommended a safe level of aluminium in water of 20 µg/l.

Most of the evidence indicates that aluminium is the cause of DE and fracturing renal osteodystrophy. Dialysis fluid is a major source of aluminium, the aluminium originating in the tap water and not from the dialysate concentrate.

Pretreatment of tap water by reverse osmosis and/or deionisers significantly reduces the level of aluminium to <5 µg/l. Most patients now use reverse osmosis or deionisers to treat their water. This is particularly important in cities known to have high tap water aluminium levels (Manchester, Newcastle, Sheffield, Leeds, Plymouth, Glasgow). Deionisers were installed in Plymouth as early as 1974, and since then they have reported only one new case of DE.

In 1982 several papers were published showing a definate correlation between the incidence of DE and fracturing renal

oseodystrophy and the level of aluminium in dialysate (43-45) and more recently by Platts et al (46). Davison et al (43) found an exponential relation (p <0.01) between the mean aluminium concentration in water used for dialysate and the time taken to die from dementia.

Cases of DE and aluminium transfer to patient have also been reported in CAPD patients (47-50). Smith et al (47) reported a case of DE in a patient after 3 years CAPD treatment, serum aluminium 244 µg/l. The patient died within 1 year of the first signs of dementia, having suffered speech difficulty and myoclonic twitches of the face.

In 1982 Cumming et al (49) observed an acute rise in plasma aluminium in fourteen patients who were routinely monitored. The acute aluminium intoxication in these patients was caused by contaminated batches of CAPD fluid. In one patient, the plasma aluminium level increased from ca. 100 to 1500 µg/l. After 5 hours haemodialysis with reverse osmosis treatment, plasma aluminium was reduced to 1000 µg/l with marked symptomatic improvement.

Rottembourg et al (50) showed that in patients treated by CAPD a positive or negative transfer of aluminium through the peritoneum was observed in relation to the levels of aluminium in dialysate and serum. In patients with high serum levels large amounts of aluminium were removed through the peritoneal route.

Hodge et al (51) showed that the rate of aluminium transfer between patient and dialysate was related to the aluminium concentration in the dialysate and bore no relation to the level of aluminium in serum. They concluded that, to ensure no aluminium uptake by the patient, the aluminium concentration in dialysate should be below 14 μg/l.

2.3. Other Sources of Aluminium Exposure

Aluminium accumulation in chronic renal failure may also result from a number of other sources such as :

(i) The contamination of blood products e.g. albumin replacement solutions (52) and sterile plasma protein solutions and coagulation factors such as factor VIII (53).

Fell et al (53) found aluminium concentration of 491 ± 159 μg/l in 73 sterile plasma protein solutions, tested and between 1000 and 3000 μg/l aluminium in factor VIII solution;

(ii) High levels of aluminium have also been reported in a variety of intravenous fluids used in total parenteral nutrition (TPN) (53-55). Several papers have been published identifying aluminium overload in TPN (56-59). For example Klein et al (56) and Ott et al (57) found high levels of aluminium in bone resulting from the use of casein in TPN. Overall, aluminium appears to be an important pathogenic factor in osteodystrophy of patients receiving dialysis and TPN.

In 1984, Klein (58) observed high aluminium levels in five children receiving long-term total parenteral nutrition. The authors suggested that aluminium deposition might play a role in its pathogenesis or exacerbate the course of liver dysfunction associated with TPN.

Sedman et al (59) reported high plasma aluminium levels in preterm infants who received intravenous therapy, 36.8 ± 45.3 $\mu g/l$ compared with normal controls of 5.2 ± 3.1 $\mu g/l$. Bone aluminium concentration in autopsy specimens from 23 infants including 6 who had received more than 3 weeks intravenous therapy showed 10 times more aluminium in infants who had received three weeks therapy, 20.16 ± 13.4 mg/kg, compared with limited intravenous therapy, 1.98 ± 1.44 mg/kg dry weight.

(iii) The dietary content of aluminium also contributes to the level of exposure and hence absorption of the metal from the gut. The level of aluminium in the diet varies from one individual to another. High levels of aluminium have been found in tea, maize, soya, and canned drink.

Other dietary constituents may affect the absorption of aluminium through the gastrointestinal wall. Slanina (60) studied serum aluminium levels in 10 healthy men during three seven-day experiments when they received:(a) citric acid (as lemon juice): (b) $Al(OH)_3$ and (c) $Al(OH)_3$ + citric acid. Serum aluminium levels increased from 5 ± 3 $\mu g/l$ to 9 ± 4

µg/l and 12 ± 3 µg/l after ingestion of citric acid only and
Al(OH)₃ only. Serum aluminium levels increased to 23 ± 2 µg/l
after ingestion of Al(OH)₃ and citric acid. These findings
suggest that aluminium absorption is greatly increased in the
presence of citric acid, probably due to the formation and
absorption of Al-citrate complexes at acid pH.

Cannata et al (61) studied three groups of patients with
"low-normal", "normal", and "high" serum ferritin. All
patients received 2.8 g/day Al(OH)₃ during the trial period.
Serum aluminium levels increased proportionally with
increased Al(OH)₃ intake in patients with "low-normal" and
"normal" serum ferritins. Patients with high serum ferritin
did not show any change in serum aluminium. These findings
were taken to indicate that in patients with high iron
stores, whose daily iron absorption is low, aluminium
absorption is also low, and vice versa. Therefore, a "common
pathway" of iron and aluminium is implicated and serum
ferritin levels might be a useful predictor of aluminium
absorption.

3. ALUMINIUM INTOXICATION.

Aluminium overload in patients with CRF may cause one or more
aluminium related conditions: Dialysis Encephalopathy (DE);
Fracturing Renal Osteodystrophy (FRO) which does not respond
to Vitamin D supplementation; and a Hypochromic Microcytic
Anaemia (MA).

3.1 Dialysis Encephalopathy.

Dialysis dementia was first described by Alfrey et al (19) in
1972. Aluminium induced encephalopathy is a progressive and
often fatal neurological syndrome characterised by dyspraxia,
myoclonus and dementia. This syndrome was found to occur in
global clusters (20, 21, 34-41). The clinical and
electroencephalographic features of this syndrome have been
well documented in the literature (19, 34, 62-67). However,
the neuropathological features have been described in few
reports (20, 21, 36, 68). Dialysis encephalopathy is
initially characterised by mild speech difficulty, with
slurring and hesitancy of speech (69), which may be
intensified during or immediately after haemodialysis. At the
onset of encephalopathy there is mild intellectual
impairment. As the disease progresses the symptoms become
more severe and speech difficulty more frequent. Facial
dyspraxia, and dysarthria become common (69). Involuntary
muscle contractions and seizures occur frequently with
myoclonic jerking of facial muscles. Progressive mental
deterioration, lethargy leading to global dementia occurs,
and death usually results within eighteen months of the onset
of the disease.

Until 1980, dialysis encephalopathy secondary to aluminium
toxicity was usually fatal (19, 20, 34, 36, 47). However,
Ward et al (21) and Poisson et al (63) reported the reversal
of encephalopathic symptoms after oral aluminium intake was
stopped. Platts et al (32) showed that dialysis with very low

aluminium-containing dialysate can result in clinical improvement in some cases. Davison et al (43), Rozas et al (38) and Elliot (39) also reported the reversal of aluminium induced encephalopathy after the installation of reverse osmosis and deionisers to remove aluminium from water which is used to prepare dialysis fluid. An effective chelation treatment, to remove and detoxify aluminium, was introduced by Ackrill et al in 1980 (70).

Epidemiological studies have shown that a number of patients at risk from DE do not, in fact, develop the disease (36, 39). This indicates that in patients who develop DE aluminium has reached and exceeded a critical threshold, and that this allows the metal to cross the blood-brain barrier and to accumulate in nervous tissue. Since the majority of aluminium in serum is considered transferrin bound (71, 72) and not ultrafilterable, only small amounts of aluminium are deposited in brain. However, in DE patients abnormalities in the barrier may cause brain deposition. In DE, aluminium accumulates mostly in the grey matter (20) where the capillary vessal density is higher than in the white matter. Severe abnormalities in the blood-brain barrier might explain the occurrence of a subacute encephalopathy in uraemic children receiving oral aluminium (24, 27, 64, 65, 73, 74) and elevated brain aluminium levels in preterm infants after TPN feeding (59).

3.2 Fracturing Renal Osteodystrophy.

There are several types of osteodystrophy associated with
chronic renal failure, including osteitis fibrosa,
osteoporosis, osteopenia, osteosclerosis and osteomalacia.
Among these osteitis fibrosa and osteomalacia are the two
most common diseases. Osteomalacia is characterised by
softening of the bone and increased flexibility which can
often lead to spontaneous fractures. This condition occurs
when there is an impairment of bone mineralisation, which is
associated with phosphate depletion.

Osteomalacia has been closely associated with dialysis
encephalopathy (21, 32, 33, 39, 40, 46, 75). The close
association between the two syndromes suggests a common
aetiological agent in both cases. In 1971 Parsons et al (17)
found high levels of aluminium in bone from patients with
renal failure; levels tended to be highest in patients who
had been uraemic or on dialysis longest. A high incidence of
osteodystrophy in patients who dialysed against high
aluminium tap water levels has been reported by many authors
(21, 32, 33, 39, 40, 45, 46, 75, 76).

Ward et al (33) correlated incidence of osteomalacia with
water aluminium levels, and after 1-4 years dialysis patients
using deionisers, as opposed to those using water softeners
only, had a much lower incidence of bone diseases. In 1981
Leather et al (42) reported that after the installation of

deionisers no new cases of osteodystrophy appeared in the Plymouth area compared to 20% of patients before deionisers were introduced. The level of aluminium in water was < 400 μg/l.

High levels of aluminium have been reported in bone in haemodialysis patients (21, 45, 75-80). Pierides et al (75) reported mean bone concentrations in 4 patients who died with severe osteomalacia, and associated encephalopathy, of 307 mg/kg of aluminium of ashed bone compared with below 10 mg/kg in normal subjects.

Analysis of the aluminium content of osteomalaic bone (81) indicates that aluminium is present in trabeculae, marrow and whole bone specimens from transiliac bone biopsies. Aluminium determinations by atomic absorption are in good agreement with histochemical methods (82).

Aluminium is mainly localised between osteoid and calcified tissue, the point where bone mineral is normally first deposited. Aluminium is thought to prevent the calcification/mineralisation of the organic matrix, resulting in the characteristic wide osteoid seams and increased osteoid volume seen in osteomalacia.

Boyce et al (83) reported hypercalcaemia in 14 patients with osteomalacia. These authors suggested that aluminium in bone may block the uptake of calcium into bone (osteoblast) resulting in high serum calcium levels coupled with the

availability of additional calcium from dialysate and vitamin
D therapy.

Charhon et al (84) found a low rate of bone formation in
patients with aluminium intoxication with or without
histological appearance of osteomalacia. From 24 patients
studied 17 had osteomalacia as defined by increased osteoid
seam thickness and in 7 patients the osteoid seam was normal.
Absence of significant increase in osteoid thickness in
patients with aluminium overload, with a low rate of bone
formation, reflects the marked reduction of bone matrix
formation at the cellular level (i.e. impairment of
osteoblast formation). The hypothesis that aluminium inhibits
parathyroid hormone (PTH) secretion, by accumulating in the
parathyroid glands, is a further possible explanation for the
reduced rate of bone mineralisation in patients with
aluminium-induced osteomalacia (85). Aluminium has been shown
to accumulate preferentially in the parathyroid (86) and
patients with aluminium-induced osteomalaia frequently have
low iPTH levels (83, 87). Some authors have ascribed low iPTH
levels to hypercalcaemia (83, 88). The decreased level of
biologically active PTH is unlikely to be the only factor
causing the mineralisation defect, however when iPTH is
elevated the bone appears to be protected against ostomalacia
in aluminium overload (87).

3.3 Anaemia.

Nearly all patients with CRF have a normochromic normocytic anaemia. Erythropoietin deficiency is probably the major cause of anaemia in chronic renal disease. This anaemia is due to the failure of the diseased kidney to produce sufficient amounts of erythropoietin to attempt to meet the increased demands for new red blood cells created by haemodialysis, blood loss and/or inhibitors of erythropoiesis present in uraemic blood (89-91).

The occurrence of microcytic hypochromic anaemia usually results from iron deficiency and disturbed haemoglobin synthesis, and the occurrence of a microcytic anaemia in haemodialysis patients has been attributed to iron deficiency (92, 93).

In 1978 Elliot and MacDougall (39) observed a severe anaemia which preceded the development of dialysis osteodystrophy and dialysis encephalopathy. These patients were found to have aluminium overload. Elliot suggested that anaemia might be a useful early indication of aluminium intoxication.

Short et al (94) studied 12 patients on intermittent haemodialysis who developed a severe microcytic hypochromic anaemia despite iron supplementation. Serum ferritin levels were normal or elevated in each of the patients indicating good body-iron stores. Seven patients later developed

fracturing osteodystrophy, indicated histologically, and one
patient died from encephalopathy. Plasma aluminium
concentrations fell, red cell morphology returned to normal
and haemoglobin increased following dialysis with
low-aluminium level dialysate. Short et al concluded that the
anaemia characterised in aluminium overload is caused by
aluminium intoxication and is reversible by removing the
source of aluminium. O'Hare and Munagran (95) and Touam et al
(96) observed similar phenomena to Elliot (39) in their
haemodialysed patient.

Ackrill et al (97) observed a significant improvement of
anaemia while treating aluminium overloaded patient with
desferrioxamine. The marked reduction of aluminium from bone
marrow during the course of treatment might account for the
improvement of anaemia. Tielemans et al (98) also reported a
decrease in transfusion needs, with an increase in Hb,
haemocrit (Hct) and mean cell volume (MCV) in 10
haemodialysis patients, with aluminium-bone disease, after
desferrioxamine therapy.

The mechanisms by which an excess of body aluminium induces
anaemia has been extensively investigated, but is still
unclear. Touam et al (96) intoxicated uraemic rats with daily
intraperitoneal injections of 30 mmol/day of aluminium. After
3 months of intoxication the uraemic rats had significantly
lower hemocrit, haemoglobin and MCV than the control group.
Serum iron and transferrin iron binding capacity remained
unchanged in both groups. Touam suggested that aluminium

fracturing osteodystrophy, indicated histologically, and one patient died from encephalopathy. Plasma aluminium concentrations fell, red cell morphology returned to normal and haemoglobin increased following dialysis with low-aluminium level dialysate. Short et al concluded that the anaemia characterised in aluminium overload is caused by aluminium intoxication and is reversible by removing the source of aluminium. O'Hare and Munagran (95) and Touam et al (96) observed similar phenomena to Elliot (39) in their haemodialysed patient.

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intoxication leads to a microcytic anaemia, possibly
interfering directly with normal haemoglobin synthesis.

In a similar study carried out by Kaiser et al (99)
microcytic anaemia was demonstrated after interperitoneal
aluminium loading in both normal and uraemic rats, and the
anaemia was preceeded by microcytosis. The occurrence of
microcytosis followed by reduced Hb and Hct, a pattern seen
in both iron deficiency and lead intoxication anaemias of
humans, suggests that the mechanism of aluminium induced
anaemia involves decreased red cell production.

Cannata et al (61, 100) studied the effect of aluminium
hydroxide intake on Hb concentrations and blood transfusion
requirements in haemodialysis patients. An increase in
haemoglobin and a decrease in the requirements for blood
transfusions and intravenous iron were observed after daily
doses of aluminium hydroxide were reduced. Cannata et al (61,
100) suggested that aluminium hydroxide might interfere with
erythropoiesis and advised that the morning dose of aluminium
hydroxide, which was in many cases unnecessary, could be
omitted. Cannata (61) discussed a possible interaction of
aluminium hydroxide with iron absorption, either through
gastroduodenal pH elevations or by its action as an iron
binder in the gastrointerinal tract, providing another factor
impairing erythropoieseis.

Tielmans (98) studied the possible influence of aluminium on
erythropoiesis in a general haemodialysis population, looking

at red blood cell parameters and aluminium status change in
serum aluminium level after DFO infusion test. Patients whose
serum aluminium changed by more than 180 µg/l were shown to
have lower haemoglobin values. As a result of these findings
Tielmans et al (98) suggested that even in mild aluminium
exposure haemoglobin synthesis may be inhibited and as a
result haemocytic anaemia in haemodialyis patients may be
aggravated even in the absence of overt aluminium toxicity
symptoms.

Gutterage et al (101) reported that aluminium salts accelerate the
peroxidation of membrane lipids stimulated by iron salts. Al(III)
ions enhanced the peroxidation of erythrocyte membranes in the
presence of H_2O_2 at pH 7.4, possibly by binding to the erythrocyte
membrane and producing a subtle change in the membrane
organisation. These two phenomena may also have a role in the
anaemia seen in CRF. Desferrioxamine decreased the H_2O_2-stimulated
peroxidation both in the presence and absence of Al(III) salts.
This response to DFO may, in part, account for the improved
anaemic state in vivo after chelation therapy (97).

The similar behaviour of aluminium and iron in vivo (102)
suggests that there may be an interaction of aluminium with
haem synthesis. However, in the haem structure iron is in the
Fe(II) form which contrasts with the invariable trivalent
state of aluminium in biological fluids. Aluminium has been
shown to inhibit the activity of two enzymes involved in
haemoglobin synthesis: (i) delta-aminolevulinic acid
dehydratase, the enzyme which catalyses the second step in

the haem synthesis pathway (103); and (ii) ferrioxidase (caeruloplasmin), the enzyme which catalyses the conversion of Fe(II) to Fe(III) in plasma, allowing the subsequent binding of Fe(III) to transferrin, the iron transport protein (104).

4.   THE USE OF DESFERRIOXAMINE IN ALUMINIUM OVERLOAD.

Currently the most effective method of removing aluminium during haemodialysis is by its chelation with desferrioxamine (DFO). Desferrioxamine is used routinely in the treatment of iron overload (105-109), and is effective in promoting the removal of iron from dialysis patients (110-112).

This chelating agent was first used to treat aluminium overload in 1980 (70), resulting in a complete reversal of symptoms in a patient with severe dialysis encephalopathy. These symptoms have not returned, and the patient remains well in 1986. This effect has been confirmed by Arze et al (113), Pogglitish et al (114), Milne et al (115) and many others. Payton et al (116) reported the successful treatment of aluminium induced encephalopathy by interperitoneal desferrioxamine; serum aluminium levels fell from 190 µg/l to 27 µg/l after 4 months DFO treatment.

4.1   Desferrioxamine Complexation In Vivo.

The effect of desferrioxamine infusion on serum aluminium levels has been well characterised (70, 117-122). Serum

aluminium levels reach a maximum 24-48 hours after infusion (123), up to 10 times above baseline levels. The change in serum aluminium level after DFO infusion is the basis of the desferrioxamine infusion test (120). Fournier has correlated the plasma aluminium increase induced by DFO with bone aluminium and cumulative dose of Al(OH)₃ (29).

Desferrioxamine is usually administered during the first two hours of dialysis (session 1), and serum aluminium levels normally rise continuously over the whole dialysis session, despite aluminium removal from the patient in the waste dialysate fluid. These observations indicate that the appearance of the dialysable aluminium-desferrioxamine is a relatively slow process, continuing over many hours after DFO infusion (up to 48 hours) and additionally, that immediately post infusion the Al-DFO complex appears in plasma at a faster rate than it is removed through the dialyser.

In subsequent dialysis sessions (2 and 3), serum aluminium levels fall during dialysis as the Al-DFO complex is removed in dialysate. Measurement of aluminium in dialysate indicates that large amounts of aluminium are removed during chelation therapy, estimated at between 300 to 1500mg aluminium over 6 to 10 months (97) depending on total body overload.
Serum aluminium levels rise between sessions 2 and 3, and often between session 3 and the following DFO infusion (session 1), as free DFO or its metabolites complex aluminium at the sites of metal storage, mobilising the Al-DFO complex into the plasma.

Milne et al (115) suggested that the improvement in EEG in patients with dialysis encephalopathy indicates that aluminium is removed from cerebral grey matter. From a study of DFO treatment in rheumatoid arthritis (124), it appears that DFO is capable of crossing the blood-brain barrier, supporting Milne's theory (115).

Desferrioxamine treatment also results in marked improvement in aluminium-induced osteomalacia both in rats (125, 126) and in renal patients (97, 117, 120, 127, 128).

Ackrill et al (97) observed a significant decrease in serum aluminium concentration and bone marrow and bone trabeculae levels during DFO treatment. These observations were coupled with an overall increase in iPTH level and an increase in haemoglobin. Histologically the bone became very active after DFO in each of the patients. The authors concluded that the resolution in calcification might be due to the removal of aluminium from sites in cells, where it might block calcium transport or active PTH secretion, and removal of aluminium from the bone marrow might account for the increase in haemoglobin concentration. Malluche et al (120) also reported a decrease in bone aluminium and osteoid volume with an increase in osteoblast activity and mineralisation rate after long-term DFO therapy.

The effects of desferrioxamine administration on aluminium

speciation in plasma and aluminium kinetics during haemodialysis have been studied (118, 119, 129). Graf et al (129) reported an overall increase of plasma aluminium levels after DFO infusion due to the mobilisation of tissue aluminium. There was an observed decrease in plasma protein binding of aluminium after DFO administration. Free dialysable plasma aluminium increased from 20% to more than 30% resulting in a significant increase of the effective concentration gradient between diffusable plasma Al and dialysate aluminium, resulting in increased aluminium removal during haemodialysis. These observations were confirmed by the same authors in 1982 (118).

Few studies of the in vivo clearance of the Al-DFO complex have been carried out. Rembold et al (130) determined the clearance of Fe-DFO (611 daltons), during haemodialysis on polyacrylonitryl, cellulose and cuprophane membranes. Ferrioxamine was dialysed most effectively through the polyacrylonitrite membrane. Chang and Barre (131) have reported similar findings for the clearance of Al-DFO through three different dialysers, although the actual clearance values, particularly with cuprophan membranes differ from those obtained by others (123, 131, 133).

Chang and Barre (131) observed that the Al-DFO clearance could be enhanced by a coated charcoal haemoperfusion system, and similarly by haemofiltration (111, 134), and during peritoneal dialysis (135). Thus the clearance rate of the Al-DFO complex is determined by the characteristics of the

dialysis being used. This suggests that maximal DFO clearance might be obtained with a highflux dialyser. However, Bonal et al (121) concluded that the rate of aluminium removal depends on the concentration gradient between dialysable-Al and aluminium in dialysate and that the high flux dialysers do not improve aluminium removal in vivo.

Side effects of long term high dose desferroxamine therapy have been reported (136-138). Presenting symptoms include cataracts, partly reversible retinal abnormalities, with cone and rod disfunction and nightblindness. These ocular side-effects are usually reversible with the cessation of chelation therapy (137, 138). Two cases have been reported of DFO side effects during aluminium chelation (139, 140). Rubinstein (140) reported only partial regression of ocular toxicity four months after DFO therapy had ceased, and expressed a need for regular opthamic examinations. Transient deafness has also been reported (141) during DFO therapy.

Blake et al (124) administered 5 x 3g DFO per week for three weeks in the treatment of rheumatoid arthritis. These authors observed cerebral and ocular side effects in their patients. They demonstrated alterations in CSF content of copper and iron. These local effects may account for the sense organ distrubance.

Segloni et al (142) studied zinc, copper and magnesium kinetics during desferrioxamine therapy. They reported a signhificiant increase in serum zinc and copper concentration

post dialysis with and without haemofiltration, while no differences were detected with DFO infusion. Magnesium decreased at the end of haemodialysis and haemofiltration, both with and without DFO infusion.

4.2 <u>Desferrioxamine Complexation In Vitro.</u>

Desferrioxamine B is a trihydroxamic acid isolated from <u>Streptomyces</u> <u>pilosus</u> which belongs to a class of naturally occurring substances called siderchromes. Siderchromes are subdivided into two groups: the sideromycins which are antibiotics; and the sideramines which are antagonists to the antibacterial properties of the sideromycins. Sideramines also promote growth and have an important role in iron metabolism in microorganisms, possibly acting as iron donors in the incorporation of iron in the porphyrin systems (143-145).

Desferrioxamine is a straight-chain molecule (Figure 1) containing one molecule of acetic acid, two molecules of succinic acid, and three molecules of 1-amino-5-hydroxylaminopentane (146). It has effectively 4 ionisable protons, with three successive ionisation constants (pK_a) 8.39, 9.03, 9.70. The oxamate groups are available to co-ordinate to metal ions, and in the case of Fe^{3+} a pseudo-octahedral hexadentate complex, ferrioxamine, forms (Figure 2). In ferrioxamine the Fe(III) is in an approximately octahedral environment and is in the high spin

Figure 1. Desferrioxamine B $(H_4 DFO)^+$
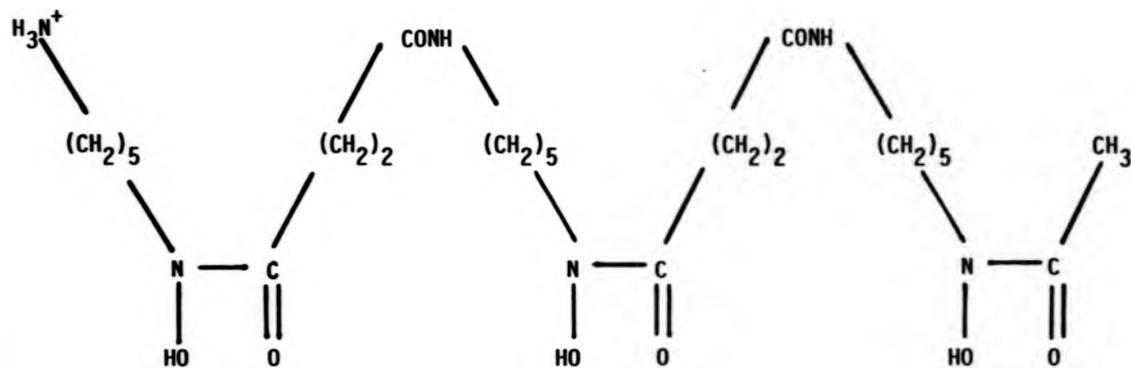
Molecular Mass = 561

Figure 2.   Ferrioxamine B
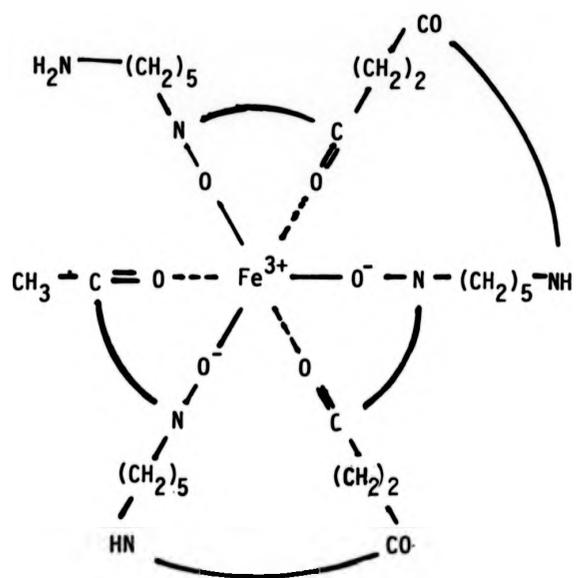
state with zero ligand field stabilization (147). The
compound has a very high formation constant (10^{31}) forming a
complex of great stability (143).

Desferrioxamine was shown to complex with aluminium by Snow
in 1969 (144) and it seems likely that a similar complex to
ferrioxamine is formed with trivalent aluminium, with a
formation constant of 10^{22} (148).

Schwarzenbach et al (149) considered the following ionisation
equilibria for desferioxamine B and calculated the
corresponding constant values.

Ionisation equilibria at 20 °C and 0.1 M NaClO$_4$.

H_4DFO^+ = $C_{25}H_{44}N_6O_8$ M.Wt. = 557 daltons

(1)	$(H_4DFO)^+$ =	H^+	+	H_3DFO	pk_1 = 8.39
(2)	H_3DFO =	H^+	+	$(H_2DFO)^-$	pk_2 = 9.03
(3)	$(H_2DFO)^-$ =	H^+	+	$(HDFO)^{2-}$	pk_3 = 9.70
(4)	$(HDFO)^{2-}$ =	H^+	+	$(DFO)^{3-}$	pk_4 > 11.0

Desferrioxamine forms stable complexes with several metallic
ions. The stability constants are given in Table 1, with the
stability constants for EDTA and Transferrin (143).
Desferrioxamine also complexes with chromium and vanadium but
the stability constants of the complexes are unknown (144).

Snow (144) reported that the chromium myobactin P complex
formed less readily than the aluminium complex, but once

formed the complex was more resistant to displacement by the ferric ion than the aluminium, copper, gallium, and vanadyl complexes. This observation suggests that the stability constant for the chromium myobactin P complex is between that of the iron and aluminium complexes, i.e. between 10^{31} and 10^{22}. The complexation of Cr(III) by DFO, however, is difficult to demonstrate in vitro (and in vivo) because of the very slow reaction kinetics.

Under physiological conditions desferrioxamine can remove iron from ferritin and haemosiderin and continues to do so until the maximum theoretical binding capacity is attained. Desferrioxamine cannot remove iron from the porphyrin ring of haemoglobin or myoglobin.

In vitro clearance measurements of the aluminium desferrioxamine complex indicated the formation of a complex with molecular weight of approximately 625 daltons, i.e. the formation of a 1:1 complex, $[Al(HDFO)]^+$ (132). The copper desferrioxamine complex formed has a dialysing molecular weight of 620 daltons indicating a 1:1 complex [Cu(HDFO)], when DFO is in excess. When a lower ratio of DFO to copper is used a 3:2 $[Cu_3(HDFO)_2]^{2+}$ complex is formed (148).

Table 1. Stability constants of various complexes (143).

Metallic Ion	DFO	EDTA	Transferrin
Fe^{3+}	10^{31}	10^{25}	$10^{27}, 10^{29}$
Al^{3+}	10^{22}	10^{16}	10^{15}
Cu^{2+}	$10^{14}, 10^{30}$	10^{19}	
Zn^{2+}	10^{11}	10^{16}	
Co^{2+}	10^{11}	10^{16}	
Ni^{2+}	10^{10}	10^{19}	
Fe^{2+}	10^{10}	10^{14}	
Mn^{2+}	10^{4}	10^{9}	
Ca^{2+}	10^{2}	10^{11}	

5. COPPER METABOLISM IN RENAL DISEASE.

5.1 Biochemistry of Copper.

Copper is sited in both proteins and enzymes, performing a
number of important and diverse metabolic functions (150).
These metabolic processes include oxygen transport, electron
transfer, the catalysis of redox reactions, and the
protection of cells against oxidation by oxygen-containing
radicals.

Metabolic Balances of Copper.

In humans, copper is absorbed from the stomach, duodenum and
all sections of the small intestine. The absorption and
retention of this metal is dependent on the chemical form in
which it is ingested and the dietary levels of other minerals
and organic substances (151). Absorption is also dependent on
the acidity of the intestinal contents in the absorptive
area. The detailed mechanism of absorption is not known
(151). Copper (and zinc) uptake is inhibited by phytate which
acts to bind copper preventing its passage across the gut
lumen (152). The presence of Zn, Mo and Cd in the diet
adversely affects copper availability due to the cationic
competition for similar binding sites on the transport
proteins at the intestinal level (151). Levels of circulating
copper are altered in diseased states. In malignant,
inflamatory and infectious conditions circulating copper is

increased (this is part of the so-called acute phase response, in which the synthesis of caeruloplasmin and other substances is stimulated in the liver), and hypocupraemia indicates total body depletion. The majority of circulating copper is bound to caeruloplasmin (95%), and this portion of copper is neither readily exchangeable nor transferable (150, 152, 153). A small fraction of plasma copper is losely bound to albumin, and the remaining copper is "free" and ultrafilterable (i.e. it is bound in various low molecular weight complexes). Non-caeruloplasmin bound copper can be identified with the metabolically exchangeable fraction. Caeruloplasmin is an important transport/storage protein and reduced plasma levels are associated with protein-energy malnutrition (153).

Albumin and amino acid bound copper is readily distributed to all body tissues and is easily transferred into the erythrocyte. In cases of copper intoxication copper enters the erythrocyte rapidly, resulting in haemolysis.

Caeruloplasmin copper is not readily exchanged or transferred and the residence time of copper within the pool is too slow to allow caeruloplasmin to be a primary transport protein (153).

The liver is the main site of copper storage and the principal target organ after copper absorption. Within the liver copper is incorporated into the mitochondria, microsomes and nuclei. Copper is either stored in these

subcellular structures or released to become incorporated into copper containing proteins and enzymes, e.g. superoxide dismutase (SOD), caeruloplasmin (Cp), cytochrome C oxidase or one of the numerous copper enzymes.

Superoxide Dismutase.

There are three known types of SOD which are characterised by having copper plus zinc, iron or magnesium as essential cofactors for the conversion of superoxide to oxygen and hydrogen peroxide.

$$2 \ O_2^- \ + \ 2 \ H^+ \longrightarrow O_2 \ + \ H_2O_2$$
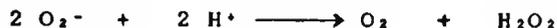
Superoxide dismutase of human origin is characterised as containing copper and zinc. The two metal sites are thought not to interact with each other, and each contains 1 Cu-Zn bridged by an histodine ligand (154). The protein has a molecular mass of 31,000 daltons and consists of two identical, non-covalently associated subunits, containing 34 µg Cu/mg protein. Superoxide dismutase comprises 60% of the total RBC copper. This enzyme is synthesised in the bone marrow, and diseases such as chronic renal failure, which effect normal bone mineralisation, may also effect SOD synthesis and the incorporation of copper into the erythrocyte.

Cytochrome C Oxidase.

Cytochrome C Oxidase is a mitochondrial membrane-bound
lipoprotein containing copper and α-haem in a 1:1 ratio. It
can be solubilised with detergents or bile salts, but tends
to aggregate in solution. Cytochrome c oxidase consists of at
least 10 subunits, some of mitochondrial and others of
cytoplasmic origin (154). This enzyme acts as the terminal
electron acceptor for the mitochondrial respiratory chain.
The enzyme is found in high concentrations in active tissue,
heart, muscle and liver mitochondria as well as brain grey
matter, and is the major generator of cellular energy through
the oxidative phosphorilation of adenosine diphosphate (ADP).
The functioning of cytochrome c oxidase as a proton pump has
been supported by a number of experimental results, but the
molecular mechanism responsible for the proton pumping is
still unknown (155).

Cytochrome c oxidase activity falls during copper deficiency,
but the reason for this response is still uncertain (156).
Copper is incorporated into the enzyme and has a functional
role there, and it is also involved in the synthesis of the
α-haem moiety of the enzyme. The degenerative changes that
appear in the membranes of copper-deficient mitochondria
could be due to either of these processes or to the direct or
indirect role of copper in the modulation of the lipid
composition (156). Reduced cytochrome c oxidase activity in
copper deficiency suggests a reduction in the amount of
energy supplied to the muscles, for muscle contraction, via

this particular respiratory chain.

Caeruloplasmin.

Caeruloplasmin is a single chain glycoprotein of mammalian blood (M.Wt. 135,000). In vitro studies indicate that Cp behaves as an enzyme (ferrioxadase) that catalyses the oxidation of Fe(II) to Fe(III) (157). The biochemistry of this protein is extensively reviewed by Frieden and Hsieh (157), and Poulik and Weiss (158). Caeruloplasmin is an essential component of copper metabolism and is required for the incorporation of iron into transferrin (the iron transport protein). Up to 95% of copper in human plasma is caeruloplasmin (Cp) bound. This portion of copper is firmly protein bound and does not exchange with the remaining 10% of plasma copper (150, 152, 153). Apo-caeruloplasmin is synthesised in the liver, and copper is incorporated into the apo-protein within the liver (159).

Caeruloplasmin is a multi-functional cuproprotein. It resembles albumin and transferrin, in that all three serum proteins are regarded primarily as transport proteins. Roeser et al (160) observed that Cp was essential to the normal movement of iron from storage cells to plasma. As copper deficiency developed, plasma Cp levels decreased rapidly, reaching less than 1% of the normal level. This reduction in plasma Cp was coupled with a reduction of plasma iron. The administration of Cp in copper deficiency was followed by a prompt and appreciable increase in plasma iron. Thus Cp

corrects the defect in cellular iron outflow which is
observed in copper deficiency. Failure to maintain plasma
iron levels at reduced Cp levels implies that the iron was
not mobilised from storage cells to transferrin, thus
indicating that Cp is essential for the oxidation of Fe(II)
to Fe(III), and for allowing an optimal rate of transferrin
formation (160). In this way copper deficiency induces a
hypochromic normocytic anaemia normally associated with iron
deficiency (161) (Figure 3). The ferrioxidase activity of Cp
is inhibited by trivalent and other metal ions (104). Huber
and Frieden (104) found that all trivalent cations tested
inhibited ferrioxidase activity but the strongest inhibitors
had an ionic radius of 0.81 Å or less. The inhibition by
Al(III) was found to be mixed competitive and uncompetitive
with respect to the substrate, Fe(II). The uncompetitive
feature of the inhibition was not due to competition by
Al(III) with the second substrate oxygen.

Another important role of caeruloplasmin is as a transport
vehicle. Copper atoms of caeruolplasmin may be released at
specific cellular sites, a prerequisite for copper
utilization in the biosynthesis of cytochrome c oxidase and
other copper proteins (157).

Caeruloplasmin plays a dual role in the prevention of deleterious
oxidations in serum. Firstly, by preventing the formation of free
radical intermediates that might be generated by non-enzymic
(non-caeruloplasmin) oxidation of Fe(II) or Fe(II)-complexes.
Caeruloplasmin also catalyses the oxidation of Fe(II), producing a

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Figure 3. Caeruloplasmin plays a Central role in regulating the Fe^{2+} and Fe^{3+} cycles its ferroxidase activity is inhibited by Al^{3+} ions (157).

rapid 4 electron reduction of molecular oxygen to water, with no intermediate formation of peroxides or superoxide anion free radicals (162).

The role of Cp as an acute-phase reactant may also be related to its antioxidant function. Serum Cp is increased two to three times in response to infection and inflammation. Lipid peroxidation products in the serum may stimulate a greater output of the acute-phase reactant. Thus, increased serum concentrations of Cp could increase its serum antioxidant function (162). Borda and Uribarrena (163) reported increased serum Cp levels in patients with haemochromatosis. This observation supports the theory that an increase in serum Cp levels reduces the lipid peroxidation induced by Fe(II) (104, 162).

Thus Cp is a copper-protein with several important functions, all of which are directly related to its oxidase activity. This oxidase activity of Cp links the complex biochemisty of copper and iron.

5.2   Copper Disturbances in Chronic Renal Failure.

There are several theories as to the cause of trace element disturbances in chronic renal failure patients, for example: (i) reduced renal function causes the retention of trace elements which are mainly excreted by the kidney; (ii) haemodialysis removes trace elements from the patient according the concentration gradient between plasma and dialysate, and thus reduces their concentration in plasma; (iii) contaminant trace

rapid 4 electron reduction of molecular oxygen to water, with no intermediate formation of peroxides or superoxide anion free radicals (162).

The role of Cp as an acute-phase reactant may also be related to its antioxidant function. Serum Cp is increased two to three times in response to infection and inflammation. Lipid peroxidation products in the serum may stimulate a greater output of the acute-phase reactant. Thus, increased serum concentrations of Cp could increase its serum antioxidant function (162). Borda and Uribarrena (163) reported increased serum Cp levels in patients with haemochromatosis. This observation supports the theory that an increase in serum Cp levels reduces the lipid peroxidation induced by Fe(II) (104, 162).

Thus Cp is a copper-protein with several important functions, all of which are directly related to its oxidase activity. This oxidase activity of Cp links the complex biochemisty of copper and iron.

5.2 Copper Disturbances in Chronic Renal Failure.

There are several theories as to the cause of trace element disturbances in chronic renal failure patients, for example: (i) reduced renal function causes the retention of trace elements which are mainly excreted by the kidney; (ii) haemodialysis removes trace elements from the patient according the concentration gradient between plasma and dialysate, and thus reduces their concentration in plasma; (iii) contaminant trace

elements in dialysate are absorbed into the plasma according to the concentration gradient between dialysate and plasma, therefore producing an increase in plasma concentrations of the trace elements; (iv) trace elements may be actively taken up by the plasma from the dialysate against a concentration gradient if the trace elements are readily bound in the blood (e.g. to proteins) - it should be noted that the effect of such binding is to reduce the thermodynamic concentration of the free metal in blood, to the extent that the thermodynamic concentration gradient is actually from dialysate to blood, opposite to the apparent concentration gradient.

Elevated plasma copper levels have been reported in dialysed and non dialysed uraemic patients (164-169). Tsukomoto et al (169) reported that plasma copper concentrations increased from 970 $\pm$ 220 to 1080 $\pm$ 250 µg/l (p<0.01, n=18) due to a single dialysis, when the dialysate contained below 15 µg/l copper. These authors concluded that this rise in plasma copper might be due to leaching of copper from the dialyser membrane. Increased plasma copper was found in non dialysed patients, indicating that the dialyser membrane was not the only source of increased plasma copper levels. Increased concentration of copper carrier proteins was not included in the study.

Blomfield et al (170) observed an increase in plasma copper concentration after dialysis against contaminated dialysate (ca. 200 µg/l). However, these authors concluded that copper deficiency would not result directly from routine dialysis

against copper free dialysate, since the metal is strongly protein bound in plasma.

Acute copper-induced haemolysis has been reported in humans after accidental ingestion, suicide attempts, and copper sulphate application to extensive burns. Acute haemolysis after haemodialysis has been reported after accidental copper-contamination of dialysis fluid (167, 171-173). Thus, Manzler et al (171) reported acute haemolysis after dialysis when treated water at pH 5 was used to prepare dialysate. The low pH of the dialysate resulted in copper being leached from copper piping leading to the dialyser. The possible mechanisms of copper appearance in the dialysate include oxidation of metallic copper to cupric ion or contamination of tap water from copper pipes. In either case the copper ion crosses the dialyser and enters the blood, significantly altering erythrocyte metabolism.

Eastwood et al (174) reported a fatal case of copper poisoning and transient illness in 12 patients after acid contamination of the water supply. Heparin inactivation leading to extracorporeal clotting, and copper poisoning were observed during and post dialysis.

Agarwal et al (175) observed that copper sulphate poisoning, even in an early stage prior to copper protein/tissue binding, can not be treated by haemodialysis. In spite of haemodialysis the patient developed haemolysis, and hepatic and renal failure.

In summary, copper concentrations in serum in renal patients, prior to or on dialysis treatment, are generally somewhat higher than in normal individuals. These elevated copper levels do not appear to have clinical consequences. Excessive copper exposure can lead to undesirable consequences, particularly haemolysis and overt copper poisoning.

6. OTHER TRACE ELEMENTS IN RENAL DISEASE.

An increasing number of acute and chronic complications have been noted arising from the long-term use of the artificial kidney in intermitent haemodialysis. Metabolic disturbance such as bone disease, hypercalcaemia, metastatic calcifications, gout, haemosiderosis and chronic potassium depletion have been observed, and the role of trace metals in their development has been questioned.

Salvadeo et al (168) analysed eleven metals in blood and dialysis fluid. These authors found increased plasma Al, As, Cd, Cu, Hg, Mn, Pb and Zn levels in uraemic patients compared to controls, no change was seen in Co, Cr, and Ni plasma levels and decreased blood lead concentrations were seen in renal patients compared with controls.

Tsukamoto et al (169) and Sandstead (176) reported decreased plasma Br levels in dialysed uraemics and normal levels in non-dialysed uraemic patients. Plasma selenium levels were unchanged in dialysed and non-dialysed patients (169).

Mansoun et al (164) and Tsukamoto et al (169) observed that
uraemic patients receiving severely restricted protein intake
and not dialysed had low plasma zinc and normal copper
levels. In dialysed uraemic patients with more liberal
protein intake the plasma zinc levels approached normal.
Non-dialysed uraemic patients had high plasma and erythrocyte
magnesium and low plasma and erythrocyte calcium levels
(164).

Cordon and Freeman (177) studied zinc metabolism in renal
failure. Manifestations of zinc deficiency include, abnormal
bone metabolism, skin lesions, impaired wound healing, hair
loss, depression, loss of libido, and anorexia, and are
frequently seen in uraemic patients (164, 177, 178). In one
study (177), plasma zinc levels were below normal in 21 of 31
patients, and hair, heart and liver levels were normal. These
authors concluded that decreased plasma zinc may be due to
redistribution rather than total body deficiency. A similar
study was reported by Mahayan et al (178). They concluded in
a later report (179) that zinc deficiency in uraemic patients
is reversible by zinc supplementation.

Bogden et al (180) reported an increase in plasma zinc level
post dialysis. They demonstrated that disposable coils within
the dialyser were the primary source of zinc in the dialysate
fluid. Gallery et al (181) reported a case of acute zinc
toxicity in a home dialysis patient after dialysing with
water stored in a galvanised tank. Subsequently the patient

suffered severe anaemia with raised plasma and erythrocyte concentrations. Patients on home dialysis with deionised water were symptom free.

In diseases where trace metal metabolism is affected e.g. chronic renal disease, anorexia, hepatic cirrhosis and haemochromatosis, there is a clinical requirement to monitor, or determine, the total body status of the metals effected by the diseased state. In most cases of trace element imbalance more than one element is affected, due to the complex interaction of metals in biochemistry, e.g copper depletion inhibits normal iron metabolism, and copper overload results in zinc deficiency, etc. Thus, the monitoring of a single element in disease, in isolation to other factors, may be of very little value, in that only a single metabolic disorder is being observed.

In renal disease a number of accidental intoxications have been reported due to contaminated dialysis fluid, e.g copper and zinc. These intoxications are in addition to the "normal" trace metal imbalances seen in chronic maintenance dialysis. Thus, the routine monitoring of trace metal body status, by serum analysis, is highly desirable (but not often achieved) in chronic renal failure.

CHAPTER 2.

METHODS OF ANALYSIS.

METHODS OF ANALYSIS.

1. INTRODUCTION.

The object of this chapter is to present the essential
experimental details for the analytical methods which have
been applied throughout the work reported in this thesis. The
work relating to aluminium, copper, and iron required the
accurate determination of these trace metals in clinical
samples, for example, serum, red blood cells, urine, faeces,
dialysate, bone and hair. Trace metal analysis was carried
out by graphite furnace atomic absorption spectrophotometry
(GFAAS) usually with Zeeman background correction. Hair zinc
levels were determined by flame atomic absorption
spectrophotometry. The determination of desferrioxamine
concentrations in dialysate samples, and caeruloplasmin
concentrations in serum, were carried out using colorimetric
methods. The details of hair sample preparation and digestion
are given in Chapter 3. Serum proteins were separated by Fast
Protein Liquid Chromatography (FPLC™), and details of the
separation techniques are found in Chapter 8.

2. ATOMIC ABSORPTION SPECTROPHOTOMETRY.

The development of Atomic Absorption Spectroscopy (AAS) has
had an unprecedented impact on the field of trace metal
analysis over the last twenty years. This method of analysis,

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2.   ATOMIC ABSORPTION SPECTROPHOTOMETRY.

The development of Atomic Absorption Spectroscopy (AAS) has had an unprecedented impact on the field of trace metal analysis over the last twenty years. This method of analysis,

with its combination of sensitivity, specificity and precision has allowed the determination of a wide variety of elements in a number of different fields of investigation. The traditional flame atomisation techniques in general permit determinations at the part per million level (mg/kg), the more recently developed furnace atomisation techniques, using graphite tubes, allows determinations at the part per billon level (µg/kg). Recently, reproducibility has improved with new furnace technology and the introduction of pyro-coated and platform (L'vov) tubes.

Many methods have been published on the determination of aluminium in biological samples (182-188). A recent paper by Slavin (189) reviews the developments in the determination of aluminium in serum by GFAAS. This review discusses background correction, matrix modification, tube type and analytical sensitivity.

Copper determination in biological samples by GFAAS has also been extensively reported, by Blomfield (170) and recently reviewed by Liska (190) and Burguera (191).

2.1 Zeeman Background Correction. (192)

Traditionally, background absorption due to light scattering, and broad band molecular absorption due to furnace and sample matrices have been compensated by the use of continuum source correction, e.g. using a deuterium discharge lamp. This type

of correction is only effective for the correction of uniform
backgrounds within the observed spectral band width (0.2 or
0.7nm). With the analyte-shifted Zeeman system, correction is
made at the specific wavelenth of the emission line of the
lamp. In this way correction can be made for non-uniform
absorption caused by atomic lines of the matrix elements, and
for rotational and vibrational absorption of molecular
species.

When an atomic absorption or emission spectral line is
subjected to a magnetic field the line splits into three or
more polarised components. A simple Zeeman splitting pattern
is shown in Figure 1a. The π component of the split spectral
line is situated at the original wavelength and polarised in
a plane parallel to the magnetic field. The two σ components
are placed symmetrically at equal wavelenth intervals above
and below the original line, and polarised perpendicular to
the magnetic field. This simple pattern is described as the
"normal" Zeeman pattern, but it is characteristic of a very
limited number of spectral transitions, and is observed at
the normal absorption lines of a few elements only e.g. zinc
and cadmium. Most transitions involve more complicated Zeeman
patterns consisting of more than one π component and several
σ components (Figure 1b). In all cases of Zeeman splitting
the sum of the +/- σ components, and the π components is
always equivalent to the intensity of the non-polarised line.

When the field is off, both analyte and background
absorptions are measured at the unshifted spectral line. When

Figure 1.   Zeeman Splitting

a)

No
Magnetic
Field

ν_0

π

Normal
Zeeman
Pattern

σ^- ν σ^+

b)

No
Magnetic
Pattern

ν_0

π
Components

Anomalous
Zeeman
pattern

σ^- ν_0 σ^+

components   components

Figure 1. Zeeman Splitting

a)

No
Magnetic
Field

ν_o

Normal
Zeeman
Pattern

π

σ^- ν σ^+

b)

No
Magnetic
Pattern

ν_o

π
Components

Anomalous
Zeeman
pattern

σ^- ν_o σ^+
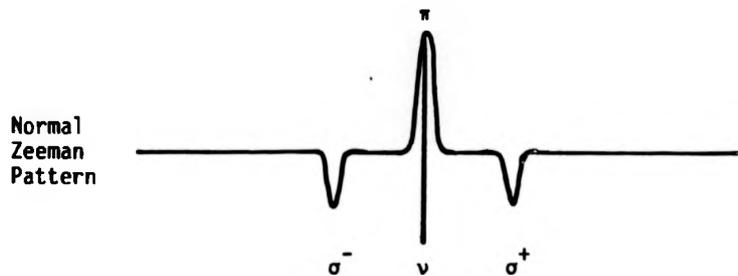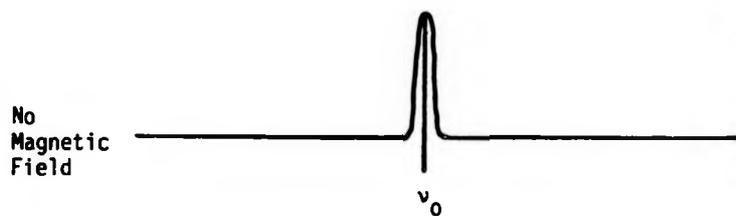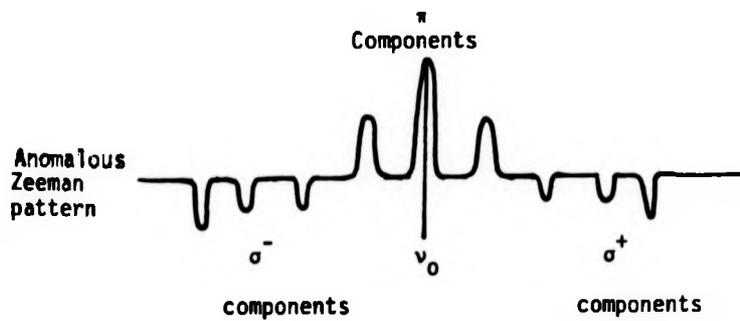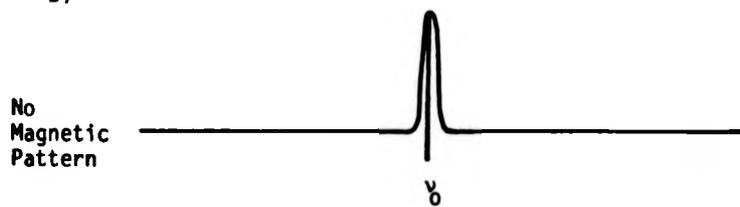
components   components
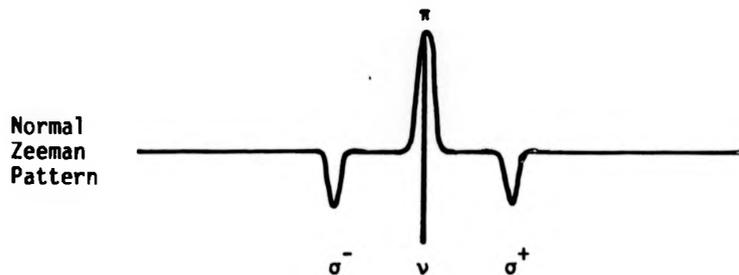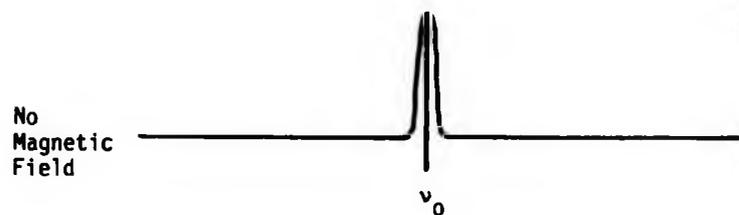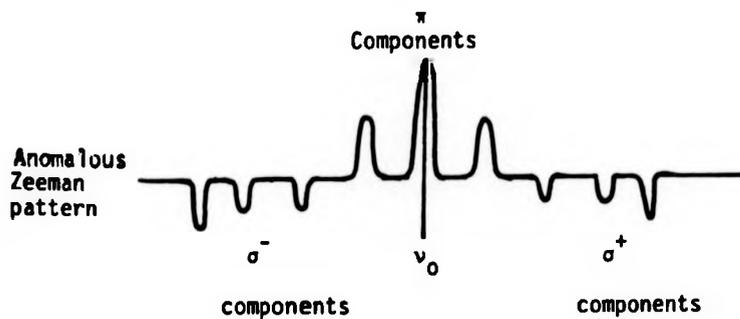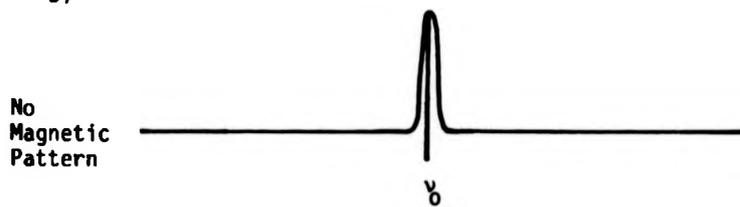
the magnetic field is on only background is measured because the σ components of the atomic absorption spectral line are shifted away from the emission line and the residual π component is not detected as the spectrometer radiation is polarised perpendicular to the applied magnetic field. Hence, by subtracting the "field-on" signal from the "field-off" signal correction for background is achieved. (In practice, a residual π component is often detected and this merely serves to reduce analytical sensitivity.)

2.2   Furnace methods.

The furnace heating programmes used for aluminium, copper and iron determinations by GFAAS are given in Tables 1-3 respectively.

The essential components of a heating programme are the three stages: Dry, Char and Atomisation. In the programmes developed for this work, the three stages are represented by multi-step programmes, designed to achieve the most effective final atomisation of the analyte element.

Drying. - First the sample is dryed in a two step drying process. The sample is heated to 80°C to drive off any water in the sample, then the temperature is ramped up to 120°C to remove any residual moisture.

Charring. - After drying the furnace temperature is increased in 2 stages to drive off the inorganic components of the

sample matrix. The second charring step ensures complete removal of any organic compenents of the sample matrix.

Atomisation. - In step 5 the furnace is rapidly heated to atomisation temperature over a 0 second ramp, thus allowing atomisation of the analyte. During atomisation the internal gas flow is reduced from 300 ml/min to 0 ml/min, which maximises the residence time of the analyte in the furnace, and thus increases sensitivity. The graphite tube is then cleaned in step 6 at a temperature of 2700°C and cooled down ready for the next sample.

This three stage heating programme is used for most element determinations by GFAAS. The actual temperatures at which each of the stages are carried out, and the lengths of the ramp and hold times used, have to be optimised for each element as the analytical method is developed. Optimisation of the heating programme results in maximum analytical sensitivity, ensuring that the analyte is not volatilised prior to the atomisation step.

Table 1. Heating Programme for HGA 600 for Aluminium.

STEP NUMBER	FURNACE TEMPERATURE	TIME RAMP	TIME HOLD	INTERNAL GASFLOW	READ
1	80	5	5	300	
2	120	10	10	300	
3	800	10	5	300	
4	1400	5	10	300	
5	2500	0	5	0	*
6	2700	1	3	300	

WAVELENGTH (NM): 309.3

SLIT WIDTH (NM):   0.7

Table 2. Heating Programme for HGA 600 for Copper.

STEP NUMBER	FURNACE TEMPERATURE	TIME RAMP	TIME HOLD	INTERNAL GASFLOW	READ
1	80	5	5	300	
2	120	10	10	300	
3	400	10	5	300	
4	1200	5	10	300	
5	2600	0	5	0	*
6	2700	1	1	300	

WAVELENGTH (NM): 324.8

SLIT WIDTH (NM): 0.7

Table 3. Heating Programme for HGA 600 for Iron.

STEP NUMBER	FURNACE TEMPERATURE	TIME RAMP	TIME HOLD	INTERNAL GASFLOW	READ
1	80	5	5	300	
2	120	10	10	300	
3	800	10	5	300	
4	1100	5	10	300	
5	2600	0	5	50	*
6	2700	1	5	300	

WAVELENGTH (NM): 248.3

SLIT WIDTH (NM):   0.2

2.3 Avoidance of Contamination.

With recent furnace atomisation techniques trace metal
determinations by GFAAS are relatively easy. A major problem
of trace metal analysis is sample contamination. There are a
number of sources which may contribute to the contamination
of the biological samples during their collection and
subsequent preparation procedures.

Contamination of samples during collection may result from:
(i) dust, industrial contamination, and cosmetics on the
surface of the body, for example, resulting in contamination
of hair and breast milk samples; (ii) disinfectants, talc and
dust on the gloves of the pathologist, resulting in
contamination of post mortem and biopsy tissue; (iii)
metallic corrosion products and residues on the instruments
from previous dissections; and (iv) dust and disinfectant on
the dissection table or trace element contamination of
collection vessels.

During sample preparation contamination may result from a
number of sources: (i) impurities in reagents used during wet
ashing procedures, and subsequent dilution with water; (ii)
pipette tips and volumetric equipment; and (iii) trace
elements in dust from the laboratory atmosphere.

Once the possible sources of contamination of a specific
element have been identified, special precautions can be
incorporated into the analytical procedures to ensure

reliable results.

3.   SERUM ANALYSIS.

Blood samples were taken using stainless steel needles and disposable syringes (Becton Dickinson & Co Ltd.) and placed in trace element free heparinised blood tubes (Lithium Heparin; Brunswick). Serum was separated from red cells by centrifugation (2700 rpm/15minutes) and stored in polycarbonated tubes (10ml). Serum can be stored at 4°C for two weeks or for several months if frozen.

Serum was diluted (x4) with an aqueous solution of nitric acid (0.1M), and Triton-X-100 (0.5%). Aluminium concentrations were determined by direct calibration against aluminium standards (0-100 μg/l) prepared in nitric acid/Triton. Triton was added to the diluting solution to improve pipetting and sample delivery, and aid sample spreading on the walls of the graphite tube after injection.

Serum samples analysed for copper and iron were diluted (x20), with nitric acid (0.1M). Metal concentrations, of the diluted samples, were determined by direct calibration against appropriate standards (0-200 μg/l) prepared in nitric acid.

Aluminium, copper and iron analysis was carried out by direct injection into the graphite furnace of an atomic absorption spectrometer (Perkin-Elmer Zeeman 3030). These methods have

previously been extensively tested (132, 193) and give recoveries ca. 100% ($\pm$ 6%).

4.   RED CELL ANALYSIS.

The method for whole blood/red cell analysis was developed in the course of this research. Whole blood was collected in trace element free heparinised blood tubes. The erythrocytes and plasma were separated by centrifugation (2700 rpm/15minutes), and the plasma layer removed. Red cells were washed in isotonic sodium chloride. Aliquots of packed red cells (0.5-1.0g), were dispersed into polycarbonate tubes and digested with an aqueous solution (2-4ml) of Triton-X-100 (1%) and EDTA (0.01M). This resulted in a clear red homogenous solution. Aluminium determinations were carried out by direct injection into the graphite furnace of an atomic absorption spectrometer (Perkin-Elmer Zeeman 3030). Digested samples were stored at 4°C if not analysed immediately. For copper analysis, appropriate further dilutions (x4) were made with Triton/EDTA solution.

Red blood cell aluminium levels were determined by direct calibration against aluminium standards (0-100 µg/l) prepared in Triton/EDTA. Standards were prepared for copper analysis, to cover the working range 0-100 µg/l, in Triton/EDTA.

The recovery of aluminium was determined by the addition of an aluminium spike to the digested red cell solution (Table

4). The trend to lower recovery at higher aluminium concentrations suggests an interference from the constituents of the digested red cells in the sample media in comparison with the Triton/EDTA calibration solution. However, the degree of error introduced is small, and all the red cell samples analysed had aluminium concentrations of < 50 μg/l.

Table 4. Recovery of Added Aluminium in Red Blood Cell Analysis.

SAMPLE	ALUMINIUM BY DIRECT MEASUREMENT µg/l	ADDED ALUMINIUM SPIKE µg/l	RECOVERY OF SPIKE %
1	1	25	107
2	1	25	105
3	1	65	108
4	6	91	98
5	11	63	91
6	11	91	93
7	18	63	90
8	18	91	88

5. URINE ANALYSIS.

Urine samples (24 hour collections), were pooled into trace
element free containers (Sterilin). Urine was diluted (x4)
with nitric acid (0.1M), for aluminium, copper and iron
analysis. Metal determinations were carried out by graphite
furnace atomic absorption spectroscopy. Metal concentrations
were established by direct calibration against appropriate
standards (aluminium 0-200 $\mu g/l$, copper and iron 0-100 $\mu g/l$),
in nitric acid. These methods have previously been
extensively tested (132).

6. FAECES ANALYSIS.

Colostomy samples were collected from the child patient
studied in Chapter 5. The total weight of sample was
determined by weighing the colostomy bag. Faeces samples were
weighed into clean, dry ashing dishes and the sample dried at
250°C to constant weight. The sample was allowed to cool and
the mass of sample was determined. An accurately known mass
of dry sample (ca. 0.1000g), was placed in a digestion tube
with concentrated nitric acid (2ml). Samples were heated to
ca. 120°C and taken to dryness. The digested sample was then
redissolved in 0.1M nitric acid (10ml) and transferred
quantitatively to a volumetric flask (50ml) and made up to
volume with nitric acid (0.1M).

Iron analysis was carried out by direct aspiration into a flame atomic absorption spectrometer (Perkin-Elmer 603). Iron concentration in solution was determined by direct calibration against iron standards (0-20 µg/ml) in nitric acid.

Aluminium and copper analysis was carried out after further dilution (x4 copper, x10 aluminium) with nitric acid. Metal concentrations were established by direct calibration against appropriate standards (0-100 µg/l copper, 0-200 µg/l aluminium) in nitric acid, by graphite furnace atomic absorption spectrometry.

7. MILK FORMULAE AND INTRAVENOUS SOLUTIONS.

Aluminium concentrations were determined, in diluted milk feeds and intravenous fluids, by graphite furnace atomic absorption spectrometry. Milk formulae and other lipo-containing solutions, e.g. Vamin and Intralipid, were diluted appropriately prior to analysis with Triton-X-100 (0.5%), EDTA (0.01M). Aqueous solutions were diluted with nitric acid (0.1M). Aluminium (0-200 µg/l) was determined by direct calibration with either Triton/EDTA or acid standards. Analytical errors were within 5%, and the percentage aluminium recovery, after addition of an aluminium spike to the sample, was between 95-105%.

8. POST MORTEM TISSUES.

Tissues samples were taken at post mortem for aluminium analysis. Samples were sectioned with a stainless steel blade and accurately known weights of brain (ca. 0.1500g), rib (ca.0.0500g), and liver (0.1500g) were placed in contamination free teflon tubes (10ml). Concentrated nitric acid (1.0ml) was added to each tube and the sample heated to ca. 100°C until all the tissue was digested. The samples were left to cool, and then transfered quantitatively to trace element free volumetric flasks and made up to volume with distilled water (25ml). The solutions were then analysed by direct injection into the graphite furnace of an atomic absorption spectrometer, against aluminium standards (0-100 µg/l) in 0.1M nitric acid.

9. DESFERRIOXAMINE ANALYSIS.

Quantitative measurements for desferrioxamine in dialysate were carried out using UV/visible spectroscopy. The method used was developed by Garstang (132), which had been adapted from a method by Fielding (194), originally used to estimate ferrioxamine and desferrioxamine in urine. This spectrophotometric method of analysis uses the highly specific chelating activity of desferrioxamine for trivalent iron, forming the six co-ordinate ferric chelate, a red-brown compound known as ferrioxamine.

A stock solution containing 0.057M citric acid (5.9574g), 0.5M sodium dihydrogenorthophosphate (39.0000g) and 0.0024M ferric ammonium sulphate (0.5696g) in 500ml deionised water was used to develop the complex colour.

The degree of dissociation and thus decolourisation of the ferrioxamine complex is pH dependent. Dissociation is most rapid between pH 2-4 and proceeds more slowly at pH 4-6. The citric acid-phosphate buffer system is used to lower the pH during chelation and restore pH before colorimetry.

A calibration curve (Figure 2) for desferrioxamine concentration was determined using a standard stock solution of desferrioxamine methanesulphonate (Desferal, CIBA) of 40mg in 100ml distilled water. Aliquots of standard desferrioxamine solution were placed into six polypropylene vials; 0, 0.25, 0.50, 0.75, 1.0, and 1.25ml, and made up to volume (5.0ml) with distilled water. Finally, 5.0ml of developing solution was added, and the colour allowed to develop for 10 minutes. These standards were equivalent to 0, 1, 2, 3, 4 and 5 mg/100ml of desferrioxamine respectively. After chelation the absorption was measured by spectrometer at 430nm.

Desferrioxamine determinations were carried out on 5.0ml dialysate solution added to 5.0ml developing solution. The solutions were shaken together and the colour allowed to develop. Dissociation and decolourisation of the standards

Figure 2.   Calibration Graph for Desferrioxamine Standards.
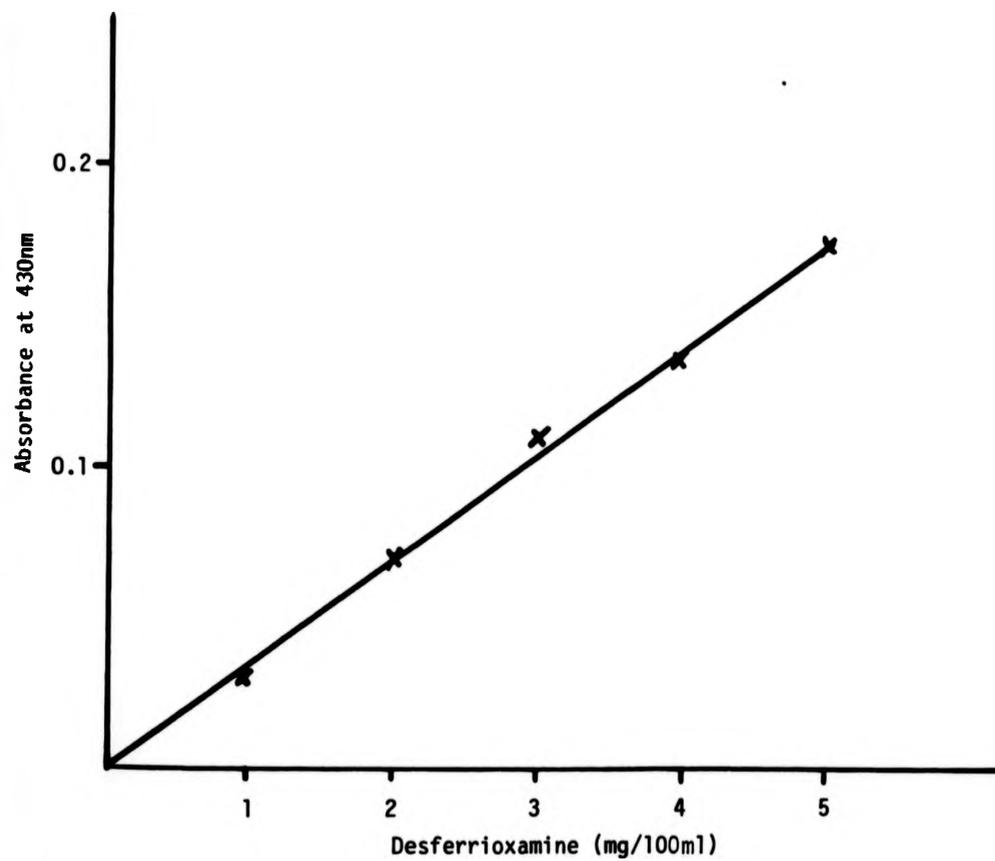
occurs after 24 hours, even in the presence of buffer, and fresh standards were prepared for each set of measurements. All samples were analysed within one hour of adding the developing solution, thus reducing experimental error, due to dissociation, to a minimum.

10.   CAERULOPLASMIN ASSAY.

The copper containing protein caeruloplasmin (ferrioxidase) catalyses the oxidation of some polyamines and its action on p-phenylenediamine was first used by Ravin (195) as a quantitative measure of its presence in serum. Rice (196), showed that the principal product of caeruloplasmin oxidation for p-phenylenediamine was Bandrowski's base (Figure 3).

A standard solution of p-phenylenediamine (5 g/l in distilled water) was prepared, and stored at 4°C. Stock solutions of 1M acetic acid (60ml glacial acetic acid/l) and 0.4M sodium acetate (54.4 g/l) were made up for the buffer solution. The acetate buffer (0.4M, pH 5.5) comprising, 1.2ml acetic acid and 20.0ml sodium acetate diluted to 50.0ml with distilled water, and stored at 4°C. Sodium azide solution, 5 g/l was used to inhibit the oxidation reaction.

A calibration curve for caeruloplasmin concentration was determined using Seronorm Protein Batch No. 103 (Nycomed (U.K.) Ltd.), containing 0.34 g/l caeruloplasmin. Aliquots of the protein were pipetted into four 10ml blood tubes; 0 ,50,

100, 150µl. Acetate buffer (8.0ml) and p-phenylenediamine
(1.0ml) were added to each tube, additionally 1.0ml sodium
azide was added to the blank solution. The solutions were
mixed together and placed in a water bath at 37°C for one
hour. After incubation, 1ml sodium azide was added to the
remaining three solutions, all solutions were shaken and
cooled at 4°C for 30 minutes. The absorption was measured at
530nm. A linear calibration graph was obtained from these
standards (Figure 4), and used for quantitative
caeruloplasmin determinations, which were carried out on
100µl serum, free from haemolysis and turbidity.

Figure 3. Oxidation of p-Phenylenediamine by Caeruloplasmin (Cp).
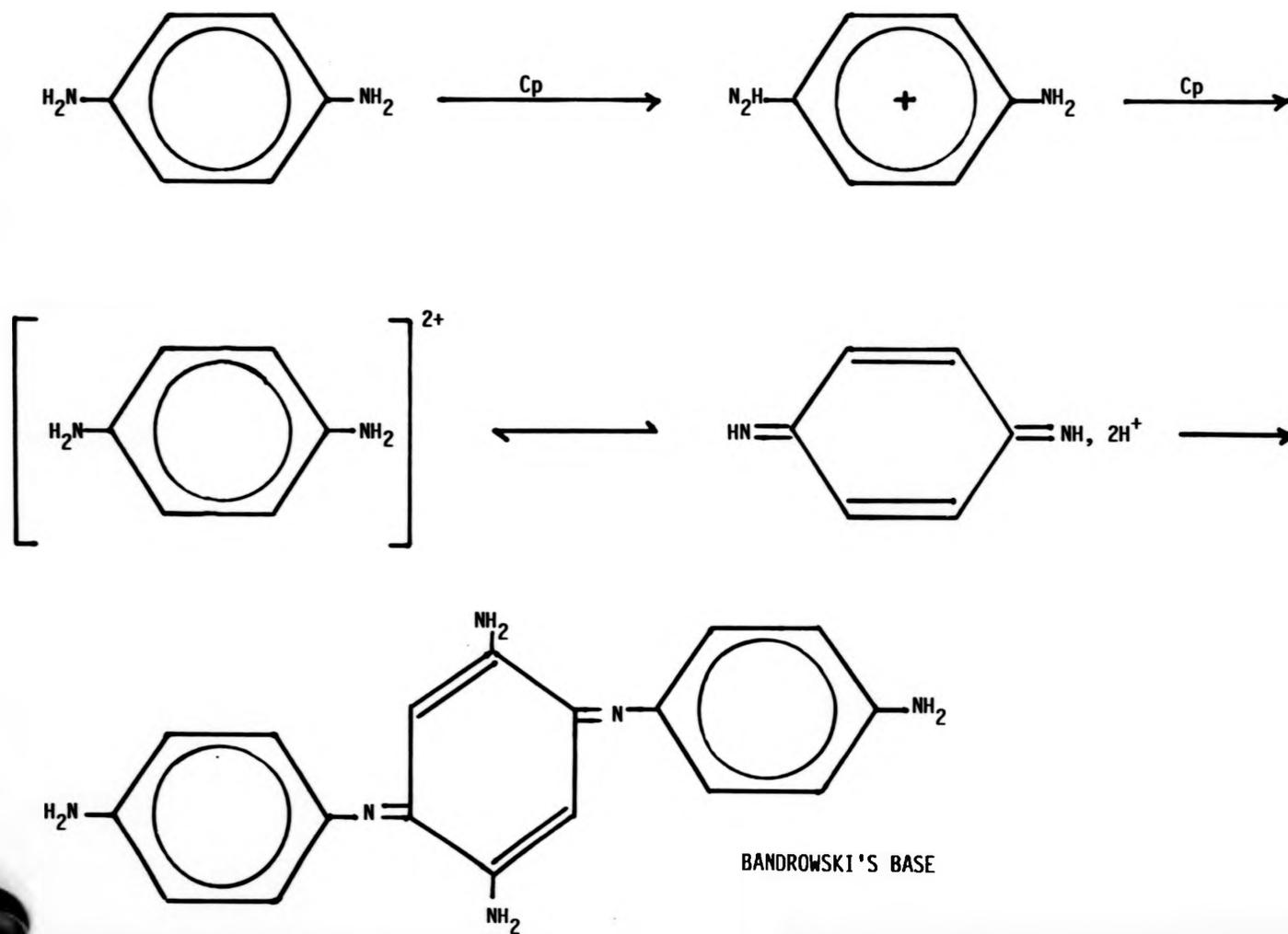
Figure 4. Calibration Curve for Caeruloplasmin Assay
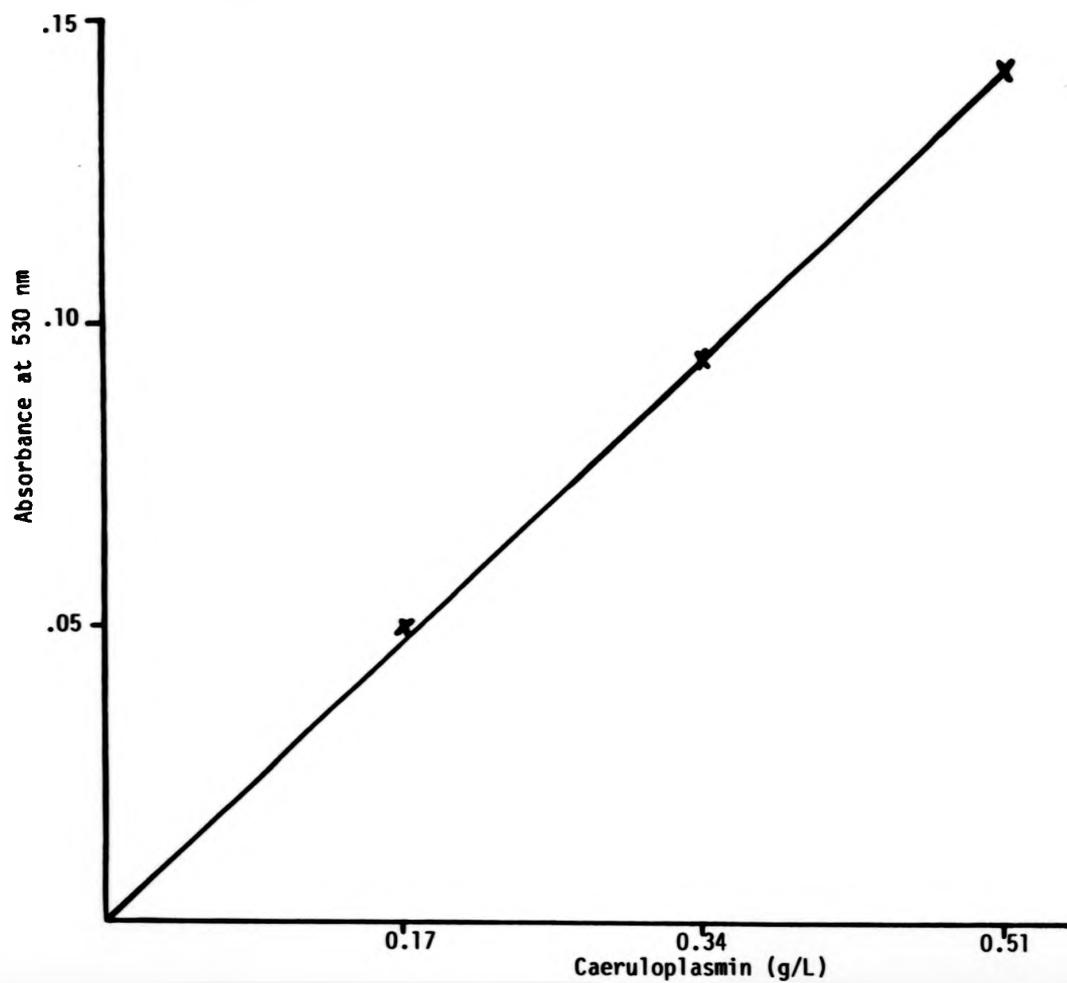
CHAPTER 3.

HAIR AS AN INDICATOR OF BODY METAL STATUS.

HAIR AS AN INDICATOR OF BODY METAL STATUS.

1. INTRODUCTION.

In diseases which affect trace element metabolism, such as renal failure, anorexia, hepatic cirrhosis and thalassaemia, there is a clinical requirement to attempt to monitor the total body loading of the metal affected by the disease. The determination of trace metal body status is usually attempted by analysis of blood or serum samples, but this procedure has several disadvantages, both practical and theoretical.

Clinically, the routine taking of blood samples for metal analysis, in addition to the regular samples required by the hospital to determine blood biochemistry, places an increased demand on red cell synthesis. It would be of benifit particularly to renal patients (who are often anaemic) if an alternative tissue could be found on which to carry out routine trace metal analysis, thus reducing red cell loss by venesection.

The use of blood analysis to derive trace metal status can also be criticised on biochemical grounds. In most cases, blood represents a transitory reservoir for an element, and blood concentrations of the element may reflect recent exposure as much as overall body load. Other tissues, for example bone and liver may offer a better measure of body load, but these require biopsy and may not be accessible for

routine analysis. Hair which is an accumulating tissue, and which is (usually) easily obtainable represents a possible compromise in this dilemma.

The usefulness of measurements of trace elements in hair has recently been reviewed by Taylor (197) and previously by authors who have indicated where hair analysis may be useful and have drawn attention to the associated problems with hair analysis (198-200).

This study of trace metals in hair was set up with two objectives:

(i) To determine whether levels of concentrations of aluminium, copper and zinc in hair are abnormal in renal patients on haemodialysis;

(ii) To determine whether hair and serum trace metal concentrations in renal patients are inter-related, with a view to using trace metal levels in hair as an indicator of body metal status.

2.   HAIR STRUCTURE AND CHEMISTRY.

Hair is a thread-like epithelial fibre formed from a cluster of specialised epidermal cells (201-203). These cells make up a soft bulb shaped follicle located in the dermis. The hair follicle is produced by extensive cell division at a point in the epidermis with the formation of a plug of cells (Figure 1). As cell multiplication continues, a tubular epidermal

structure is formed growing downward into the dermal layer.
The rounded base of the follicle begins to flatten and the
sweat and sebaceous glands begin to develop. The base of the
follicle then turns inward, enclosing specialised dermal
cells which form a dome-like papilla, this is the site of
further cell division and hair formation (Figure 1). During
the growth phase of the matrix cells there is intensive
metabolic activity within the follicle bulb which produces up
to 0.5mm of hair growth per day. As hair is formed it is
exposed to the metabolic environment of the matrix cells,
circulating blood and lymph and extracellular fluids.
However, hair is only in equilibrium with the matrix cells
during the time of its formation, 2 to 3 days.

Keritinisation. - The hair fibre is comprised of a thin outer
cuticle and an inner cortex (202, 203) (Figure 2). Hair cells
when formed are soft fluid-filled structures containing
nuclei. As the hair develops and moves up the hair follicle
and approaches the skin surface, the outer layer of cell
hardens and becomes impermeable, enclosing the metabolic
products of the matrix cells. As the inner cortex forms the
hair cells shrink due to dehydration, and the cell membrane
and nucleus degenerates. This occurs in the region of
keratinisation. Once the cell membrane has deteriorated,
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Figure 1. Hair Follicle Development
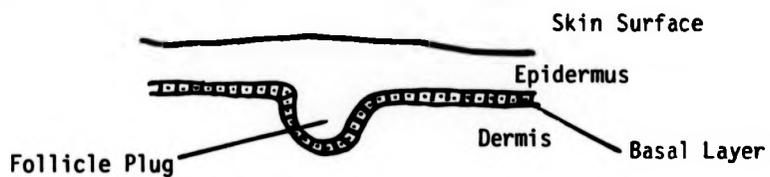
First stage of follicle development: downgrowth of epidermal cells into the dermis.
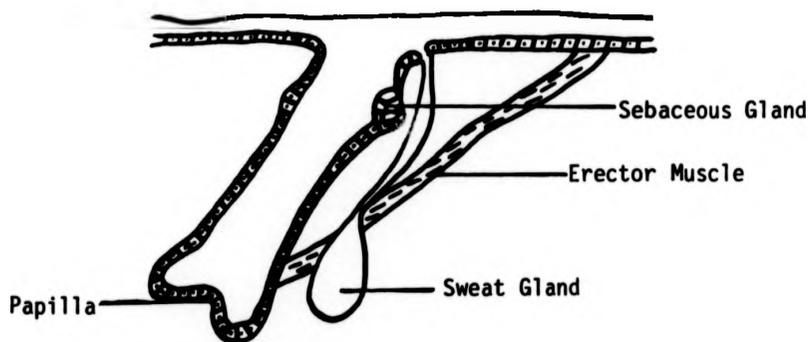
Later stage in follicle development: formation of the dermal papilla.

Figure 2.   Internal Structure of the Hair Shaft.

The cortical cells of the upper part of the follicle bulb are rich in ribosomes, structures vital for protein synthesis. The protein synthesised in the bulb is thought to be in the form of randomly distributed molecular coils. This protein contains thiol groups (-SH) but its overall sulphur content is low. The coils gradually become arranged in helices which form the fibrilar component of keritin. Further up the follicle another protein is synthesised, rich in sulphur; this forms the poorly organised matrix in which the helices are embedded.

Keritin is comprised of 18 different amino acid residues, the sulphur-containing cystine being the most abundant. Keritinisation is the oxidative process in which dithiol cross linkages form between adjacent pairs of cystine groups lying in separate long chain proteins.

Keritin is a highly stable fibrous protein, which is remarkably resistant to enzymic digestion and all but the most vigorous reagents, e.g. strongly oxidising or alkaline solutions. The keritin matrix is able to absorb water vapour up to 35% of its mass, before beginning to feel wet (202). This water is exchangeable, and the amount taken up is dependent on environmental humidity. As moisture is absorbed into the hair fibre, trace elements in solution may be incorporated into the matrix. This exogenous source of trace metals is difficult to remove. Washing methods which remove these bound metals may also remove endogenous metals from the keritin matrix.

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The Hair Follicle Cycle. - Individual hairs from the same person, even when in close proximity to each other, may have different trace element compositions. This is due to their different growth cycles.

Human hair follicles have independent growth cycles (202, 203). In any given region of hair there are follicles in each of the main stages of activity. Anagen is the stage of follicle regeneration and active hair growth. Catagen is the retrogressive stage when the hair fibre is no longer growing. This lasts for about 2 days. Telogen is the final inactive stage, commonly called the resting phase. This is the longest phase in the cycle. As a result of this cyclical activity, only hairs which grow in follicles that are in the growth phase at the same time will have similar trace element composition.

Endogenous Sources of Trace Elements. - The glandular components of the hair follicle are the principal sources of endogenous trace elements in hair (201). Glands are conditioned by metabolic and environmental variations. The sweat glands are affected by temperature, humidity and the degree of internal hydration and the type of sebaceous secretions are dependent on age and sex. Sweat glands remove large amounts of copper from the body, and the more active the gland the greater the amount of copper removed by this route, and perhaps in turn, incorporated into the hair filament.

Natural hair colour also affects the levels of trace metals found in the hair (200). Blond hair has less iron and manganese than brown hair, and red hair contains more iron, copper and zinc than any other colour hair. Copper in hair is associated with melanin, the complex responsible for hair pigmentation. Copper is also present in hair in two metaloenzymes, tryosinase and lysyl oxidase (202). The former catalyses the first step of the conversion of tryosine to melanin, via Dopa (204). This suggests that hair containing no pigment would have lower copper levels than hair with pigment. Zinc deficiency produces depigmented hair, which on supplementation, is restored to its natural colour (205). The role of zinc in melanin formation is not known. The high level of zinc in melanised structures and the restoration of pigmentation following zinc therapy suggests that zinc may have a more critical role in melanin synthesis than copper.

Exogenous Contamination. - Exogenous trace elements found on the hair shaft originate from a number of different sources: the atmosphere (206-210), solutes present in water used for washing or swimming (211-213), and hair treatments used for cosmetic, medical and hygenic purposes (200, 211, 213-215). Elements deposited directly onto the hair shaft become incorporated into the hair and are not easily removed by washing. For example, this results in very high selenium (211) and zinc (213) hair levels when scalp medications are used regularly containing these metals. Green hair has also been reported, resulting from high levels of copper in

swimming pool water (when copper sulphate is present as a
algaecide) (212).

Effect of Cosmetic Treatment. - The longitudinal variation of
certain elements is largely caused by cosmetic treatment of
hair. The term "cosmetic treatment" includes any preparation
applied to the hair, such as shampoo, hair and scalp
medication, bleaching, dying and permanent waving. A number
of studies report the effect of various cosmetic treatments
on hair trace metal levels (200, 211, 213-215). These studies
yielded variable results. Hinderbrand (215) confirmed that
the treatments used in his study were representative of the
many types of treatment available, and stated that different
brands might have given different results.

Bleaching and permanent waving have a marked effect on both
hair zinc and copper levels (213-215); these treatments are
more commonly, but not soley, attributed to the female
population. In this way changes in trace element levels due
to hair treatment complicate the interpretation of results
compared for sex differences.

3.   TRACE METALS IN HAIR.

There is considerable interest in the use of hair as an index
to body trace metal status, and in the exposure of
individuals to trace metals at work and from the environment.
The hair shaft has been termed "the recording filament of the

body" (216), and can provide a history of trace element levels of individuals and their exposure to certain elements. This has been used in forensic investigation (217). In some respects, hair is an ideal tissue for use in diagnosis, it is readily obtainable without pain or trauma, and it requires no special equipment for sampling or storage. Once sampled hair does not deteriorate and can be stored for many years.

Most body tissues are in a state of dynamic equilibrium. Unlike every other body tissue, hair and nail tissues once formed are no longer in a state of equilibrium with the rest of the body. Thus, the endogenous components of a hair shaft reflect the metabolic events that occurred during the relatively short time of its formation. In principle, the hair components comprise an historical record covering several weeks/months.

A wide range of hair aluminium levels have been reported, in humans (210) and in rabbits (218) following aluminium exposure. Rees (210) reported elevated hair aluinium levels in severely delinquent, psychotic and prepsychotic children living down-wind from an Alcan aluminium factory in Albany, California. She concluded that children become mentally disturbed when exposed to high levels of aluminium in the environment. Yokel (218) observed a rise in hair aluminium levels in rabbits injected with aluminium lactate. The increase in hair aluminium did not correlate with the dose of aluminium given nor with the amount of fur produced. Similar

doses of aluminium produced variable uptake and storage in hair. However, animals exposed to excessive aluminium showed very large increases in hair level. Yokel (218) concluded from his animal studies that hair could be a useful indicator of aluminium loading in humans in aluminium induced conditions such as dialysis encephalopathy. Maurumo (219) found raised hair aluminium levels in dialysis patients but no correlations between hair and serum levels were indicated.

McBean et al (220) determined zinc concentrations in paired hair and plasma samples in 75 Iranian children on zinc deficient diets, and in 26 age matched American children on adequate zinc diets. The correlation between zinc levels of paired plasma and hair was insignificant, and he concluded that hair analysis is not a reliable indicator of body zinc stores in children. Chittleborough and Steel (201) found no significant increase in beard zinc level when oral zinc sulphate was administered over a period of two months. They concluded that the concept of hair as a trace element monitor was a simplistic one.

In contrast, Prasad (221) showed that marginal zinc deficiencies in the diet can be identified by below-normal concentrations of zinc in hair. Other investigators have also related low zinc intake with decreased hair zinc levels both in rats (222, 223) and humans (223-225). Variations in hair zinc levels have also been reported according to age, sex, race and geographical location (200, 205, 220, 226, 227). Severe zinc deficiency is generally characterised by

extensive hair loss, with any remaining hair having normal zinc concentrations (221). Prasad concluded that hair zinc levels may only be of use in determining marginal zinc deficiency.

Normal hair copper levels have been reported in two cases of gross copper deficiency in children under one year of age (228, 229). Copper supplementation restored serum copper levels to within normal range with no increase in hair copper concentration. Similarly, in Wilson's disease (230) (an inherant defect in copper metabolism which results in the accumulation of excessive amounts of copper) hair copper levels remain within the normal range. The considerable variation seen in hair copper levels is probably independent of body stores and may be more closely related to the rate of hair growth (229). Copper containing enzymes are known to play key roles in hair formation and in the production of melanin pigments (202, 231). Bradfield (229) noted that in periods of copper toxicity, hair and nails act as an additional excretory pathway. He concluded that this observed response to toxicity may be of use in diagnosis, but should not be interpreted as a linear response between body stores and hair metal content.

Petering et al (226) reported that zinc and copper concentrations in hair varied with age and sex, with higher concentrations of hair copper being found in female subjects compared with male subjects (200, 205, 226, 232). Deeming and Weber (31) reported that hair copper levels were decreased

and serum copper levels increased in women taking oral
contraceptives, paralleled with increased hair zinc levels
and decreased serum zinc levels. These changes in serum
copper and zinc concentration are due to the well documented
antagonistic relationship between the two metals (151, 153).
Similarly, other drugs may also affect trace metal metabolism
resulting in increased complexity in the interpretation of
tissue trace metal analysis.

Longitudinal trace metal variation of the hair shaft has been
reported (200, 203, 214, 217, 226, 233-235) in addition to
variation due to age, sex and geographical location (200,
205, 220, 226, 227). Alder (233) found increased zinc levels
towards the distal end of the hair shaft with fairly constant
levels of cobalt and copper. This was in contrast to the
findings of Renshaw (217), and Hambidge (235) who observed an
increase in distal end copper levels. In a multi-element
study of human scalp hair, analysing for Ni, Co, Si, Mn, Al,
Cu, Ag, Cr and Fe (234), in almost all cases trace element
levels were lower near the root compared to distal levels,
copper being the only exception. Hair is metabolically
inactive after it emerges from the hair follical. This
suggests that longitudinal variation of the hair shaft is
from external sources such as environmental contamination and
cosmetic treatment, and not due to endogenous sources.
Longitudinal variation is not unexpected because of the
ion-exchange nature of the hair fibre.

The most reliable results indicative of environmental

exposure have been from heavy metal pollutants such as lead, arsenic, cadmiumn and mercury (205, 206, 208, 209).

4.   METHOD OF ANALYSIS.

Standardised Pre-treatment. - The question of whether or not to wash hair samples prior to analysis is a subject of great debate. A wide range of washing procedures have been discribed in the literature, from the use of distilled water only to complicated methods using organic solvents to remove oil, followed by detergents and chelating agents to strip and sequester complex trace metals.

The International Atomic Energy Agency (IAEA), in an attempt to standardise hair analysis and to make the comparison of results feasible, has recommended a wash procedure using acetone and water only. The use of acetone has been critisised (236) because other solvents remove more trace elements. The use of more stringent washing procedures may in fact remove endogenous trace elements. A number of comparative studies on washing procedure have been carried out (215, 218, 233, 236, 237). Although these experiments provide a comparison between washing agents no conclusion can be drawn as to which method provides the best wash. The IAEA method may not be ideal but it does facilitate the comparison of results from other studies when the same pre-treatment wash is used.

Comparison of Washing Procedures. - In the present study two washing methods were selected to compare the effect of different washing procedures on hair trace metal levels. The two methods used were:

1) A simple acetone, water (x3), acetone wash. (This wash method was later selected as the standard wash procedure, and is the method suggested by the IAEA and used by Stevens (238));

2) A two stage wash procedure consisting of an organic and detergent wash, acetone, chloroform, acetone, water, 5% Decon 90, water (x2).

In method 1, the initial acetone wash was intended to remove the exogenous oily film from the hair, then water to wash off trace metal contamination and finally acetone to remove the water and aid drying. The first step in method 2 was an organic wash to remove the oily layer from the hair shaft, and the second step was an inorganic detergent wash to remove positively charged ions adhered to the hair filament, and finally water to wash off the detergent.

Experimental. - Two batches of hair were taken from different subjects KH and CH. The batches of hair were then subdivided into five portions. Each of the five portions were subjected to one of the following pre-treatment procedures:

i) No treatment, unwashed hair sample.

ii) Acetone wash only.

iii) Acetone, chloroform, acetone.

iv) Acetone, chloroform, acetone, water, 5% Decon, water

x2.

     v) Acetone, water x2, acetone.

Washed hair samples were dried at 60°C and digested in 5% TMAH (238). Full details of this digestion procedure are given later in this Chapter.

    Results. - Unwashed hair samples contained 10.0 and 14.9 µg/g aluminium in samples CH and KH respectively. The percentage aluminium removal in each washing procedure varied between 63-66% for sample CH, which lies within the analytical variation of the analysis and between 61-81% from sample KH, which lies outside the precision of the analysis and may be due to external contamination of the original sample.

Washed hair samples contained between 25.9 and 30.8 µg/g copper compared to 25.8 µg/g in the unwashed CH sample, and wash samples from KH contained between 13.1 and 18.7 µg/g copper compared to 17.2 µg/g before washing. These variations in copper levels lie within the analytical precision of the experiment and indicate that copper is not removed during any of the washing methods.

Unwashed hair contained 184 and 154 µg/g zinc from CH and KH respectively, washed samples ranged between 182-186 µg/g for CH and between 147-166 µg/g for KH. This comparison showed that no zinc was removed from the hair during any of the washing procedures. These results are summarised in Table 1.

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Table 1. Results for the Comparative Study of Different
Washing Procedures.

SUBJECT	TRACE ELEMENT µg/g	(i)	WASHING	PROCEDURE		
			(ii)	(iii)	(iv)	(v)
	Al	10.0	3.7	3.4	3.7	3.4
CH	Cu	25.8	30.8	26.6	26.6	25.9
	Zn	184.0	183.0	186.0	182.0	183.0
	Al	14.9	2.8	4.0	4.2	5.4
KH	Cu	17.2	13.1	14.0	14.1	18.7
	Zn	154.0	153.0	147.0	154.0	166.0

Discussion. - From these results it was concluded that the majority of aluminium found in an unwashed hair sample may be exogenous metal contamination. This environmental contamination may be removed from the hair sample by washing. A sample treated with the two step washing method (method 2) lost 63% of the total during washing, compared with 65% removed in the single step washing method (method 1). Since the same amount of aluminium is removed in both methods, the simplest and quickest method was selected for future sample preparation (method 1). Washed hair samples contained effectively the same levels of copper and zinc as unwashed hair samples. This finding suggests that copper and zinc are endogenous to the hair shaft and are not leached from the hair during washing. These results indicated that there was little or no variation between the two washing methods compared. Thus, method 1 was selected as the future washing procedure, enabling us to compare our results with those of other workers who use the IAEA procedure.

Method Employed. - In this study hair samples were collected from 12 renal patients on haemodialysis and 12 healthy volunteers. Hair was taken from the nape of the neck, close to the skin. All sampling and sub-division was done using stainless steel scissors. The sub-samples were washed sequentially in acetone, water (x3) and acetone (IAEA method). Each wash comprised a 10 minute soak followed by 10 minutes ultrasonic agitation. The washed samples were finally dried at 60°C.

Prepared hair samples (ca. 200mg) were treated with 5%
tetramethyl-ammonium hydroxide in water (TMAH; 2ml) and
incubated at 60°C for 2 hours until sample digestion was
complete. The cooled samples were then diluted with 10ml
absolute ethanol. The final solutions were analysed for
aluminium and copper by direct injection graphite furnace
atomic absorption spectrometry (GFAAS; Perkin Elmer model
HGA-500 furnace and 703 spectrometer) against combined Al and
Cu standards perpared in TMAH/ethanol. Zinc was determined by
flame AAS, also against TMAH/ethanol standards.

Blood samples were also collected from each of the renal
patients at the time of hair sampling. Serum was diluted
appropriately (aluminium, x4; copper, x20) prior to analysis
by GFAAS. Details of serum analysis are given in Chapter 2.

Reproducibility of Analytical Method. - A large sample of
hair was obtained from a single person and washed and dried
by the standard washing procedure. Samples (ca.200mg) were
weighed into individual polypropylene vials. Each sample was
digested with TMAH (2ml) and made up to volume (12ml) with
ethanol. The six samples were analysed in duplicate for
aluminium, copper and zinc. The results of this in-batch
analysis are presented in Table 2.

Two samples were then reanalysed five times each to determine
the precision of the analysis. These results are shown in
Table 3.

Table 2. Within-batch Precision of Hair Analysis.

	ALUMINIUM	COPPER	ZINC
MEAN VALUE ($\mu g/g$)	21.87	28.66	217.1
S.D. ($\mu g/g$)	1.51	0.82	17.8
C.V.(%)	6.9	2.9	8.2

Table 3. Between-batch Precision of Hair Analysis.

	SAMPLE NUMBER	ALUMINIUM	COPPER
MEAN VALUE ($\mu g/g$)	1	21.43	27.60
	2	20.82	27.60
S.D. ($\mu g/g$)	1	1.56	0.69
	2	1.18	0.75
C.V.(%)	1	8.0	2.8
	2	6.3	3.1

Table 4. Recovery of Added Aluminium and Copper in Hair Analysis.

SAMPLE	ALUMINIUM (µg/l)			COPPER (µg/l)		
	ORIGINAL SOLUTION	SPIKE	% RECOVERY	ORIGINAL SOLUTION	SPIKE	% RECOVERY
A	6.19	82.1	86.6	55.7	83.7	87.2
B	6.19	83.8	92.3	55.7	85.3	81.9
C	6.19	82.1	90.9	55.7	83.3	87.4

Known amounts of aluminium and copper were added to three
different hair samples and the percentage recovery was
determined. The results of this investigation are shown in
Table 4.

5.   RESULTS.

Aluminium Analysis. - Aluminium levels were widely
distributed in the renal patients and control groups. Control
values ranged between 0.3 and 16.6 µg/g and from 0.5 to 11.9
µg/g in renal patients. Mean hair aluminium values were 6.17
± 4.21 µg/g and 5.34 ± 3.83 µg/g for controls and patients
respectively. This difference was not significant, and hair
aluminium levels were found to be randomly distributed,
(Figure 3). A plot of hair aluminium (µg/g) against serum
aluminium (µg/l), in renal patients showed no correlation (p
= 0.05)   (Figure 4).

Copper Analysis. - Hair copper levels were more widely spread
in the group of patients compared to controls. Patients hair
copper levels ranged between 8.0 and 37.0 µg/g and controls
from 8.0 to 25.0 µg/g   (Figure 3). Mean hair copper values
were 14.35 ± 9.56 µg/g and 12.24 ± 5.32 µg/g for patients and
controls respectively,showing no significant difference.
There was no correlation between hair and serum levels when
hair copper (µg/g) is plotted against serum copper (µg/l)
(Figure 5).

Figure 3. Hair Aluminium, Copper and Zinc in Renal Patients and Control Subjects.
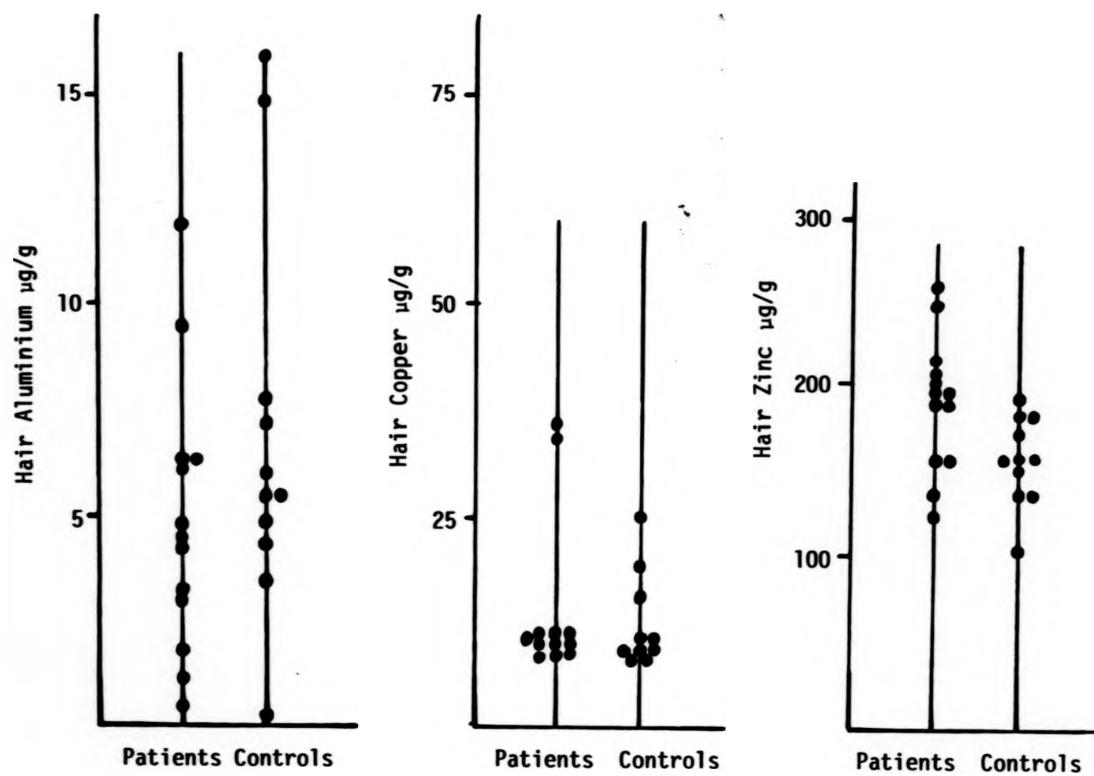
Figure 4.   Serum Aluminium (µg/L) Plotted Against Hair Aluminium (µg/g).
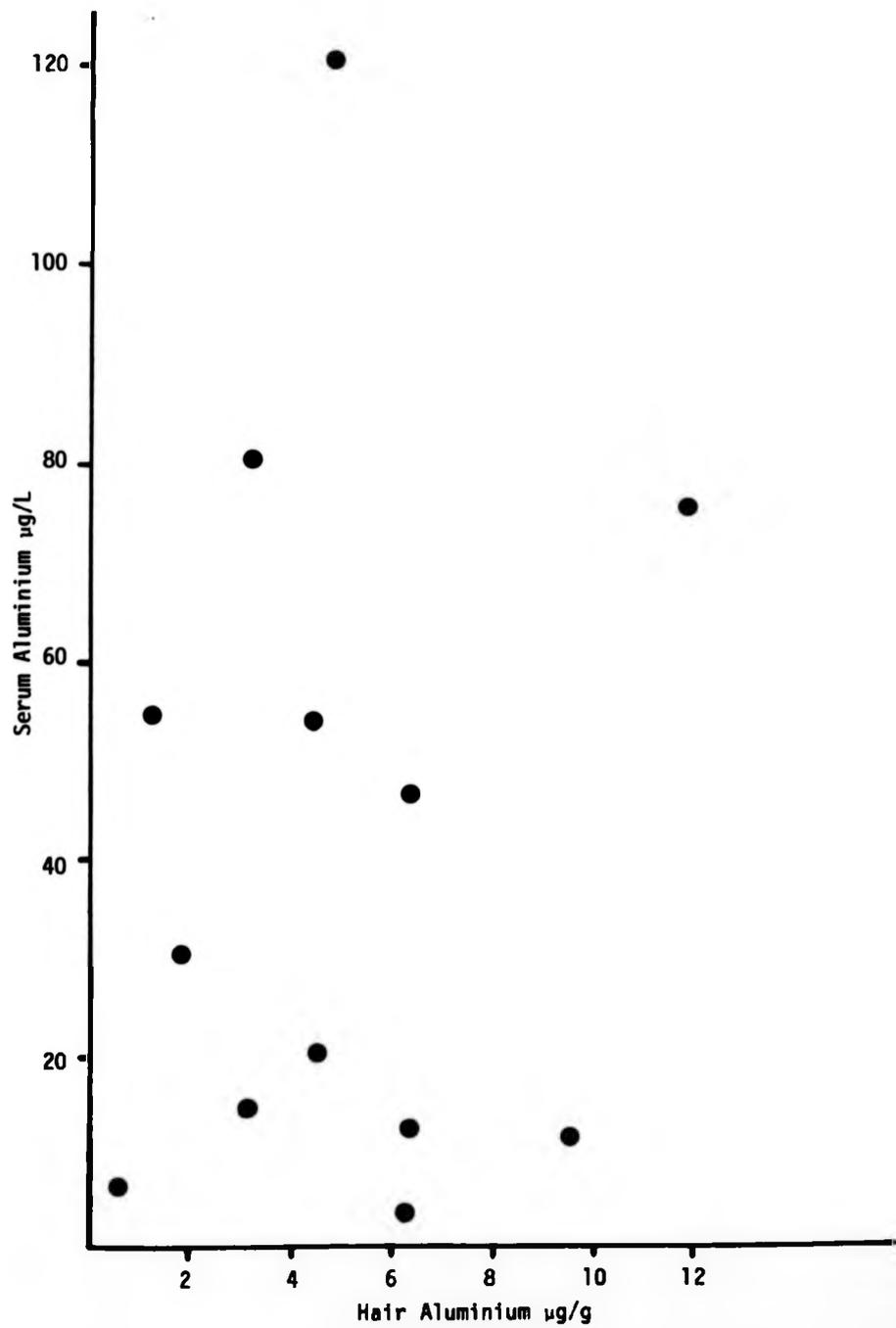
Figure 5. Serum Copper (μg/L) Plotted Against Hair Copper (μg/g)
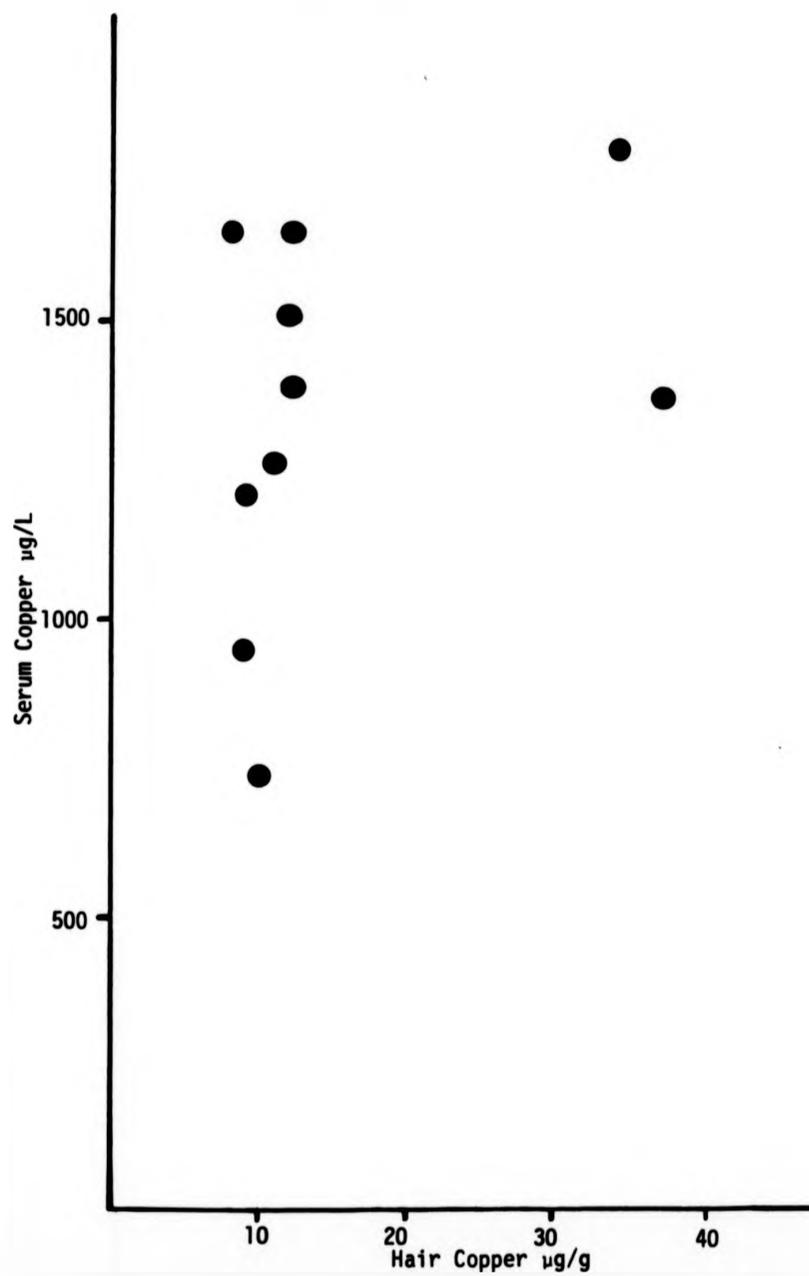
Zinc Analysis. - Hair zinc levels were more widely
distributed in renal patients compared with controls, 128.0
to 262.0 µg/g and 107.7 to 196.0 µg/g for patients and
controls respectively (Figure 3). Mean hair zinc values were
also higher in patients (195.1 ± 38.8 µg/g) than controls
(161.2 ± 25.5 µg/g). This difference was just significant, at
the 0.05 level of probability.

6. DISCUSSION.

Aluminium, copper and zinc concentrations can be determined
reproducibly in human hair by straightforward sample
preparation and analysis procedures. This method could be
used for routine clinical monitoring.

Concentrations of aluminium and copper in hair were found to
be widely distributed (Figure 3), with no apparent
distinction between patients and controls. These observation
are in contrast with those of Yokel (218), who found a
correlation between hair aluminium and increased exposure due
to injected aluminium solutions in rabbits, and who suggested
the possible use of hair for aluminium monitoring in humans.
Additionally Maurumo et al (219) reported raised hair
aluminium in dialysis patients, although no correlation
between hair and serum was suggested.

No correlation was found between hair and serum copper levels

(Figure 5). This finding is in agreement with Graham (228) and Bradfield (229), who reported normal hair copper levels in children who were severly copper deficient. The lack of correlation between hair and serum levels, and the wide range of hair copper values suggests that hair copper is independent of body stores and trace metal status and may be more closely related to hair production needs.

The increased level of hair zinc in renal patients over the control group was unexpected. Renal patients on dialysis are often reported to be zinc deficient (153, 164, 165, 177, 178), and zinc supplementation in renal failure improves symptoms often related to zinc deficiency (poor apatite, reduced taste acuity and poor wound healing) (179-240).

In the case of aluminium, serum concentrations for renal patients ranged from 3 to 120 $\mu g/l$. Patients with serum levels above 60 $\mu g/l$ are normally regarded as being at risk from aluminium accumulation. Several of the patients in this study were known to have had high levels of aluminium exposure in the past with elevated serum aluminium levels, but this increased exposure was not detected in hair. The almost total lack of correlation between hair and serum aluminium indicates that hair is not a suitable material to replace (or augment) serum as a substrate for analysis for aluminium monitoring. An increase in hair aluminium is more likley to have resulted from environmental exposure or sample contamination then increased body loading.

CHAPTER 4.

ALUMINIUM AND COPPER MOBILISATION IN RENAL PATIENTS USING
DESFERRIOXAMINE CHELATION TO REDUCE ALUMINIUM OVERLOAD.

ALUMINIUM AND COPPER MOBILISATION IN RENAL PATIENTS USING DESFERRIOXAMINE CHELATION TO REDUCE ALUMINIUM OVERLOAD.

1. INTRODUCTION.

The iron chelating agent desferrioxamine (DFO) is now used extensively for the treatment of renal patients suffering from aluminium overload (70, 97, 120, 127, 241). The first use of desferrioxamine for aluminium chelation (70) was prompted by the chemical similarities between Al(III) and Fe(III), together with the existing knowledge of the properties of desferrioxamine in relation to iron chelation (146, 242) and haemodialysis. Since that time, a number of investigators have examined the chemistry of aluminium complexation by desferrioxamine, both in vitro (117, 148) and in vivo (118, 122, 243), and the mode of action of the drug is now more fully understood.

We have observed that serum copper levels are raised during chelation therapy, and that copper is removed in the dialysate during haemodialysis. The increase in serum copper levels probably results from indirect mechanisms, e.g. induction of an acute phase response by DFO, rather than from desferrioxamine-copper complexation per se. The interaction of DFO with the metabolism of other elements suggests that there may be possible side effects resulting from the depletion of essential elements, during chelation therapy. By monitoring the effect of desferrioxamine on essential trace

element metabolism, deficency side effects can be avoided.

2.   DESFERRIOXAMINE COMPLEXATION IN VITRO AND IN VIVO.

Desferrioxamine B is a trihydroxamic acid obtained from the
Actinomycete, Streptomyces pilosus (143). The molecular
structure of desferrioxamine is shown in Figure 1. The
compound is obtained as the methane sulphonate, $(H_4DFO)^+$
$(CH_3SO_3)^-$. The cation can undergo progressive deprotonation
and at pH 7 the major species in solution is the doubly
charged anion, $(HDFO)^{2-}$. This anionic species is the most
important in the formation of complexes with the dipositive
and tripositive metal ions in neutral solution (244). When
desferrioxamine complexes with Fe(III), the high spin complex
$[Fe(HDFO)]^+$, ferrioxamine (FO), is formed in which the metal
ion is chelated by the three oxamine groups to give six
co-ordinate Fe(III), in an approximately octahedral
arrangement (Figure 2). The formation constant (10^{31}) is
unusually high for this type of complex (which will have zero
ligand field stabilisation energy (147)), and the high
stability presumably arises from the electrostatic
interactions between the metal cation and the
negatively-charged O-Donor atoms. Factors which stabilise the
ferrioxamine complex might also apply to the formation of a
complex between desferrioxamine and the non-transition metal
aluminium. The Al(III) ion has no ligand field stabilisation,
is of identical charge and of similar ionic radius to that of
Fe(III), and therefore could plausibly form a 1:1 complex of

Figure 1. Desferrioxamine B $(H_4 DFO)^{\pm}$
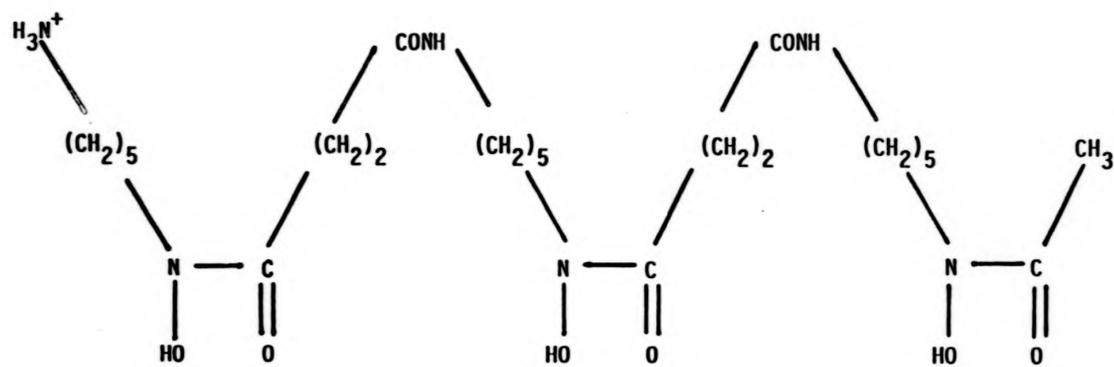
Molecular Mass = 561

Figure 2. Ferrioxamine B
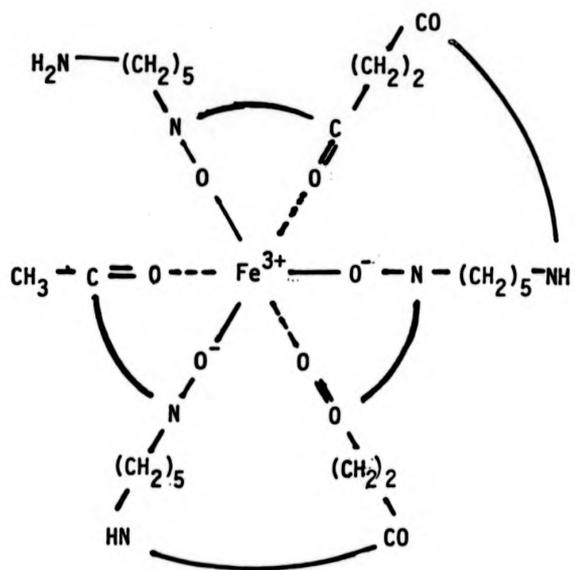
Figure 2. Ferrioxamine B
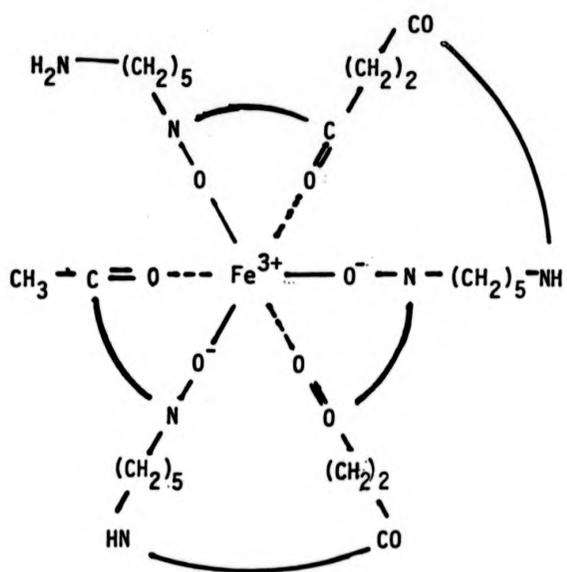
similar structure and stability to ferrioxamine. On this
basis the clinical use of desferrioxamine was suggested for
aluminium chelation.

The stability constants for complexes of desferrioxamine with
various metal ions are given in Table 1 (146, 148, 149). The
data substantiates the chemical arguments for the formation
of a stable Al-DFO complex in vivo. and additionaly indicate
the possible complexation of other physiologically important
cations, eg. copper. In vivo these three metals, Fe(III),
Al(III) and Cu(II) are strongly bound to various proteins and
other potential ligands, such as amino acids, ionic citrate
and phosphates. The appearance of metal-desferrioxamine
complexes in extracellular fluid depends ultimately
therefore, on the relative values of the various stability
constants, and kinetic factors such as rates of release of
metal ions from their binding sites, and the degree of
saturation of metal carrier proteins, i.e. the site of the
metal storage pool (245). The stoichiometries, stabilities
and dialysis properties of complexes of desferrioxamine with
Al(III) and Cu(II), have been studied under physiological
conditions in vitro (117, 132, 148,). Al(III) forms a 1:1
complex analogus to ferrioxamine, but with rather lower
stability (Table 1). The measured clearance of this complex,
in standard dialysate solution using a calibrated
haemodialyser, corresponds to an effective molecular mass of
ca. 600 daltons, close to the theoretical value for the 1:1
complex (132, 246).

Table 1. Stability Constants of Various Metal Complexes (143).

METALLIC ION	DFO	EDTA	Transferrin
Fe^{3+}	10^{31}	10^{25}	10^{27}, 10^{29}
Al^{3+}	10^{22}	10^{16}	10^{18}
Cu^{2+}	$10^{14}/10^{20}$	10^{19}	
Zn^{2+}	10^{11}	10^{16}	
Co^{2+}	10^{11}	10^{16}	
Ni^{2+}	10^{10}	10^{19}	
Fe^{2+}	10^{10}	10^{14}	
Mn^{2+}	10^{4}	10^{9}	
Ca^{2+}	10^{2}	10^{11}	

In contrast, copper (II) forms two complexes with desferrioxamine of stoicheometries 1:1 and 3:2 (Cu:DFO). At physiological concentrations, if chelation occurs, the 1:1 complex would be the more likely to form. In view of the high binding constants of Cu(II) for its carrier proteins (e.g. caeruloplasmin), amino acids and other ligands (157), copper-desferrioxamine complexation in serum seems unlikely. However, in this study we report observations of copper mobilisation during chelation therapy for aluminium overload.

3.   CASE STUDIES.

Four patients (BD, AM, GB and RP) who had been on dialysis for over seven years were found to have one or more of the aluminium-related diseases, dialysis encephalopathy, osteodystrophy and microcytic anaemia. Each of the patients had taken aluminium hydroxide ("Aludrox" or "Alucaps") as a phosphate binder since commencing dialysis therapy. These patients had undergone home dialysis without water treatment until about 1980. Initially each patient was prescribed a twelve week course of desferrioxamine therapy, and their clinical status was assessed after this period.

Prior to desferrioxamine treatment, patient BD exhibited signs of dialysis encephalopathy with speech disturbance, and signs of bone disease. He complained of generalised bone pain, suffering fractures of the ribs and right thumb and showed signs of proximal myopathy. His serum aluminium level

was ca. 30 µg/l before chelation therapy, which increased to a maximium value (ca. 180 µg/l) after the initial loading dose of DFO.

Patient AM had symptoms of dialysis encephalopathy, speech disturbance and deafness. This patient also had a microcytic anaemia and required regular blood transfusions to maintain a haemoglobin level above 4 g/dl. Serum aluminium was ca. 20 µg/l before desferrioxamine, rising to ca. 280 µg/l after the first DFO infusion.

At the start of chelation therapy, patient GB had signs of osteomalacia, with a heavy deposit of aluminium demonstrated histochemically by solachrome staining (82). This patient commenced treatment with only mild dialysis encephalopathy and no signs of anaemia. He reported no bone pain and had a haemoglobin level of 14 g/dl. His serum aluminium concentration was ca. 50 µg/l, which increased to ca. 250 µg/l after the DFO loading dose.

Patient RP showed signs of dialysis encephalopathy with speech impairment before chelation therapy, and suffered from osteomalacia and osteofibrosis. This patient also had a long history of convulsions. His serum aluminium level was ca. 10 µg/l before desferrioxamine, this level increased markedly to ca. 320 µg/l after the DFO loading dose.

4. DESFERRIOXAMINE TREATMENT.

Before commencing chelation therapy with desferrioxamine, it
is now standard practice to administer an initial loading
dose. Fournier et al (29) concluded that the DFO test gives a
good indication of bone aluminium levels in uraemic patients
who were moderately overloaded with aluminium from the use of
phosphate binders. Thus, the four patients were given a
loading dose (6g) of DFO initially, followed by 2g each
dialysis (3 times per week). DFO was infused in 500ml normal
saline into the arterial line of the dialyser during the
first two hours of each six hour dialysis. The mobilisation
and removal of both aluminium and copper was studied, and the
removal of desferrioxamine through the dialyser and changes
in serum caeruloplasmin levels were monitored.

Blood samples were taken pre and post dialysis, starting one
week before DFO treatment commenced. Serum and red cells were
separated by centrifugation. Serum was diluted with nitric
acid prior to trace metal determination by GFAAS. Red cells
were washed and digested prior to analysis. Metal transfer
during dialysis was determined from the analysis of dialysate
solutions collected pre and post dialyser over successive 10
minute intervals using an automatic fraction collector.
Desferrioxamine removal was determined by spectrophotometric
analysis of waste dialysate solution, and serum
caeruloplasmin levels were determined at intervals during
chelation therapy. Full analytical details are described in
Chapter 2.

5. RESULTS.

Serum Metal Levels. - The changes in serum aluminium and copper during DFO treatment in patient BD are shown in Figure 3; similar behaviour was observed in the other three patients. Both aluminium and copper concentrations returned to pre-treatment levels within nine days of the last DFO infusion.

A rapid rise in serum aluminium occurred during the first 24 hours following the loading dose. Subsequently, serum aluminium levels fell during a dialysis session, and rose again between sessions. This cyclical pattern of variation has been seen previously and is typical of a thrice weekly DFO infusion. Serum aluminium rises between dialysis sessions as free DFO removes aluminium from its storage sites and mobilises aluminium into serum. As the DFO complex, high serum aluminium levels are well tolerated until the following dialysis session, when the Al-DFO complex is removed through the dialyser.

Serum copper also increased immediately following the loading dose, but the highest levels of serum copper (in the case shown ca. 1500 µg/l, an overall increase of 50%) were not reached for two to three weeks. In contrast to aluminium, serum copper levels did not show the same short term varation.

Figure 3.   Serum Metal Levels During DFO Treatment.
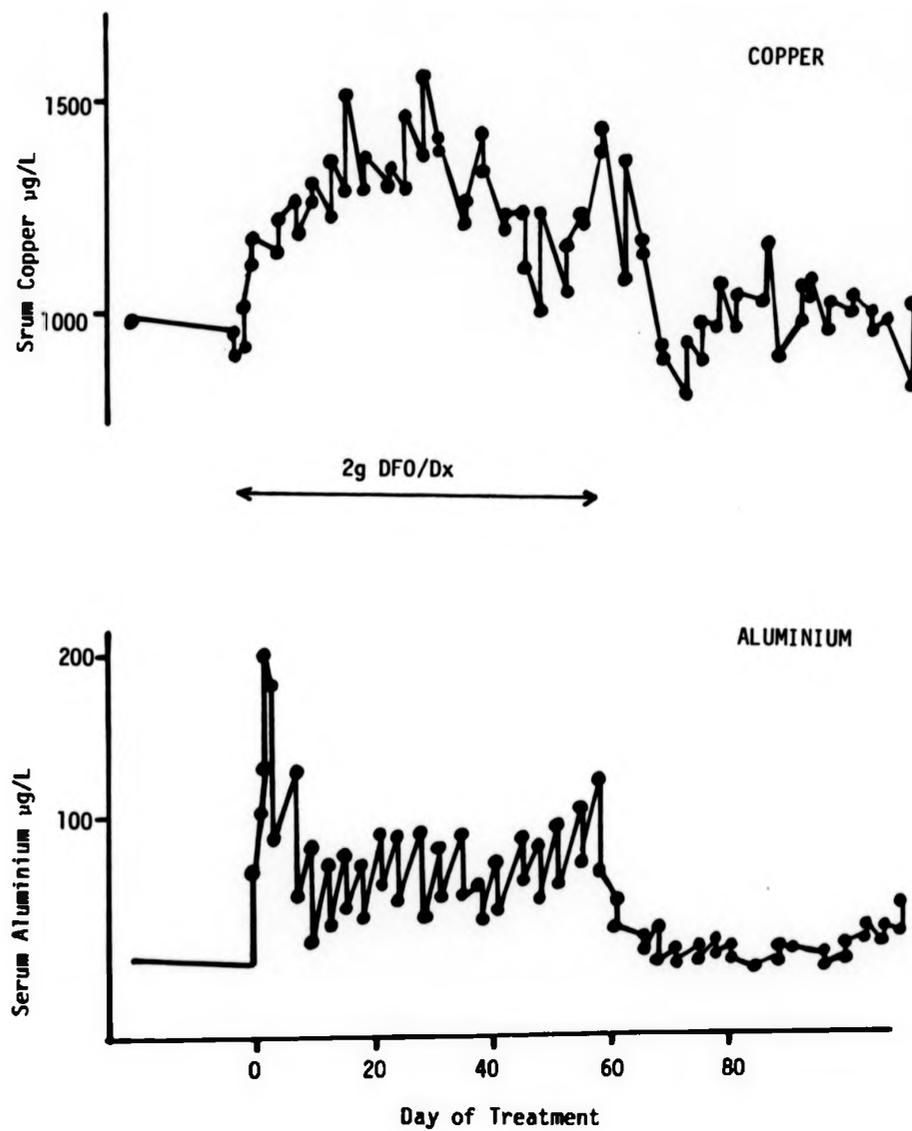
Red Cell Metal Levels. - Aluminium and copper levels in red blood cells (RBC) were also determined. A plot of red cell aluminium against serum aluminium before DFO therapy indicates a direct relationship between serum and erythrocyte concentrations (Figure 4). Over the wide range of aluminium loading shown by these patients the aluminium concentration ratio (erythrocytes:serum) stays approximately constant. This suggests a mechanism for aluminium equilibration between red blood cells and serum, although not necessarily a direct transfer of aluminium across the red cell membrane.

During the period in which the four patients were being treated with DFO, serum aluminium levels rose; there was no apparent correlation between erythrocyte and serum levels (Figure 5). The administration of DFO resulted in an increase in serum aluminium, as shown in the previous section, of up to 10 fold over pre-treatment levels, whilst erythrocyte aluminium concentrations remained essentially unchanged.

In patient BD, RBC-aluminium levels were ca. 30 µg/l, before DFO, gradually decreasing to ca. 5 µg/l during the first two months of chelation therapy (Figure 6). In contrast to serum levels during DFO treatment, red blood cell aluminium levels showed little variation, pre and post dialysis,

During the same period of time (2 months), RBC-copper concentrations were ca. 300 µg/l and gradually increased to ca. 700 µg/l (Figure 7). We also noted that RBC-copper levels

Figure 4. Red-Cell Aluminum vs. Serum Aluminium for 12 Patients on Maintenance Haemodialysis.
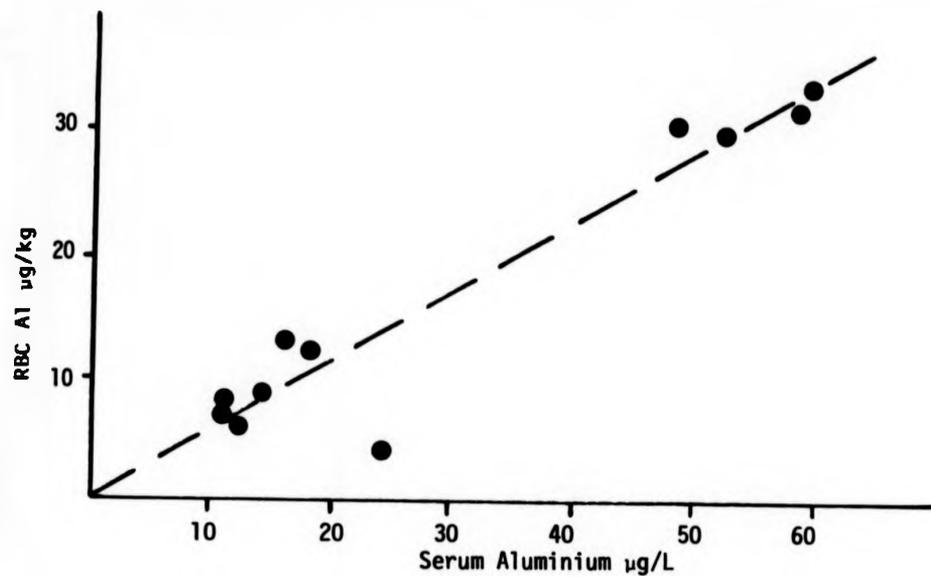
Figure 5. Red-Cell Aluminium vs. Serum Aluminium for 5 Dialysis Patients on DFO Treatment.
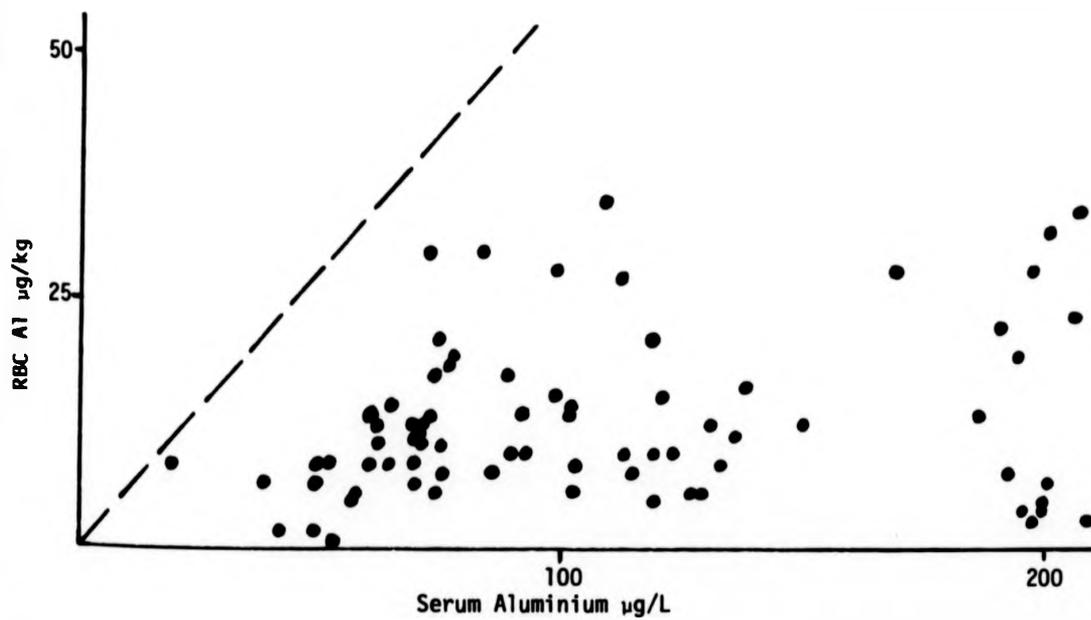
(The plotted line shows the red cell: serum ratio from Figure 4).

Figure 6.   Aluminium Concentrations in Serum and Red Cells During DFO therapy.
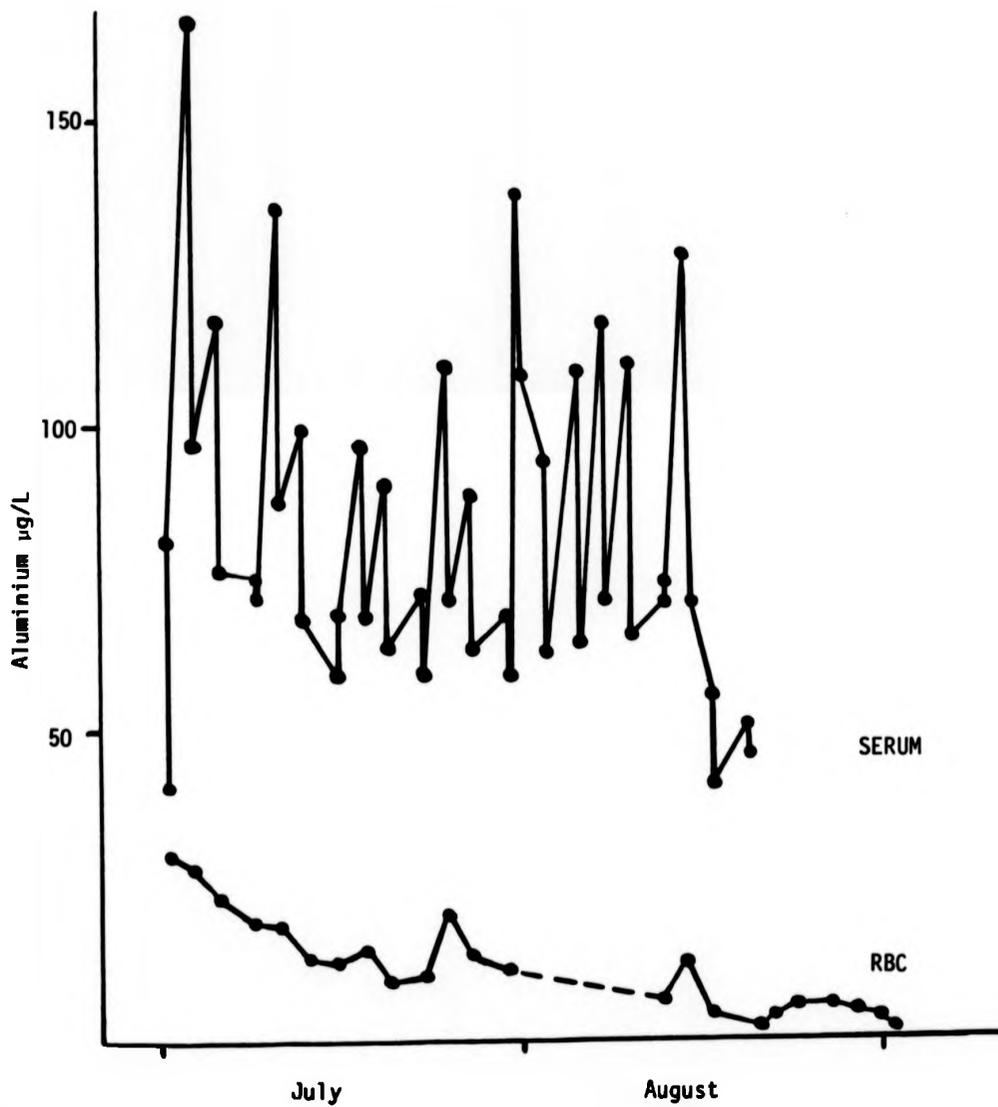
Figure 7. Copper Concentrations in Serum and Red Cells during DFO Therapy.
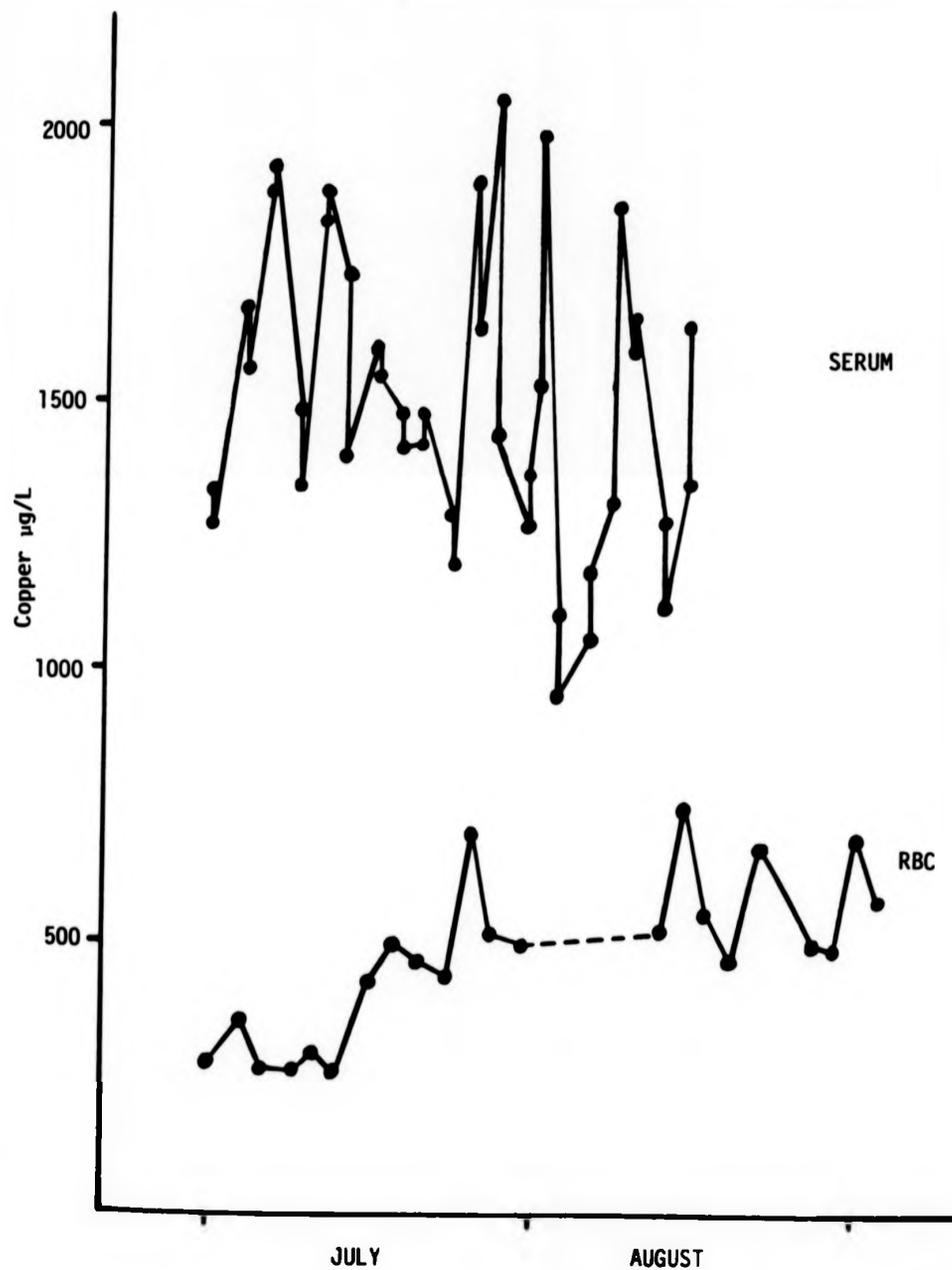
in most of our chronic renal failure patients fall in the range 300-600 µg/l, which is low in comparison with the accepted normal range of 790-1410 µg/l, for healthy individuals, (247). Previous studies of RBC aluminium and copper levels have not been reported in renal patients. These changes in RBC metal levels could be related to the microcytic hypochromic anaemia sometimes seen in CRF patients (94-96, 98, 248). (This will be discused in the final chapter.)

Metal Removal in Dialysate. - During chelation therapy, both aluminium and copper were removed in the dialysate. For aluminium the rate of removal was relatively constant over the whole dialysis session (Figure 8), although often reaching a maximum at the point corresponding to the end of DFO infusion. This resulted in the removal of between 1-2mg of aluminium per dialysis session. Copper in contrast was always removed most rapidly during the first 30-40 minutes of dialysis (Figure 9), declining to a constantly low removal for the remaining part of the dialysis session. The total removal of copper in dialysate during DFO treatment was ca. 1.5mg. Baseline studies, prior to treatment, showed a copper removal of ca. 0.5mg, thus during chelation therapy the additional removal was 1mg/dialysis. It seemed possible that this additional amount of copper could have been leached from the dialyser by the DFO in the patients blood. The dialysers used have "Cuprophan" membranes, consisting of a polysaccharide film produced by the cuprammonium process. However, in separate in vitro leaching experiments using both

Figure 8.   Aluminium Removal in Dialysate.
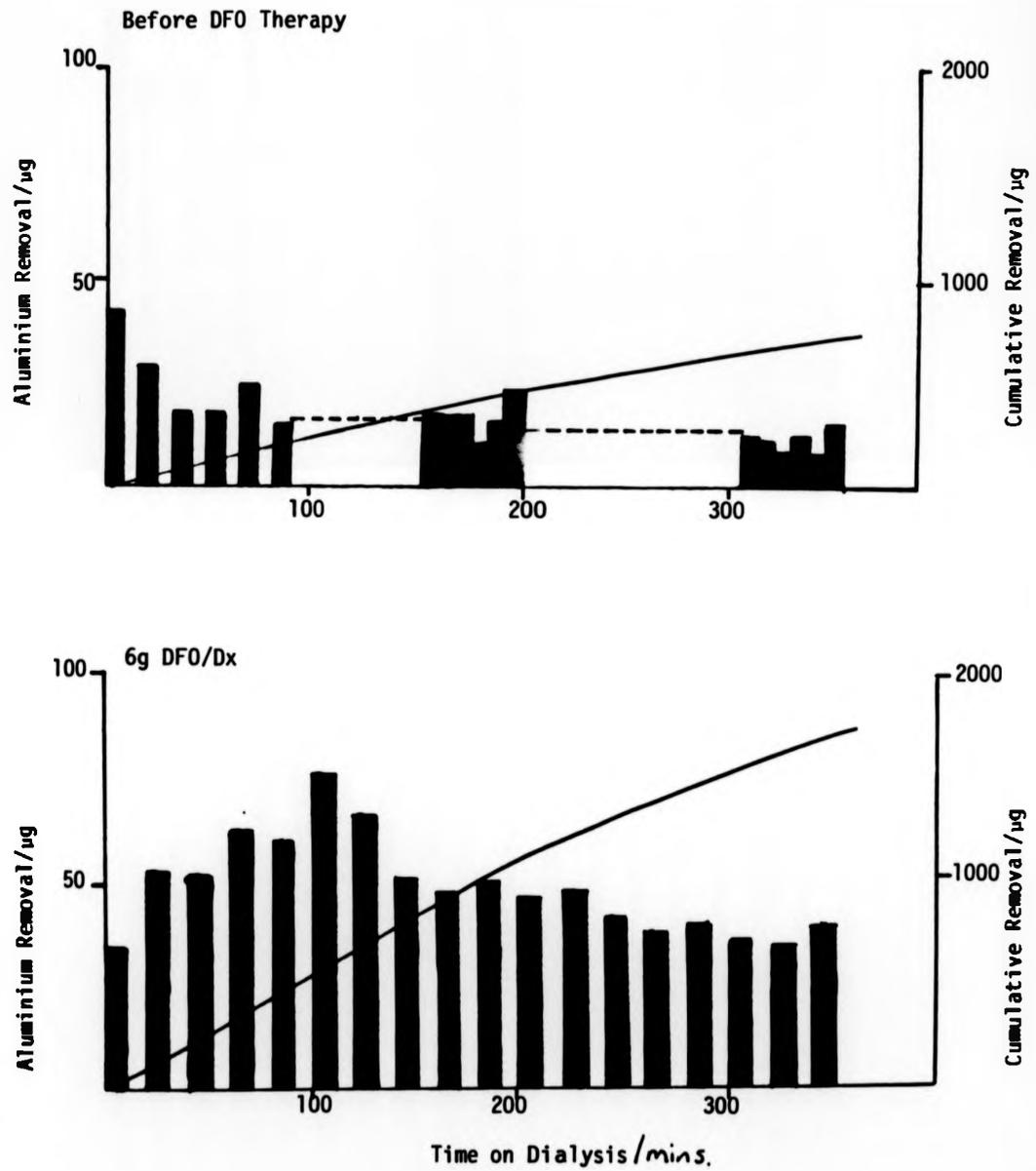
Figure 9. Copper Removal in Dialysate
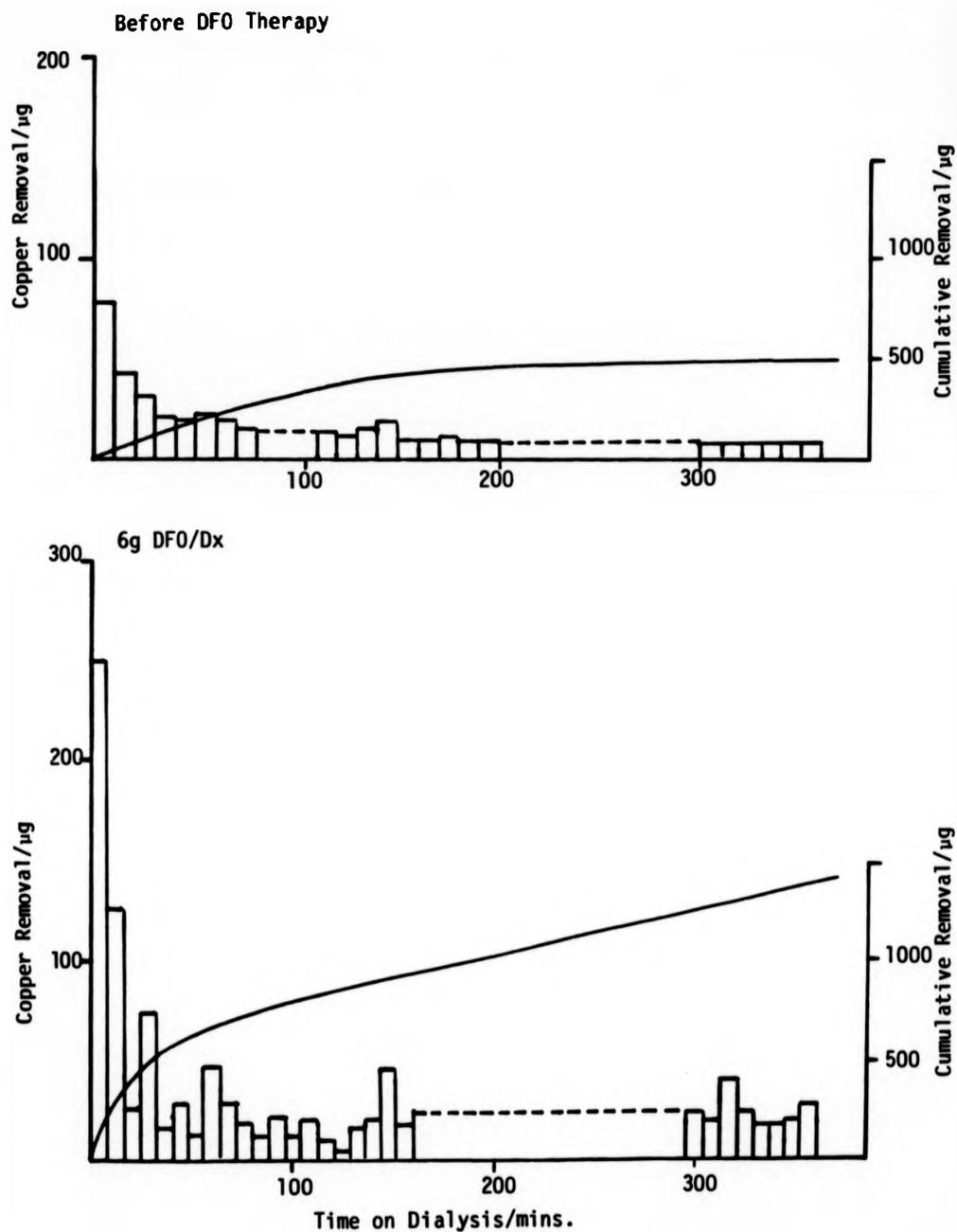
EDTA and DFO, the total leachable copper from the dialyser was found to be ca. 0.15mg. Thus, we concluded that the residual copper ca. 0.8mg, apparently removed by dialysis, does represent an actual loss from the patient.

Removal of Desferrioxamine in Dialysate. - Free DFO was detected in dialysate throughout the dialysis session (Figure 10). The rate of removal was at a maximium during the time of infusion (first two hours of dialysis), which indicates that a large fraction of infused DFO is dialysed through the kidney membrane before entering the patient. DFO remaining in the blood after the first pass through the dialyser, enters the patient, and is rapidly dispersed into a volume approaching that of the extracellular volume (249). The DFO remaining within the circulating blood volume continues to pass through the dialyser, but because the concentration is now lower the rate of removal is also much less. The actual loss can be calculated; for an assumed extracellular volume of 14 litres, and DFO clearance of 25 ml/min, the loss of DFO during a 6 hour dialysis is ca. 25% of the infused dose.

The total removal of uncomplexed DFO during a six hour dialysis was calculated to be between 25-30% of the infused dose, i.e. only 1.2-1.5g of a 2g dose is available for complexation at the end of dialysis.

Figure 10. Desferrioxamine Removal in Dialysate.
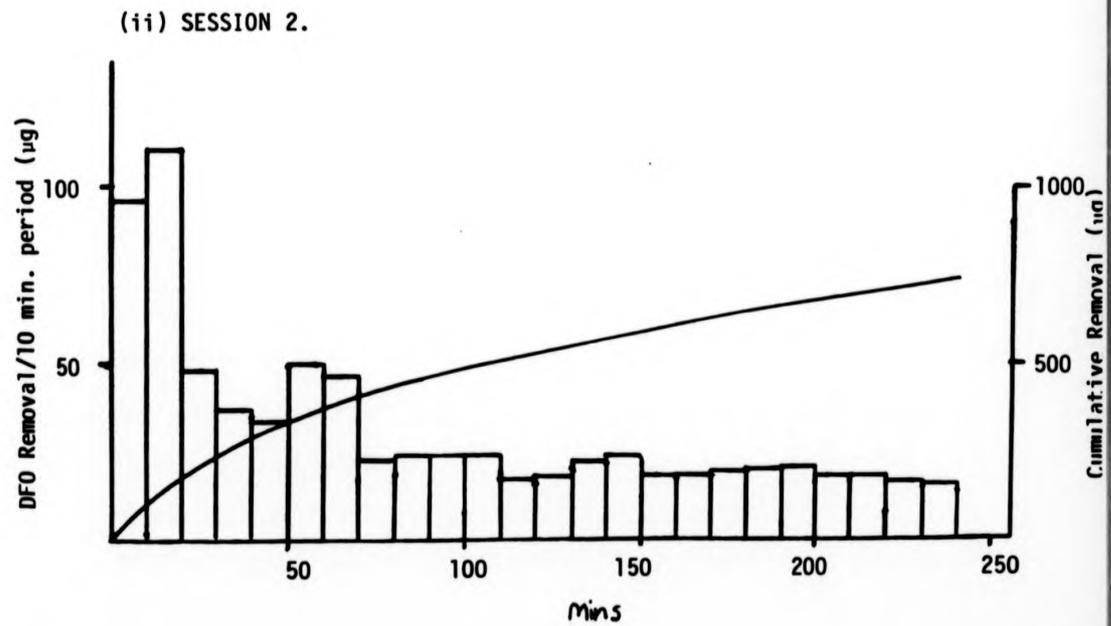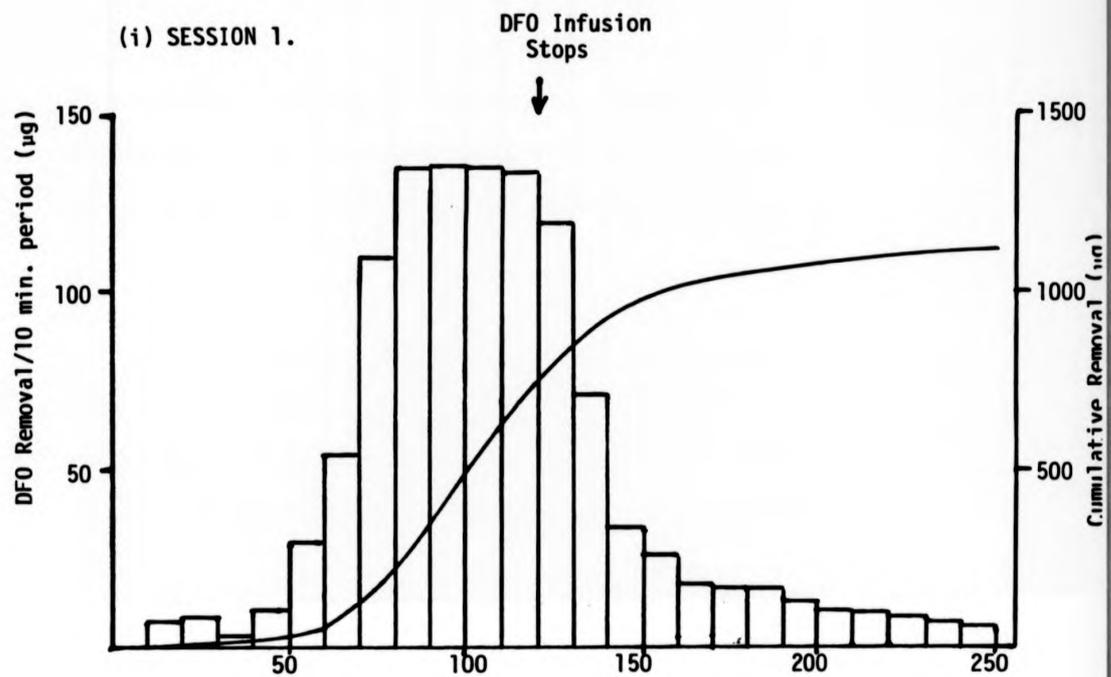
<u>Changes in Serum Caeruloplasmin.</u> - Serum caeruloplasmin
levels were monitored on patient BD during the DFO treatment
(Figure 11). The protein assay was carried out by the method
described in Chapter 2, and the normal range of serum
caeruloplasmin by this method is reported as 0.38 ± 0.08 g/l
(247).

Before DFO therapy, serum caeruloplasmin levels appeared to
be elevated above normal levels, ca. 1.7 g/l, compared with a
serum copper level within the normal range, 790-1410 µg/l
(247). During chelation therapy the serum caeruloplasmin
level increased overall, reaching a maximium level of 2.7 g/l
seven weeks after the first DFO infusion. This general rise
in caeruloplasmin was accompanied by a rise in serum copper
concentration (Serum Metal Levels; this Chapter).
Caeruloplasmin is the major copper containing serum protein,
and it seems likely that the dominating factor in the
observed copper concentration changes was the caeruloplasmin
concentration itself. This protein is too large to be removed
by dialysis; the copper is held too firmly for removal by
DFO. We thus conclude that the changes in serum copper
concentration seen during DFO therapy were caused by the
interaction of DFO and/or other operators with the so-called
acute phase response (i.e. caeruloplasmin, an acute phase
protein, is synthesised in the liver in response to the
stimulation of DFO treatment and dialysis itself).
Caeruloplasmin levels varied randomly pre and post dialysis.

Figure 11.   Serum Caeruloplasmin Levels During DFO Treatment.
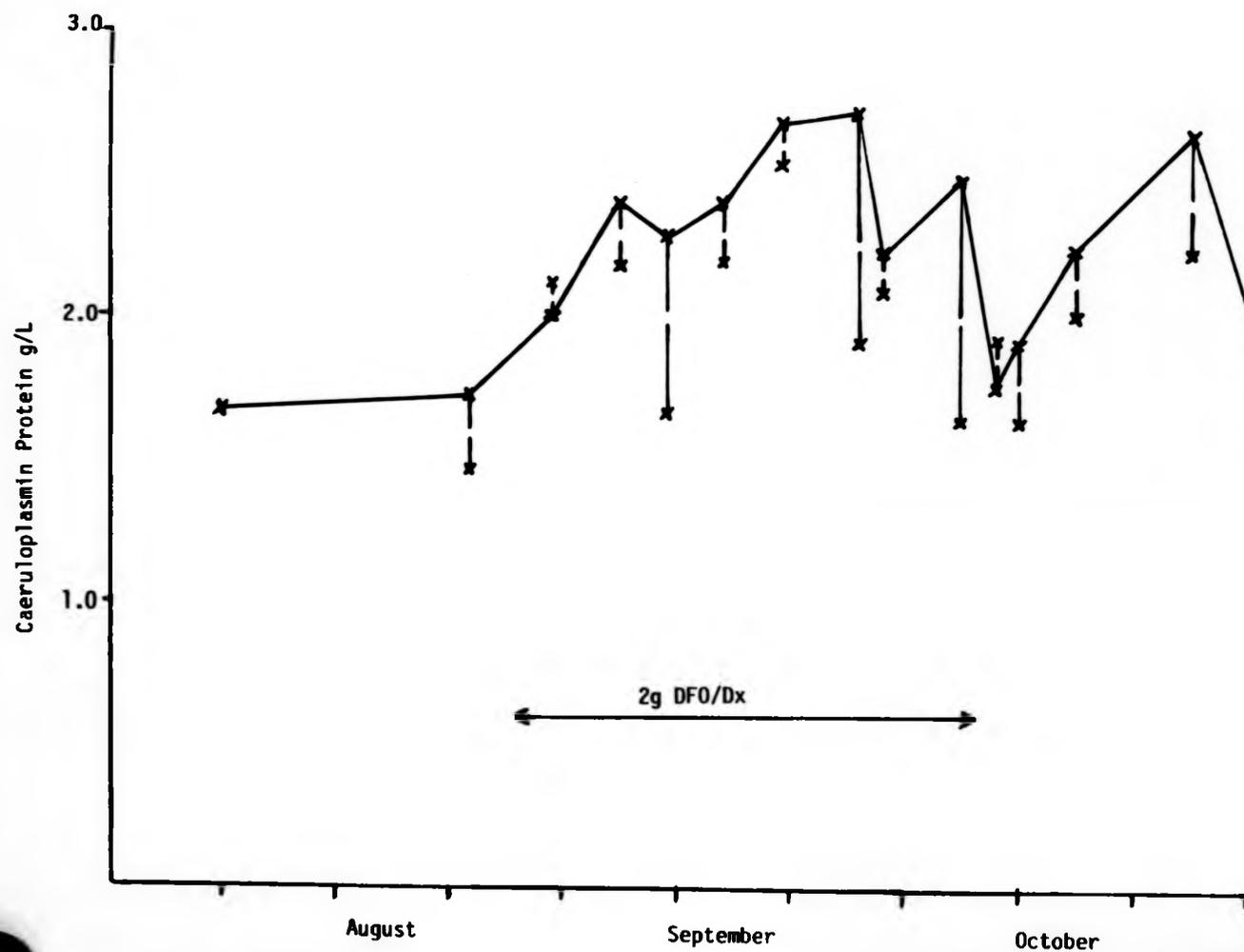
Bone Analysis. - The bone aluminium and copper concentrations
for three (other) patients with aluminum-related
osteomalacia, before and after desferrioxamine treatment, are
given in Table 2. The removal of aluminium by dialysis (up to
1300mg from the patient, in dialysate, over 10 months
chelation therapy) was accompanied by a large reduction in
aluminium in both bone marrow and mineralised bone (117). We
also observe a small reduction in bone copper levels. This
reduction is most marked in the bone marrow. Normal levels of
copper in bone are 1.0-25.7 µg/g (250).

Table 2. Depletion of Aluminium and Copper in Bone During
Desferrioxamine (DFO) Treatment.

PATIENT	ALUMINIUM (µg/g)		COPPER (µg/g)	
	Apatite	Marrow	Apatite	Marrow
GO Pre-DFO	245	488	16	61
Post-DFO (1)	192	225	3	3
Post-DFO (2)	55	53	5	12
WA Pre-DFO	242	110	19	17
Post-DFO	53	60	30	5
GH Pre-DFO	527	252	67	131
Post-DFO (1)	213	91	-	-
Post-DFO (2)	149	57	4	16

6. CONCLUSIONS.

Desferrioxamine treatment stimulates a marked increase in serum copper levels over the first two to three weeks of chelation therapy. This increase in serum copper is maintained throughout the course of treatment, but returns to pre-treatment levels when therapy ceases. This rise in serum copper is due, at least in part, to the increased level of serum caeruloplasmin. Caeruloplasmin is one of the acute phase proteins, and an increase in the rate of its synthesis, in the liver may be a direct result of desferrioxamine treatment.

RBC-aluminium levels were elevated in aluminium overloaded patients (5-35 µg/l), compared to normal subjects (<5 µg/l). During DFO treatment, no variation was seen in RBC-aluminium concentration pre and post dialysis, in contrast to serum-aluminium which fell after dialysis. The gradual decrease in RBC-aluminium, with no short term variation, suggests that the erythrocyte membrane is not premeable to DFO and/or the Al-DFO complex. The most probable mechanism for the reduction of RBC-Al during DFO treatment is through the natural turn-over mechanism for these cells. If the presence of DFO in the bone marrow is sufficient to prevent the uptake of aluminium in newly forming red cells, the observed decrease in RBC-Al results from the degradation and removal of Al-loaded cells from circulation.

Erythrocyte copper concentrations, which were found to be low in aluminium-overloaded patients, increased towards the normal levels during desferrioxamine treatment. Bone copper was depleted during prolonged chelation therapy. A greater decrease was seen in bone marrow copper levels compared with mineralised bone. The bone marrow is the site of red cell formation. The gradual increase in red cell copper levels might be accounted for by the formation of new red cells containing higher concentrations of copper. Likewise, the reduction of bone copper levels might be due to the increased incoporation of copper into red cells from the bone marrow during DFO treatment. These two observations suggest that in aluminium-overload, aluminium in the bone marrow may either block the route by which copper enters the forming red cell, or enters the red cell in place of copper. During chelation therapy, DFO binds aluminium and mobilises it into serum, and in this way may remove the inhibitory effect it has on copper mobilisation, with an overall increase in red cell copper levels. Alternatively, during DFO treatment aluminium is complexed by DFO and mobilised from the bone into serum from where it is removed by dialysis, allowing copper to enter the red cell by its normal metabolic route, with out competition from aluminium.

Copper removal from patients by dialysis is low in relation to total serum copper levels. Only a small fraction (at most 10%) of serum copper appears to be dialysable under these conditions. This is probably because the rise in serum copper can be accounted for by the increased level of serum

caeruloplasmin. Copper is strongly bound to this high
molecular weight protein and can not be removed in the
dialysate. This contrasts with aluminium, where during a
period of desferrioxamine treatment, between 30-40% of serum
aluminium appears to be dialysable (118, 129). Aluminium is
usually present in serum largely bound to transferrin
(molecular mass ca. 65 000 daltons), but is chelated by DFO
from storage sites to form the low molecular mass (ca. 600
dalton) Al-DFO complex, which is removed by dialysis.

Although we have demonstrated a considerable rise in serum
copper and caeruloplasmin levels during aluminium chelation
therapy, together with long-term reduction in the bone copper
content, we note that even in patients who have received
several courses of desferrioxamine treatment, covering a
period of five years, no signs of copper deficiency have been
observed.

CHAPTER 5.

DESFERRIOXAMINE TREATMENT OF A CHILD WITH ALUMINIUM OVERLOAD:

A CLINICAL STUDY.

DESFERRIOXAMINE TREATMENT OF A CHILD WITH ALUMINIUM OVERLOAD:

A CLINICAL STUDY.

1.   INTRODUCTION.

Over the last five years there have been a number of reports
of encephalopathy in children with chronic renal failure (24,
27, 64, 65, 73, 74). High plasma and bone aluminium values
have been reported in uraemic children ingesting
aluminium-containing phosphate binders prior to dialysis
treatment (24, 27, 65, 73, 74, 251, 252). Children who
receive high doses of aluminium hydroxide appear to be
susceptible to aluminium intoxication. There have been two
cases of aluminium overload in children being treated with
desferrioxamine (251, 252). Desferrioxamine was administered
in conjunction with continuous ambulatory peritoneal dialysis
in both cases.

This study concerns the desferrioxamine treatment of a child
patient with encephalopathy, renal osteodystrophy and
microcytic anaemia. The case was brought to our attention by
Dr. Mary McGraw from the Royal Manchester Children's
Hospital, Pendlebury, in July 1985. At that time the patient
was nearly seven years old.

The child had a serum aluminium level of 270 µg/l (reported
by the Regional Unit for Toxicology, Dudley Road Hospital,

Birmingham), and had received aluminium hydroxide as a phosphate binder for about five years. She was well known at the hospital because of her chronic renal failure, which was secondary to congenital renal abnormalities. The child had a single abnormal left kidney which, by July 1985, was unable to maintain satisfactory blood biochemistry. Her glomerular filtration rate (GFR) was 3-4 ml/min/1.73m^2 (normal 50-85 ml/min/1.73m^2). Due to this poor kidney function her creatinine and urea levels were elevated, 511 µmol/l (normal 30-80 µmols/l) and 29.4 mmol/l (normal 2.5-7.5 mmols/l), respectively. At that time her serum phosphate level was acceptable, being controlled by the use of phosphate binders.

This child had suffered seizures since the beginning of 1985, which had been fairly well controlled by the use of anti-convulsants until July 1985. She also suffered episodes of "going blank" and "just dropping off to sleep". In addition to the seizures, the patient developed marked tremor and drowsiness suggesting aluminium encephalopathy.

At the start of the clinical study this child had not received any form of dialysis treatment. Her ureamic state required hospital admission for preparation for the dialysis-transplant programme, with insertion of a CAPD catheter. On the child's admission, the paediatricians decided to assess her aluminium overload with a trial of desferrioxamine therapy. It was not known whether her residual renal function would be sufficiant to remove the aluminium released from body stores as a result of chelation

by desferrioxamine. Previously desferrioxamine therapy had
been used in conjunction with haemodialysis (70, 113-115,
117, 127) and continuous ambulatory peritoneal dialysis
(CAPD) (116, 251-253), which allowed an acceptable rate of
aluminium removal in the dialysis fluid, but the potential
for removal by the (partially functioning) kidney was
unknown.

2. CASE HISTORY.

This patient (EG) was a fullterm baby girl, birth weight
2.58kg, born with multiple congenital anomilies including,
renal hypoplasia and complex structural abnormalities of the
urogenital tract. She remained in chronic renal failure with
a GFR of 15-20 ml/min/1.73m² during her first year of life.
At 2 years she was found to have radiological evidence of
severe osteodystrophy. Blood biochemistry revealed: urea 52
mmol/l, calcium 1.6 mmol/l, phosphate 3.7 mmol/l, with a
predicted GFR of 9.5 ml/min/1.73m². With dietry restriction
alone the serum urea fell to 25 mmol/l and serum phosphate to
1.9 mmol/l. She was commenced on aluminium hydroxide as a
phosphate binder (20 mg Al/kg/day).

There was further deterioration of her renal osteodystrophy
with marked deformities of both radii and tibiae, and vitamin
D, 1-alpha-hydroxycholecalciferol (1-HCC, 50 ng/kg/day) was
commenced at 2 years 6 months. The dose of aluminium
hydroxide was increased to a maximium of 80 mg Al/kg/day.

When EG was 2 years 9 months old, surgical osteoclasis was performed on both left radius and ulna because of severe deformities. Despite satisfactory biochemical control she showed continuing clinical and radiological deterioration of her renal osteodystrophy. At the age of 4 years, the dose of aluminium hydroxide was reduced to 30 mg Al/kg/day due to hypophosphatemia and at 5 years of age, the 1-HCC was reduced to 50 ng/kg/day, because of hypercalcaemia. Subsequently her PTH levels rose (5.9 ng/ml), with normal calcium, and the 1-HCC was increased to 65 ng/kg. This resulted in suppression of PTH and a return to normal values (0.4 ng/kg) over 6 months.

The patient also developed partial motor seizures, which increased in frequency to several attacks per day by 5 years 6 months. An electroencephalogram (EEG) was performed, demonstrating features comparable with aluminium encephalopathy (62, 67). Progressive encephalopathy has been reported (64, 65) in infants and children with chronic renal disease before dialysis and in some cases prior to the use of aluminium-containing phosphate-binders (65). At this time (February, 1985) her serum aluminium level was 268 μg/l, and aluminium hydroxide was discontinued. Over 3 months, serum aluminium level fell to 150 μg/l, but there was increased difficulty in controlling seizures despite anticonvulsant therapy. In addition she developed ataxia, marked tremor and increased drowsiness.

3.   DESFERRIOXAMINE TREATMENT.

Following a conference on this case, between Dr. McGraw and
Dr. Ackrill (July, 1985), it was decided to commence
desferrioxamine therapy; initially the programme of treatment
was to be followed by analysis of serum, urine and faeces for
aluminium content (the child was not on dialysis at this
time). Desferrioxamine (DFO: Desferal-CIBA), 80 mg/kg, was
given intravenously in 200ml normal saline over 4 hours, in
two trial doses 14 days apart. The first dose of DFO was well
tolerated. During the second infusion she developed
hypertension and vomiting, and generally tolerated the dose
badly. In retrospect, this dose (80 mg/kg) was considered to
have been a little too high, even though the dose takes into
account total body weight and was thought to be suitable in
this case. No child dose has been reported in the literature
for chelation without dialysis (Andreoli et al (251)
successfully used a dose of 22.5 mg/kg/24hr with CAPD on a
seven year old boy which resulted in the reversal of
osteomalacia and microcytic anaemia and an overall
improvement in encephalopathy). Changes in serum aluminium
and copper were monitored during chelation therapy and the
amounts of aluminium, iron and copper excreted in urine and
faeces were separately determined.

After the second dose of DFO there was further deterioration
in the child's neurological condition, and she became
semicomatose. Following two unsuccessful attempts to commence

continuous ambulatory peritoneal dialysis, haemodialysis was
started at 6 years 2 months (urea 34 mmol/l). A regular
pattern for dialysis was established, 4 times per week (2
hour dialysis sessions, using a 0.28m^2 dialyser and blood
flow 50 mls/min), and she received weekly DFO therapy, 40
mg/kg. Over the subsequent 3 months treatment, the patient's
neurological condition dramatically improved; she returned to
normal conscious level, and a marked decrease in both tremor
and ataxia were observed with the abolution of seizures.

Prior to chelation therapy, this patient had a microcytic
microchromic anaemia (haemoglobin 5.4 g/dl) and had become
transfusion dependant. There was no decrease in this
patient's transfusion requirement during DFO therapy.
However, her requirements remained stable despite increased
venesection (2% total blood volume per week). An increase in
haemoglobin levels has been observed in patients during
chelation therapy (251, 254).

4.   TRACE METAL ANALYSIS.

Samples of serum, urine and faeces were analysed for
aluminium, copper and iron by atomic absorption spectrometry
using graphite furnace atomisation with Zeeman background
correction (Perkin- Elmer Zeeman 3030). Serum samples were
taken pre and post dialysis on the day of infusion (session
1) and 24 hours later (session 2), and pre dialysis only
(sessions 3 and 4). Serum was separated by centrifugation,

and diluted (aluminium, x4; copper, x20) with 0.1M nitric acid prior to metal determination, and urine samples were diluted x4 before analysis. Urine and faeces samples (24 hour collections, sub sampled for analysis) were obtained over an 8-day period, 1 day prior to and then during the initial trial doses. Faeces samples were ashed at 120°C and then sub samples digested in concentrated nitric acid. The digested samples were diluted to volume (100 ml) with distilled water before direct injection into the graphite furnace. All samples were run against matrix matched standards. Full details are given in Chapter 2.

5. RESULTS.

Serum. - During the two trial doses of DFO (no dialysis) serum aluminium levels increased rapidly above baseline levels within the first 24 hours post DFO infusion, from 110 µg/l to 850 µg/l, and from 90 µg/l to 840 µg/l (Figure 1). Serum aluminium levels returned to baseline levels between trial doses.

During the weekly chelation therapy (with dialysis), aluminium levels showed a typical cyclical variation (70, 118, 129) (Figure 2). Serum aluminium rose during session 1, indicating that the DFO was mobilising aluminium from the body tissues. In the following dialysis session, 24 hours later, the pre-dialysis serum aluminium level was higher than at the end of session 1, indicating that DFO had

Figure 1. Serum Aluminium and Copper Levels During Two Trial Doses of Desferrioxamine.
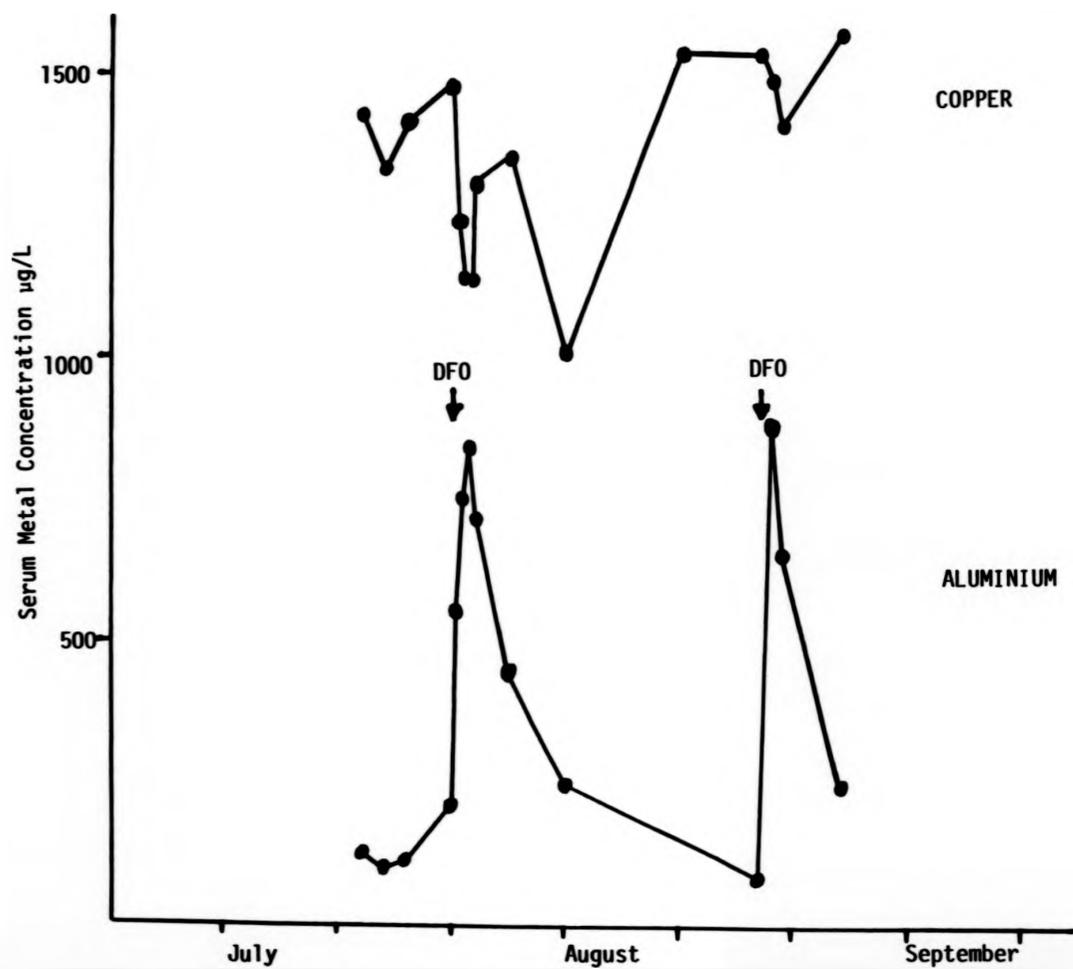
Figure 2. Serum Aluminium and Copper Levels During Weekly DFO Therapy.
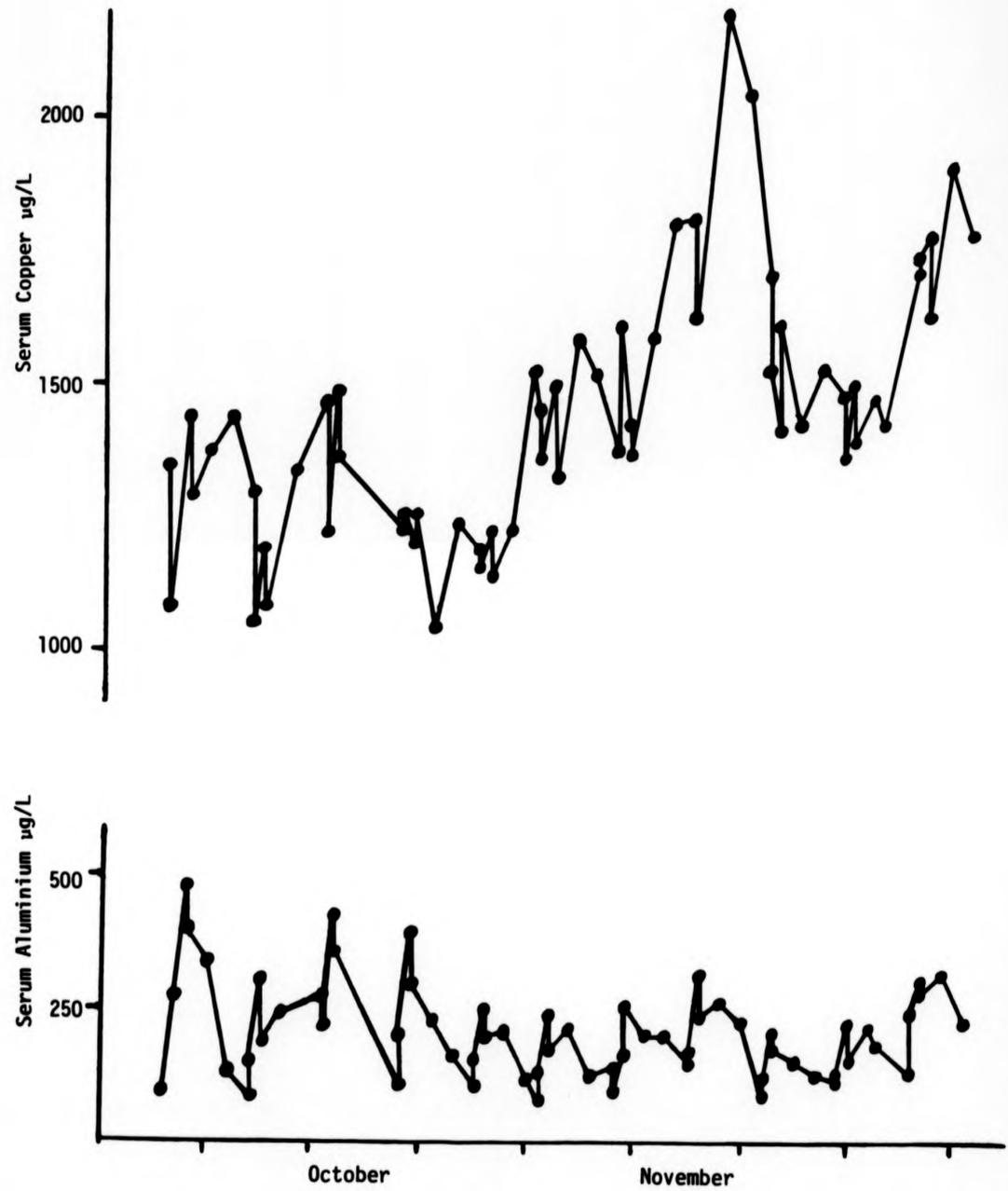
mobilised aluminium from storage sites between sessions 1 and
2, and increased the concentration of serum aluminium. During
session 2 the serum aluminium level fell as the Al-DFO
complex was removed by dialysis, and similarly during session
3 and 4. During DFO therapy, serum copper levels increased
gradually, with no regular variation. This effect probably
results from the acute phase response induced by DFO, one
such response being the release of caeruloplasmin from the
liver.

Urine. - The concentrations of aluminium and iron in urine
during the trial doses of DFO increased rapidly within 24
hours of the DFO infusion, from 32 µg/l to 632 µg/l and from
39 µg/l to 1044 µg/l for aluminium and from 5 µg/l to 312
µg/l and 19 µg/l to 1007 µg/l for iron (Figure 3). The marked
rise in urine aluminium levels increased urinary excretion by
approximately 3mg above baseline levels, on each infusion.
Urine aluminium levels returned to baseline levels within 9
to 10 days of each infusion, and iron levels returned to pre
treatment levels within 8 days.

There was little or no change in urine copper excretion after
DFO infusion (Figure 3).

Faeces. - The amounts of iron and aluminium excreted in
faeces showed marked increases over baseline levels,
following the two DFO trial infusions (Figure 4 and 5). Both
these elements would have been present in the diet, and the
baseline levels reflect the dietry load. After DFO infusion,

Figure 3. Metal Removal in Urine During Two Trial Doses of Desferrioxamine.
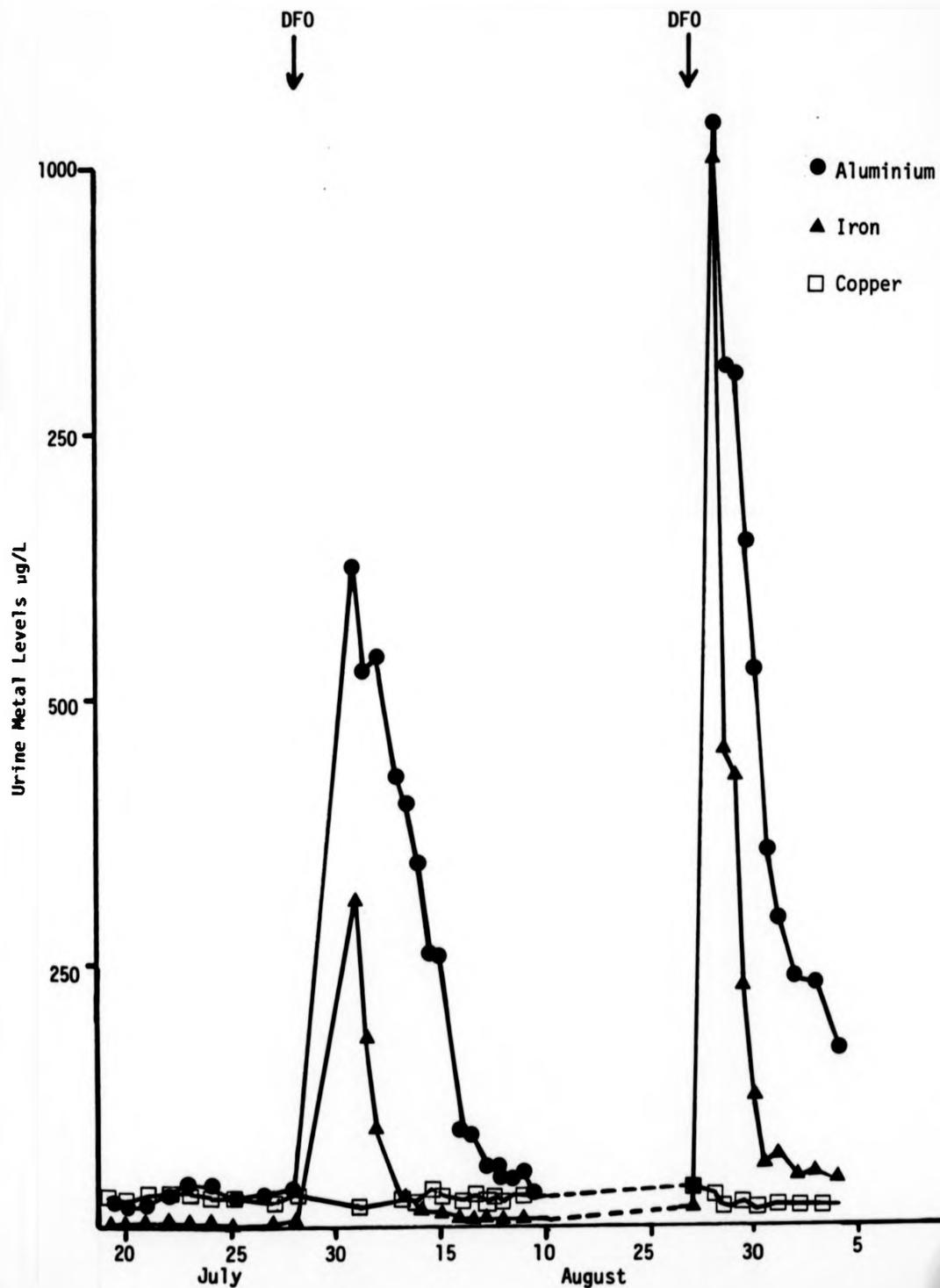
Figure 4.   Iron Removal in Faeces During Two Trial Doses of Desferrioxamine.
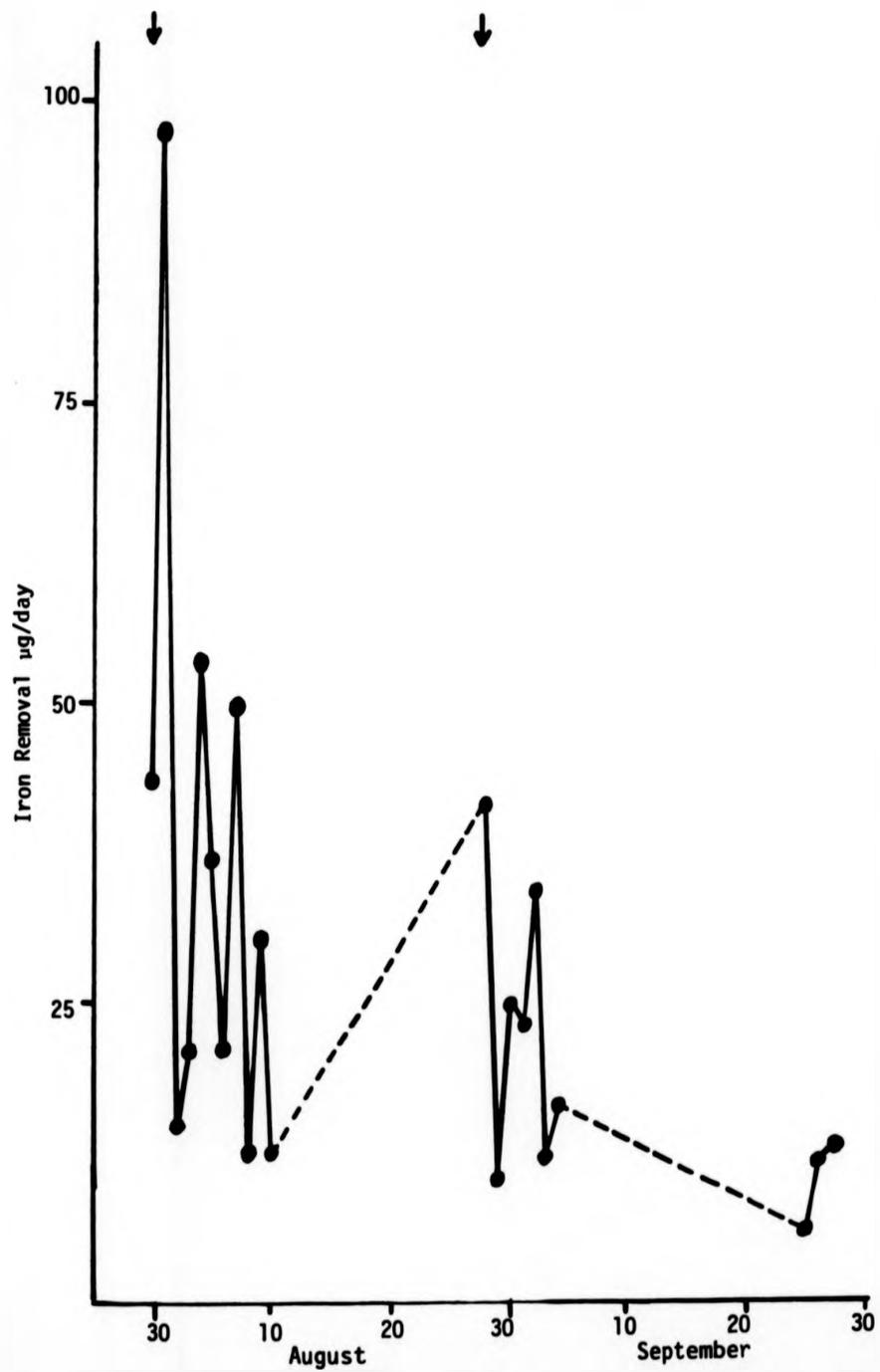
Figure 4. Iron Removal in Faeces During Two Trial Doses of Desferrioxamine.
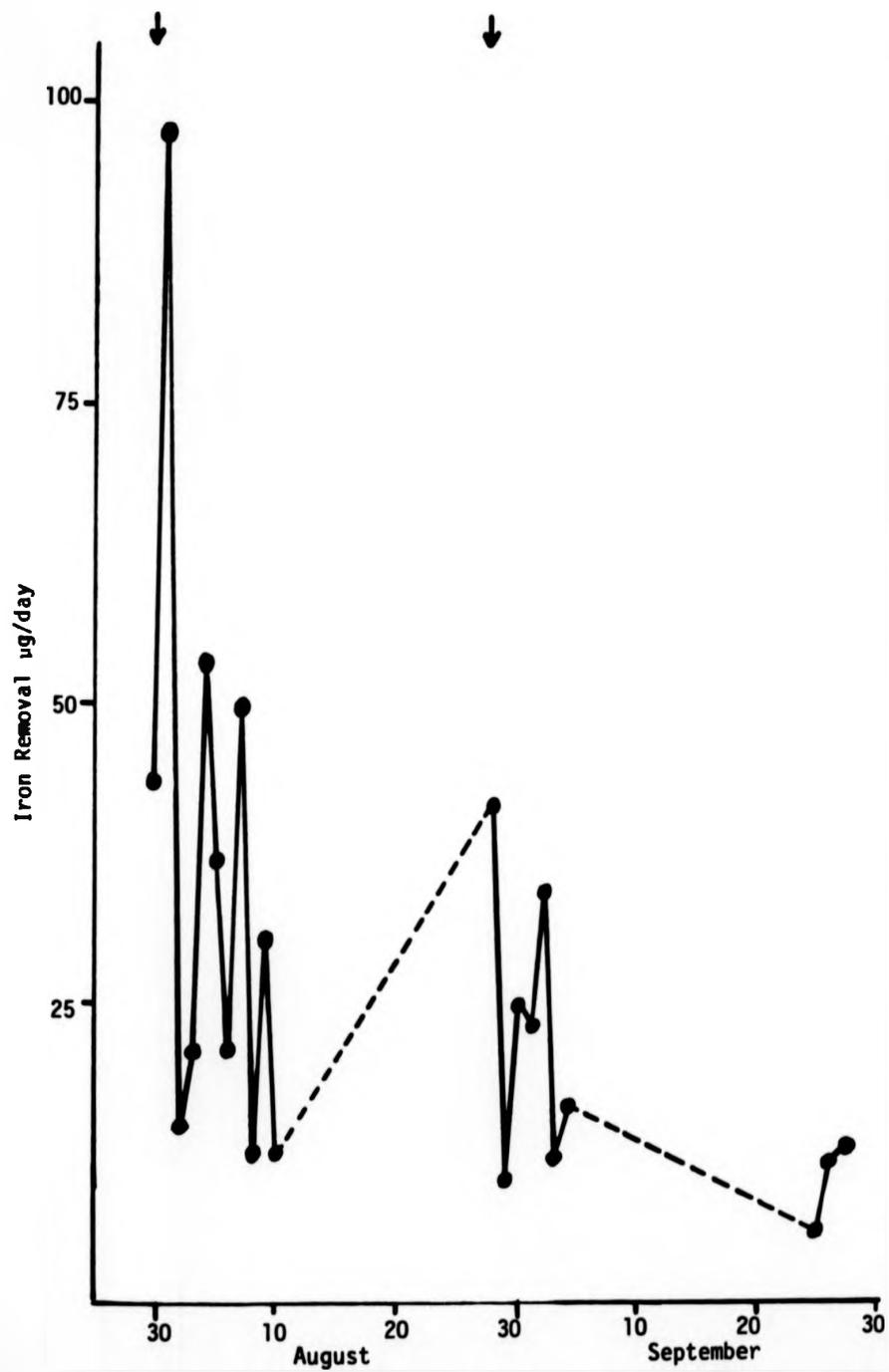
Figure 5. Aluminium Removal in Faeces During Two Trial Doses of Desferrioxamine.
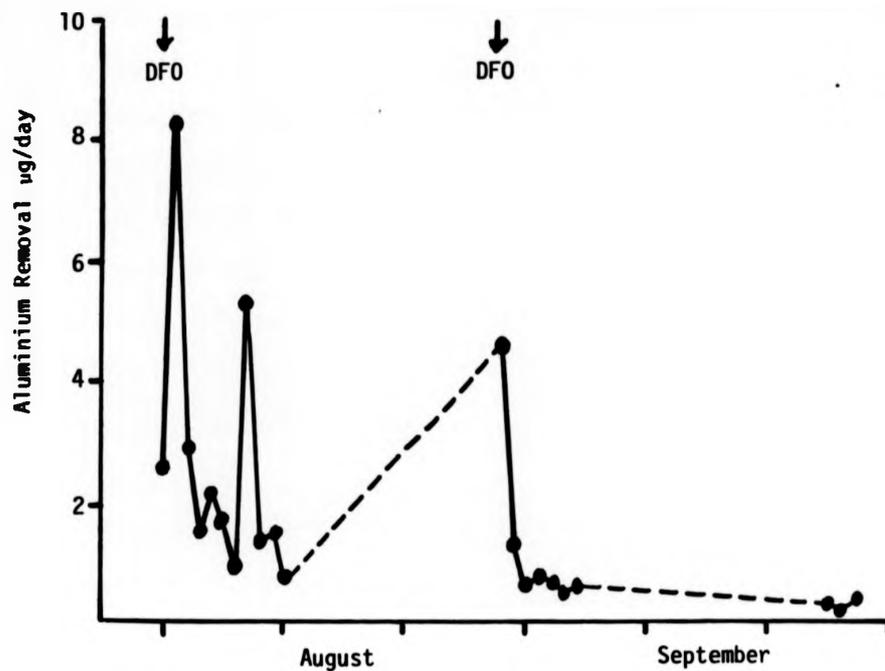
Figure 6. Copper Removal in Faeces During Two Trial Doses of Desferrioxamine.
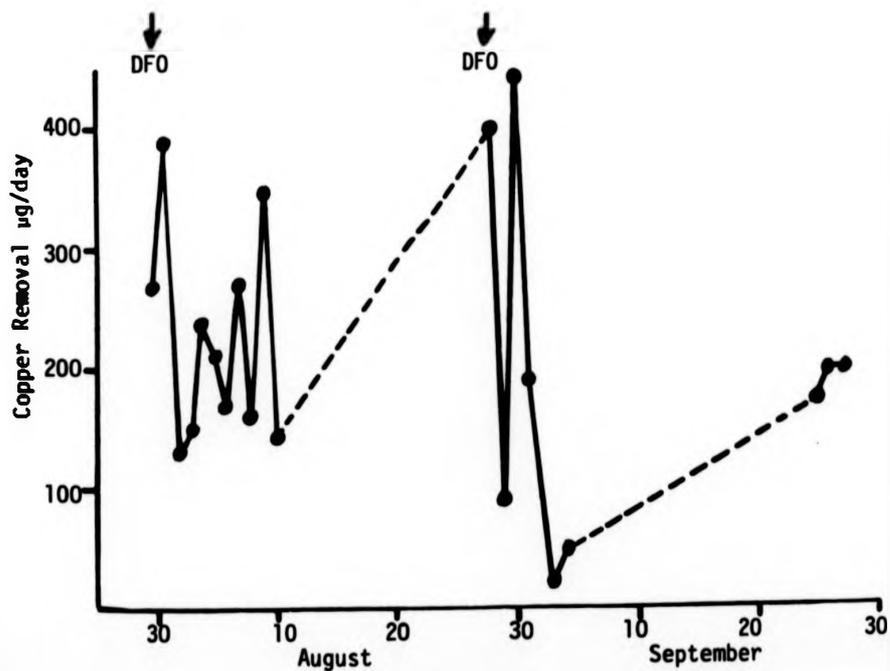
the metals excretion increased over the following 24 hours. Assuming baseline levels for the trial doses of ca. 30 and 15 mg Fe/day, then iron excretion increased 3-fold immediately following DFO infusion. There was a 4-fold increase in aluminium excretion above baseline levels post DFO infusion. This marked increase of metal excretion in faeces represents a greater reduction of body aluminium and iron loading via the faecal route, compared with removal by the kidney; i.e. the gut is the major route for the excretion of chelated aluminium and iron (Table 1).

There was no change in copper excretion during DFO treatment (Figure 6).

Table 1. Aluminium and Iron Removal by DFO (mg over 8 days).

	DFO - 1		DFO - 2	
	URINE	FAECES	URINE	FAECES
ALUMINIUM	3	11	3	5
IRON	1	90	1	45

6. DISCUSSION.

Desferrioxamine administered intravenously, whether during dialysis or without dialysis, is rapidly dispersed in a volume that approaches that of total body water (249). The effect of DFO infusion on serum aluminium levels has been well characterised (70, 118, 129). Although a variety of modes of administration are used, following DFO infusion serum aluminium concentrations rise to a maximium within 24-48 hours of infusion. This rise in serum aluminium indicates a rapid release of aluminium from intracellular stores (muscle, liver, brain, bone and other tissues), and is accompanied by the formation of a dialysable Al-DFO species (117, 123, 129). The high stability of the Al-DFO complex formed, may account for the fact that the very high serum aluminium concentrations are generally well tolerated during chelation therapy. During regular dialysis and weekly DFO treatment, serum aluminium levels show a short term cyclic variation, clearly related to DFO administration.

In this patient, marked changes were observed in the amount of aluminium and iron excreted via the kidney after each loading dose of DFO. Urine aluminium and iron levels increased immediately after DFO infusion. Metals excreted in the urine are directly removed from the blood (plasma) by the nephron and must be low molecular weight bound (< 10,000 daltons). The increase in aluminium and iron excretion via the kidney indicates the formation of either i) stable Al(III)- and Fe(III)-DFO complexes of ca. 600 daltons, or ii)

aluminium- and iron-containing proteins or protein fragments
of low enough molecular weight to be cleared by the kidney,
or iii) an increase in concentration of low molecular weight
complexes of Al(III) and Fe(III) (e.g. phosphato-, citrato-).
In this case, the rapid rise in serum aluminium resulting the
formation of the Al-DFO complex is accompanied by the rapid
removal of aluminium from plasma by the kidney and the
presumed excretion of the Al-DFO complex. It seems likley
that the removal of iron in urine follows a similar mechanism
(see below).

Desferrioxamine has been used as an iron chelator in iron
loading anaemias, notably the thalassaemia syndromes, for
many years. The nature and amount of iron excreted after DFO
infusion has been studied by several research groups (105,
106, 249, 255-260). By considering aluminium excretion
alongside iron excretion after DFO infusion, we have been
able to see a number of similarities between the removal of
these two metals. After parenteral administration, DFO has
been shown to enter the hepatocyte and complex with hepatic
iron. Gastrointestinal ferrioxamine (FO) is largely derived
from bile (105, 260), though a small contribution may arise
from DFO excreted in bile complexing in the gut with dietary
iron. In contrast, after FO administration the iron chelate
is confined to the extracellular fluids (107); the complex is
unable to enter the hepatocyte or bile and is excreted
through the kidney, (255). Thus, it is inferred that nearly
all faecal iron loss arises directly from the intracellular
compartment of the hepatocyte.

Urinary iron excretion is increased after DFO infusion (255).
One possible source of this chelated iron is the processing
of haemoglobin and transferrin iron in the hepatocyte (249).
Pippard (249) reported a reciprocal relationship between
concentrations of urine and stool iron. The proportion of
iron appearing in urine was lowest at low levels of
erythropoiesis (the production and release of red cells). An
increase in urinary iron excretion was observed with active
erythropoiesis, which might reflect either the release of
iron in chelatable form as it is mobilised for red cell
production (i.e. by the release of iron from transferrin to
haemoglobin), or an increased delivery of iron to the
tissues, where it would be available for chelation. Brown et
al (257) noted a direct correlation between percentage
transferrin saturation and DFO-induced urinary iron excretion
($r = 0.353$, $0.2 < P < 0.10$). DFO has no effect on plasma iron
turnover, and therefore cannot compete directly with
circulating transferrin (143, 256, 262). Chelatable iron is
thought to be derived from an intracellular exchangeable pool
located largely in the reticuloendothelial system (245). The
amount of iron available to desferrioxamine from this source
is related to the size of the pool and the rate at which iron
is flowing through the pool. Thus the amount of iron which
DFO can chelate is increased in patients with increased iron
stores. Summers (106) reported higher plasma ferrioxamine
concentrations in iron loaded subjects treated with DFO, and
high DFO concentrations in normal iron loaded subjects.
Plasma FO levels increased gradually up to 12 hours after DFO

infusion, and the peak concentration of FO in plasma and urinary iron excretion were found to be correlated (r = 0.736, P < 0.001).

The selective labeling of Reticuloendothelial (RE) and parenchymal iron stores in the hepatocyte with 59-Fe revealed two alternative pathways for the chelation of iron in vivo by DFO (258):

(i) The intracellular chelation of iron within the hepatocyte, with the chelated iron excreted through the bile only;

(ii) Extracellular excretion, only activated after the iron binding capacity of transferrin has been saturated, chelated iron being removed by the kidneys.

The reciprocal relationship between faecal and urinary iron with changes in erythropoiesis (249) suggests that the mobilisation of hepatocyte storage iron may be the most significant factor producing the altered pattern of chelate iron excretion. This would be consistent with a change in the distribution of chelatable iron between the two major transit-iron pools within the hepatocyte; one being related to iron stores (from which iron chelated enters the biliary tract, parenchymal iron) and the other to exchange indirectly with circulating transferrin (comprising the greater proportion of iron excreted in the urine).

The simultaneous increase in iron and aluminium excretion in urine in our patient suggests that the infusion of DFO mobilised aluminium from body stores into plasma by a similar

pathway to iron. We have not seen this increase in iron excretion, post infusion, previously. However, in the past we have always administered loading doses of DFO during the first 2 hours of dialysis, and in cases where there has been no renal function.

Aluminium in liver is largely in the hepatocytes (263), the site of iron storage. It also seems likely that the actual storage site for aluminium will be the principal iron-storage protein, ferritin, and in cases of iron overload, haemosiderin. The observed rise in faecal iron, paralleled with an increase in faecal aluminium removal, suggests that intracellular aluminium is removed from the liver in bile in a similar way to iron.

Serum copper levels show a long term rise during chelation therapy (264). This variation has also been seen in other patients, and long term changes in serum copper are probably due to changes in copper metabolism, as seen by the rise in serum caeruloplasmin during DFO therapy, Chapter 4. Short term changes in serum copper are not well characterised and appear to vary from patient to patient. This lack of cyclic variation suggests that, in contrast to aluminium, copper does not form a very stable complex with DFO and that changes in serum copper may be due to the indirect, rather than the direct, effect of Cu-complexation. Thus, the unpredictable variations in serum copper levels probably reflect the complexity of copper metabolism, the multiplicity of copper-containing enzymes and proteins, and their role in the

acute phase response.

The increase in high molecular weight protein bound copper, does not result in an increase in urine copper excretion, since high molecular weight proteins are not cleared by the kidney. There were no changes in urine copper after DFO infusion, probably because the Cu-DFO complex is not very stable (formation constant 10^{12}) compared to the Al-DFO and Fe-DFO complexes (10^{31} and 10^{22} respectively) and compared to the stabilisation of Cu-protien binding. Faecal copper excretion remained unchanged after DFO infusion. Caeruloplasmin is synthesised in the liver, and therefore, any copper released in the liver is more likely to be incorporated into newly synthesised caeruloplasmin than to be excreted in bile. An increase in caeruloplasmin concentration is usually attributed to the acute phase response induced by DFO.

7. CONCLUSIONS.

Effects on metal biochemistry. - The use of DFO in a patient who retained some renal function resulted in the removal of aluminium and iron in both urine and faeces. Of the total 22 mg aluminium removed, following the two large intravenous doses of DFO, 27 % was via urine and 73 % via faeces. Iron removal was 137 mg, 1 % and 99 % in urine and faeces respectively. Continued treatment with smaller weekly doses of DFO, accompanied by haemodialysis, resulted in a continuous

depletion of aluminium stores, a reduction in serum aluminium levels, and a reversal of the symptoms of aluminium toxicity. Iron depletion was rectified by oral iron supplements. Although serum copper levels were elevated by DFO therapy, no abnormal amounts of copper were removed, nor were symptoms of copper-depletion observed. We conclude that Al(III)- and Fe(III)-DFO, but not Cu-DFO, were formed in vivo and removed by dialysis. The Al and Fe complexes formed in the liver, were removed by the bile, and excreted in faeces. This latter route of removal is quantitatively most significant.

Clinical. - We conclude that desferrioxamine may be used to treat children with severe alum inium intoxication. Desferrioxamine may be safely administered intravenously without dialysis treatment when there is residual renal function. In this case we saw marked improvement in the child's neurological condition within three months of chelation therapy.

CHAPTER 6.

REVERSAL OF OSTEOMALACIA IN CHRONIC DIALYSIS PATIENTS WITH

HEAVY IRON AND ALUMINIUM LOADING.

REVERSAL OF OSTEOMALACIA IN CHRONIC DIALYSIS PATIENTS WITH HEAVY IRON AND ALUMINIUM LOADING.

1. INTRODUCTION.

The accumulation of iron in bone following repeated blood transfusions has been demonstrated both in patients with normal renal function (265, 266) and in patients on dialysis (267, 268). Aluminium accumulation in bone is a recognised cause of osteomalacia in chronic dialysis (17, 39, 40, 76, 78, 84, 127, 269, 270). The presence of aluminium in bone is associated with low bone formation (76, 84, 269). However, a direct relationship between aluminium and mineralisation has not yet been demonstrated, and the precise mechanisms whereby aluminium interferes with bone mineralisation remain unclear (76, 84). The major sources of aluminium accumulation for dialysis patients are dialysate fluid (11, 26, 39, 40, 45, 50, 271) and aluminium-containing phosphate binders (26, 28, 31). The iron-chelating agent, desferrioxamine, is increasingly used in end-stage renal disease to eliminate iron and/or aluminium during haemodialysis, (40, 70, 110-112, 120, 121, 130, 265, 267). Iron and aluminium, deposited in body stores, are chelated by desferrioxamine and the metal complex formed is removed from the patient during haemodialysis (70, 114, 118). Desferrioxamine has been shown to remove aluminium from bone with reversal of the calcification defect (97, 120), but the removal of iron from the bone has not yet been demonstrated.

A group of four patients who had been exposed to large amounts of both iron and aluminium were presented to Dr. Ackrill, at Withington Hospital, Manchester, by Dr. Ahmed from Sefton General Hospital, Liverpool. The present study was set up to determine whether, in cases of heavy iron and aluminium overload, desferrioxamine is able to complex both metals and remove them by dialysis, thereby reducing the overall body burden of iron and aluminium.

2. CASE STUDIES.

Each of the four patients had been on haemodialysis for over eight years. These patients were chronically anaemic and had received multiple blood transfusions, and in one case monthly intravenous iron was given. All patients received oral iron while on haemodialysis (Table 1).

These patients had been exposed to aluminium in dialysate prepared from tap water treated by water softener only, water-aluminium levels having been in the range ca. 20-70 $\mu g/l$. Each patient had received aluminium-containing phosphate binders to control serum phosphate throughout their haemodialysis treatment.

All patients presented with severe bone pain and three with fractures and proximal muscle weakness.

Table 1.

| PATIENT | TIME ON DIALYSIS (yrs) | FRACTURES | PROXIMAL MYOPATHY | IRON LOADING | | | ALUMINIUM LOADING | |
				BLOOD TRANSFUSION (units)	I-V	ORAL	WATER TREATMENT	ORAL
HB	10	+	+	>100	−	+	Softener	+
JR	10			>100	−	+	Softener	+
CY	8	+	+	67	−	+	Softener	+
JH	11	+	+	20	Imferon (100mg/wk)	+	Softener	+

3. DESFERRIOXAMINE TREATMENT.

Transiliac bone biopsies were obtained, and examined
histologically at the Department of Rheumatology: serial
sections were stained for iron and aluminium using Perl's
reagent and acid solochrome azurine (82). Staining of
cancelluos bone pre and post DFO is shown in Plate 2. Bone
marrow and trabeculae were separated by oxygen plasma ashing
and analysed for iron and aluminium by GFAAS in our
laboratory (81).

All patients were treated with DFO, patient HB received 2g
(ca. 40 mg/kg body weight) then 4g three times per week, the
other 3 patients received 4g (ca. 80 mg/kg) three times per
week. Desferrioxamine was infused in normal saline (200ml)
over 2 hours at the beginning of each dialysis.

Serum and erythrocyte aluminium levels were determined at
intervals during the course of DFO therapy. The removal of
iron and aluminium in dialysate was estimated by repeated
sampling of dialysate from the in-flow and the out-flow lines
of the dialyser. Iron and aluminium concentrations in
dialysate were determined by atomic absorption spectroscopy.
Analytical details are given in Chapter 2.

4. RESULTS.

The bone biopsies of all patients showed osteomalacia and
increased area and thickness of osteoid seams (except patient
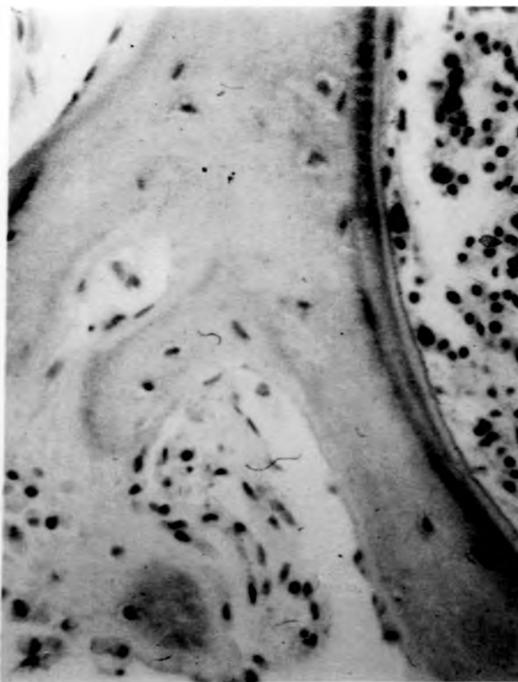
- 151 -

Plate 2.   Stained Sections of Cancellous Bone.   Aluminium in Trabeculae is stained purple.   (i) is pre-DFO treatment
(ii) is post-DFO treatment.

(i)

(ii)

Plate 2. Stained Sections of Cancellous Bone. Aluminium in Trabeculae is stained purple. (i) is pre-DFO treatment (ii) is post-DFO treatment.

(i)   (ii)
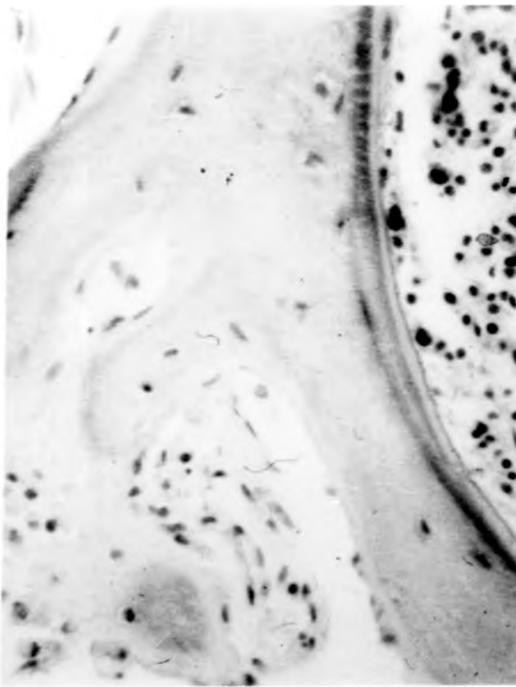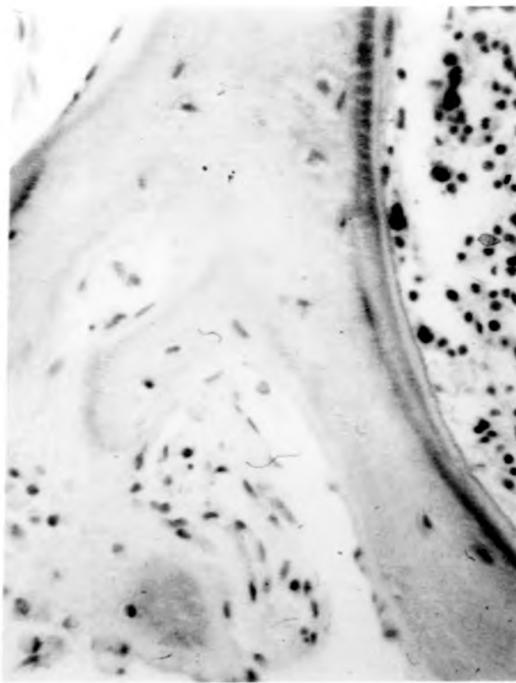
Plate 2.   Stained Sections of Cancellous Bone.   Aluminium in Trabeculae is stained purple.   (i) is pre-DFO treatment
(ii) is post-DFO treatment.

JH with normal osteoid seams). Aluminium staining was positive in each of the patients at the osteoid mineralised bone junction, and within the bone and at cement lines in three patients. Iron was demonstrated at the junction in two patients and at the cement lines in two patients (Table 2).

Before DFO treatment, the aluminium content of trabecular bone was high, ranging between 175-355 $\mu g/g$ and the iron content was very high, between 650-2125 $\mu g/g$. The trabecular iron content of non-iron overloaded chronic dialysis patients is usually less than 100 $\mu g/g$, ranging between 3-221 $\mu g/g$ (46 $\pm$ 54 $\mu g/g$) (26). All patients showed very heavy iron loading of the bone marrow, 1321-4569 $\mu g/g$ (Table 2).

Following DFO infusion there is a rapid rise in serum aluminium concentration. Serum aluminium levels fell gradually during chelation therapy, indicating a reduction in the body burden of chelatable aluminium (Figure 1).

After three months of chelation therapy iron removal in the dialysate was between 6-30 mg/dialysis in each of the patients studied. Aluminium removal was seen in three patients (Table 3). In one patient there was an apparent uptake of aluminium during dialysis. This overall gain of aluminium during dialysis is possibly related to the use of contaminated dialysate (ca. 30 μg Al/l) prepared from untreated water. After eight months of chelation therapy, and a change to reverse osmosis (R.O.) water treatment, iron and aluminium were detected in dialysate in all patients. The

Table 2.

PATIENT	BONE HISTOLOGY	BONE BIOPSY SOLOCHROME STAIN J	BONE BIOPSY SOLOCHROME STAIN CL	PERLS' STAIN J	PERLS' STAIN CL	BONE CONTENT ALUMINIUM (ug/g) T	BONE CONTENT ALUMINIUM (ug/g) M	IRON (ug/g) T	IRON (ug/g) M	SERUM FERRITIN (ug/l) Pre DFO	SERUM FERRITIN (ug/l) Post DFO	SYMPTOMATIC IMPROVEMENT
HB	OM	+	+	+	+	355	87	1413	4569	8188	5000	+
JR	OM	+	−	−	−	175	48	650	2263	1566	1200	+
CY	OM	+	+	+	−	278	76	1424	2322	2133	2800	+
JH	Aplastic	+	+	−	+	270	134	2125	2151	1184	4000	+

OM = Osteomalacia   J = Osteoid mineralisation bone junction   CL = Cement lines

T = Trabeculae   M = Marrow

Table 3.   Metal Removal in Dialysate During DFO Treatment.

| | REMOVAL IN DIALYSATE | | | |
| | AFTER 3 MONTHS | | AFTER 8 MONTHS | |
PATIENT	Al(mg)	Fe(mg)	Al(mg)	Fe(mg)
HB	1.0	17.0	2.7	12.0
JR	-0.9	16.0	0.5	0.6
CY	1.6	6.0	0.4	0.9
JH	0.7	30.0	0.8	1.9

Figure 1. Serum Aluminium Levels During Prolonged Desferrioxamine Treatment.
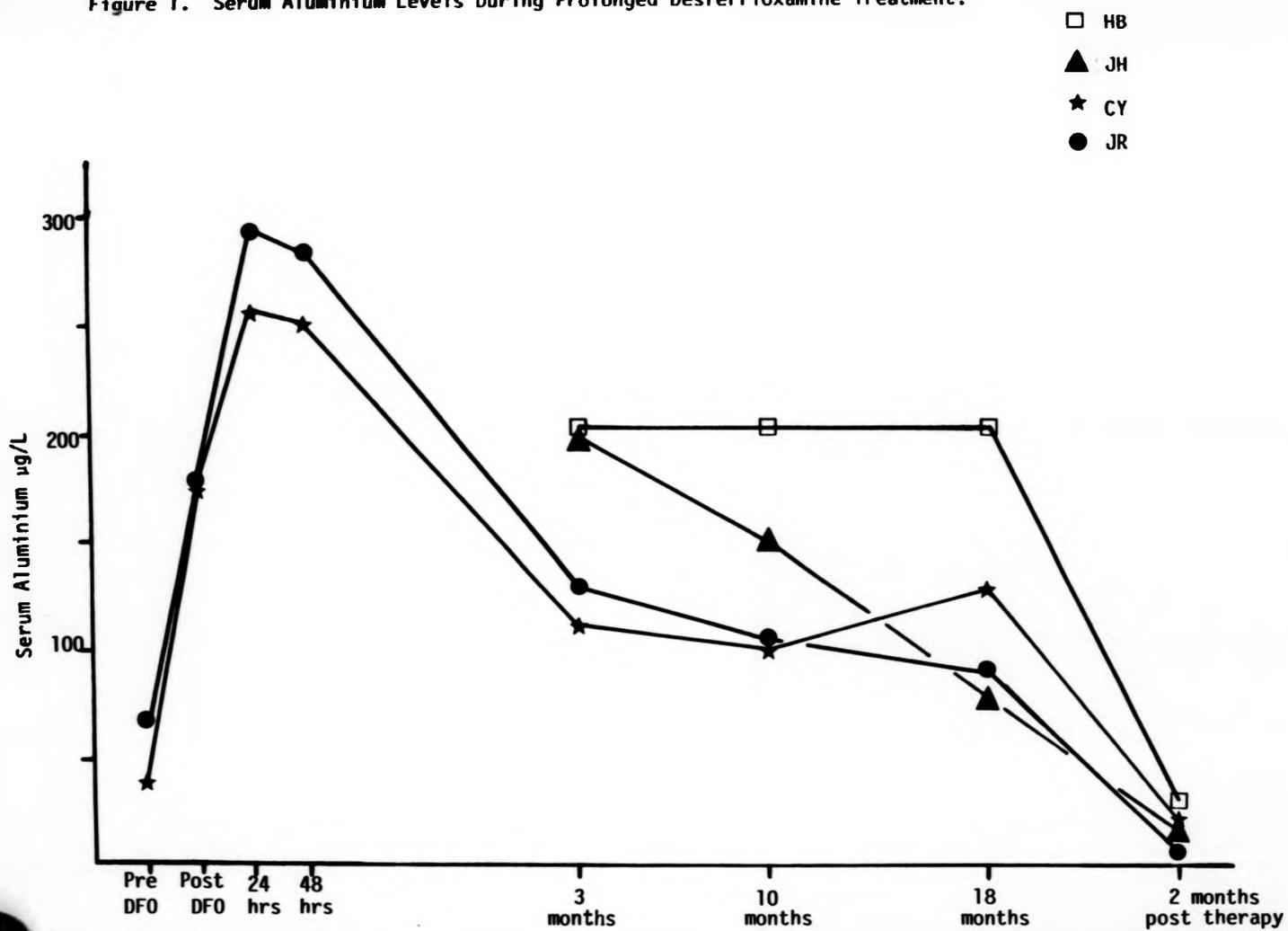
total amount of iron removed was greater after three months chelation therapy than after eight months; this suggests that the total amount of iron available for chelation had been reduced. After changing to R.O. water treatment (dialysate <2 μg Al/l) a negative aluminium balance was demonstrated in each of the four patients, and the amount of aluminium removed during dialysis increased.

Two patients (CY and JH) showed increased haemoglobin levels after 15 months DFO treatment (Figure 2). The other patients (HB and JR) are anephric and their anaemia did not improve during chelation therapy.

All patients showed marked symptomatic improvement within weeks of starting DFO treatment, despite the continued requirement for regular blood transfusions.

Patient HB was observed on DFO treatment for 2 years. After only three months, bone pain and proximal muscle weakness disappeared and she was able to walk unaided. There was a marked reduction in the iron and aluminium content of both bone marrow and trabeculae despite repeated monthly blood transfusions and the continuation of oral aluminium (Figure 3). Liver biopsy after 12 months of chelation therapy indicated gross iron overload. Iron deposits were found in the hepatocytes and Kupfer cells. Bone biopsy showed a reduction in both iron and aluminium staining at the mineralisation front, but was unchanged within the bone marrow. Histologically she remained osteomalacic. A further

Figure 2.   Improvement of Anaemia During DFO Treatment (4g DFO/Dx).
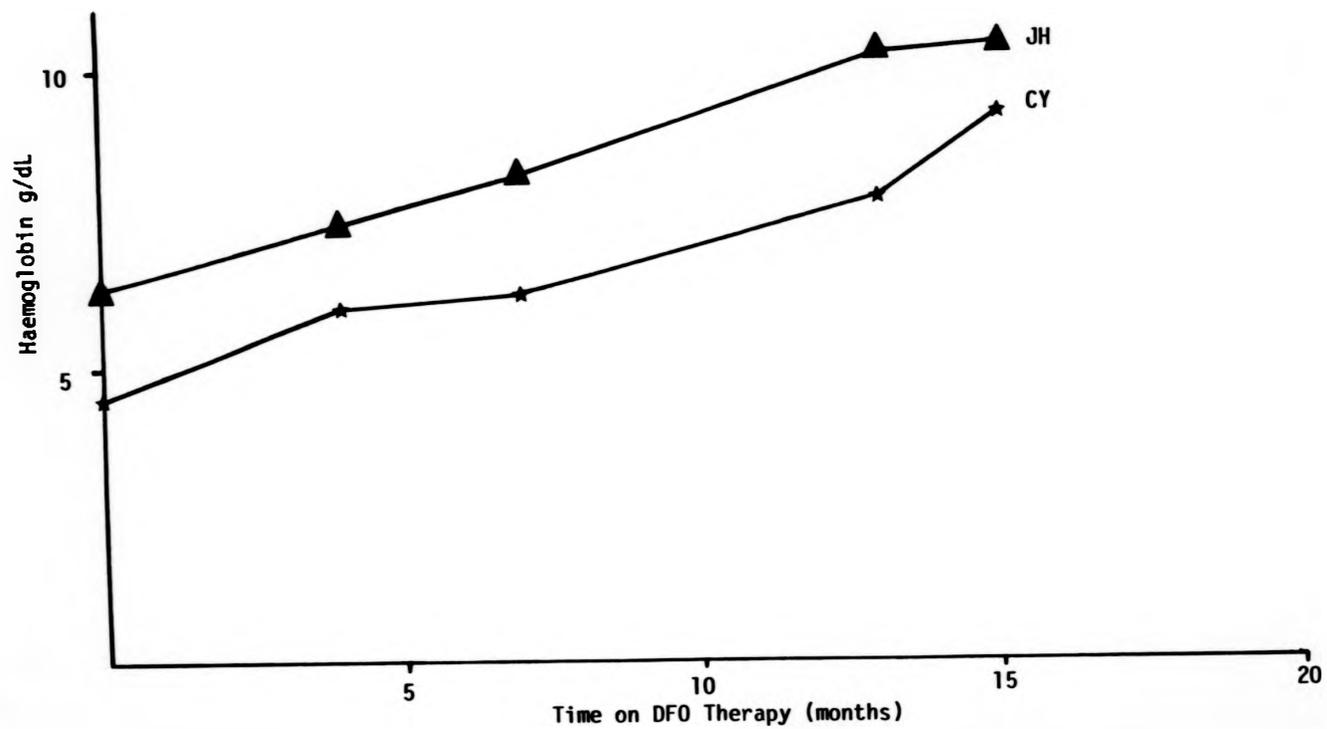
Figure 3. Response of Bone Aluminium and Iron Content to DFO Therapy in Patient HB
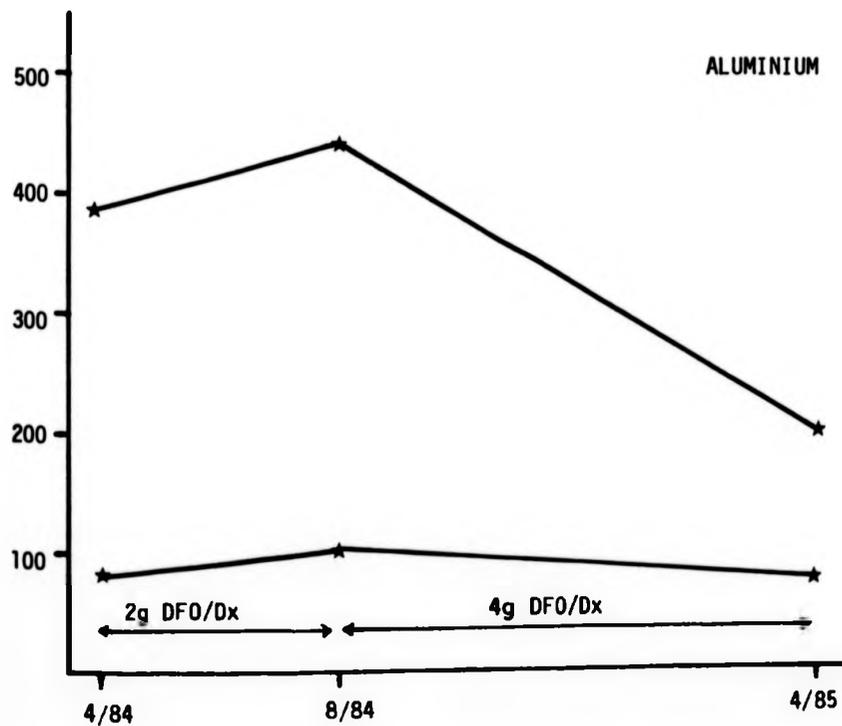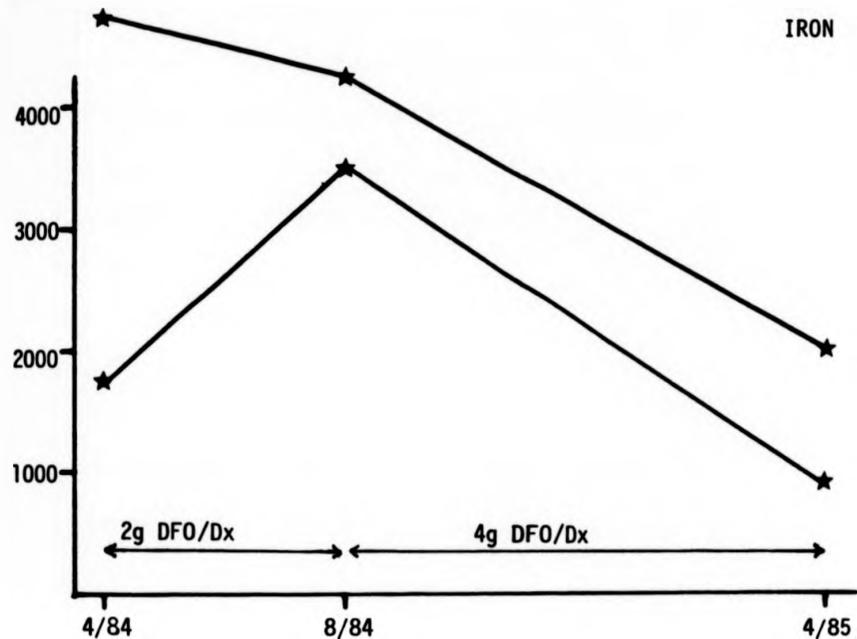
bone biopsy was obtained after 2 years DFO treatment; little stain was found at the fronts, and that present appeared to be beneath the active seams, indicating that the iron and aluminium present in bone had not been laid down recently. The continued mineralisation defect despite the absence of staining from fronts suggests that the failure of mineralisation is not related to metal overload. There was persistent gross bone marrow overload even after prolonged chelation therapy.

Bone biopsies taken from the other three patients at the end of treatment indicated a reduction in bone iron and aluminium at the mineralisation fronts, with heavy iron staining in the marrow in two patients and moderate staining in the third patient.

5. CONCLUSIONS.

The prolonged administration of DFO with haemodialysis appears to reduce the iron and aluminium content of bone of chronic dialysis patients with iron and aluminium overload, resulting in marked symptomatic improvement. But even after 16 months chelation therapy iron and aluminium still remained within the bone. This reduction of body stores was seen despite repeated blood transfusions and continued oral aluminium during chelation therapy.

In these patients the infusion of DFO produced a marked rise

in serum aluminium. This increase in serum aluminium was seen
even in the presence of severe iron overload. Desferrioxamine
complexes with iron, forming the very stable Fe(III)-DFO
complex, ferrioxamine (formation constant 10^{32}), and forms a
less stable Al(III)-DFO complex (formation constant 10^{21})
under physiological conditions (117). The marked rise in
serum aluminium indicates that aluminium complexation does
occur in patients treated with DFO who also have large iron
stores. Even though the ferrioxamine complex would be
preferentially formed in aqueous solution, in vitro, this
does not appear to be the case in vivo. It may be inferred,
therefore, that the binding of iron (III), in vivo, is
stronger than the binding of aluminium (III) by more than the
ratio between their in vitro stability constants (10^{11}).

The presence of iron and aluminium can be demonstrated in the
dialysate fluid during dialysis. The amount of iron removed
in dialysate is possibly related to the total body iron
store, i.e. more iron was found in dialysate after three
months of chelation therapy than after eight months. At the
beginning of chelation therapy serum aluminium levels were
high, but the amount of aluminium removed in dialysate was
small (in one case aluminium was taken up by the patient
during dialysis). This observation is probably related to the
use of high aluminium level dialysate (ca. 30 µg/l) prepared
from water treated by a water softener. A number of other
reports have indicated the transfer of aluminium from the
dialysate to the patient during dialysis when high aluminium
concentration dialysate was used (11, 46, 96, 271). After

changing to the R.O. water for dialysate preparation a
negative aluminium balance was observed in all four patients.
Aluminium removal from the patient has been demonstrated
previously with the use of very low aluminium level dialysate
(46, 51, 272). The total amount of aluminium removed was
greater after the introduction of adequate water treatment
even though serum aluminium levels had fallen. This
observation indicates that aluminium removal during dialysis
is more efficient when a dialysate of low aluminium
concentration is used. Baldamus et al (111) studied the
removal of aluminium and iron during desferrioxamine
treatment by haemodialysis and haemofiltration.
Haemofiltration was found to be the more efficient treatment
when iron and aluminium clearances were related to urea
clearances.

The simultaneous reduction of iron and aluminium from the
bone and the presence of both metals in the dialysate fluid
indicates that the dose of desferrioxamine administered to
these patients (4g) was sufficiently large to complex with
both iron and aluminium deposited in body storage sites.

CHAPTER 7.

EXPOSURE OF INFANTS TO ALUMINIUM FROM INTRAVENOUS FLUIDS AND MILK FORMULAE.

EXPOSURE OF INFANTS TO ALUMINIUM FROM INTRAVENOUS FLUIDS AND MILK FORMULAE.

1.   INTRODUCTION.

Man is continuously exposed to aluminium from drinking water, food and dust particles. In health the element is poorly absorbed through the gut, skin and lungs which act as natural barriers to the uptake of aluminium. Aluminium that does enter the body is efficiently excreted by the kidneys, in the urine, which results in a low body burden of aluminium in a healthy person.

The problem of aluminium intoxication in chronic renal failure is well recognised. The accumulation of the element may result from a number of sources including:

(i) Contaminated water supplies and solutions used for dialysis (32, 33, 37, 40, 43, 72, 273);

(ii) Aluminium-containing phosphate binding gels, which are taken orally, often in large doses (up to ca. 10 g/day) by renal patients to control serum phosphate levels (24-27, 64, 65, 73, 74, 251, 274);

(iii) Blood products, such as whole blood and albumin (52, 55), given to patients in transfusions.

Concerted efforts have now been made to control the exposure of these patients from pharmaceutical products and water used

for dialysis. A number of offical recommendations have been
made regarding a "safe" limit for aluminium concentrations in
water. The CEC proposed a limit of 10-15 $\mu g/l$ aluminium in
water used for dialysis in 1982 (275), whereas the National
and Regional Health Authority recommendations in Britain are
at 30 $\mu g/l$ (276). The CEC recommendations are in keeping with
the findings of Hodge et al (51), who noted a net transfer of
aluminium from dialysate to patient when aluminium in
dialysate exceeded 14 $\mu g/l$.

However, renal patients are not the only group of people
susceptable to aluminium accumulation. Premature infants born
with immature gut, kidneys and metabolic systems are also at
risk from aluminium exposure and accumulation. Elevated
plasma, urine and bone aluminium levels have been reported in
premature infants who received several weeks of total
parenteral nutrition (TPN), and the source of exposure was
ascribed to aluminium-contaminated intravenous fluids (56,
59). Two fatal cases of aluminium toxicity in uraemic
neonates have been described (277). These infants had been
fed orally entirely on milk formulae, and had received
neither intravenous fluids nor phosphate binders. The source
of exposure was thought to be from the powdered milk
formulae, containing 230 $\mu g(Al)/l$. At post mortem they were
found to have elevated brain aluminium levels. In another
case (278), severe aluminium toxicity in a uraemic baby
resulted in thoracic wall instability and respiratory
failure; the suggested source of exposure was from the use of
aluminium-containing phosphate binders.

The overall aim of the present study was to determine the
level of aluminium exposure of infants receiving nutritional
support, in the form of total parenteral nutrition and
specialised milk formulae, compared to breast-fed infants;
and to determine what effect, if any, this aluminium exposure
has on body aluminium loading. This study was carried out in
four parts, and the aims of each part were:

(i) To determine the levels of aluminium in intravenous
solutions commonly used for total parenteral nutrition and
baby milks fed to fullterm and preterm infants, and hence
calculate the resultant aluminium exposure in both oral and
parenterally fed infants;

(ii) To compare infants' serum aluminium levels at the
time of birth and sequentially during the first weeks of
life, comparing aluminiumn levels of babies fed with formula
feeds and TPN;

(iii) To determine the range of aluminium levels in
breast milk from mothers with normal serum aluminium levels;

(iv) To determine brain, bone and liver aluminium levels
in a child who had died after receiving TPN for several
weeks, compared with children under one year of age who had
died from non-aluminium related diseases.

In the first part of the study aluminium was determined in
samples of most of the milk feeds and intravenous fluids used
in our local hospitals. The selection covers various
manufactures and batches. The samples analysed, though not
exhaustive, enabled us to calculate the exposure of patients

on nutritional supplements to aluminium. The other three
parts of the study were intended to be exploratory only, to
give an indication of which infants were at most risk from
aluminium accumulation. The intention was that a large
long-term study could perhaps be set up on the basis of these
preliminary findings.

2. ALUMINIUM IN MILK FORMULAE AND INTRAVENOUS FLUIDS.

2.1 Nutritional Support.

Humans require a balanced intake of protein, fat,
carbohydrate, minerals and vitamins to maintain normal
metabolic processes and health (279). Nutritional
requirements vary from one individual to another and are age
dependant. These nutritional requirements may change in
disease and in such circumstances daily intake must be
adapted to body demands.

Preterm infants, born before the gastrointestinal tract and
other metabolic systems are prepared for extrauterine
nutrition, and patients in intensive care, require continuous
nutritional support. The nutritional status of most of these
patients is normally maintained by the use of currently
available commercial formulations for parenteral and enteral
feeding.

The care and feeding of infants is a major consideration of

the Paediatrician. Nutritional management of paediatric patients requires attention to a number of unique problems not encounted in adults. In addition to requiring nutrients to maintain life and health, children, and especially babies, have very large requirements of vitamins (280, 281), minerals (282, 283), proteins and energy for growth (279). In infants the nutritional requirements imposed by disease are superimposed on the limited endogenous nutrient body stores, immature metabolic processes and relatively large requirements for normal developement. In the case of premature neonates, nutritional support must replace the interuterine feeding normally received from the mother at a time of rapid growth and developement.

Nutritional support may be administered by the parenteral or enteral routes. Parenteral nutrition is given intravenously by catheter, and is used when feeding via the gastrointestinal tract is either impossible or undesirable. In the case of preterm infants the intestinal tract is underdeveloped and feeding by this route may often be undesirable. If the gastrointestinal tract is functional either in part or fully but nutritional support is still required, then entral feeding is used. In the case of preterm infants with gastric function, specially formulated milk feeds are given. These feeds comprise a specially balanced diet suitable for premature babies, and in particular contain elevated levels of calcium and phosphate to promote bone formation and growth.

2.2 Results and Discussion.

The levels of aluminium we found in milk feeds and fluids
used for parenteral nutrition are given in Table 1 and 2
respectively.

Milk Formulae. - Aluminium concentrations in milk formulae
ranged between 35 and 2080 µg/l (Table 1). This range is
similar to that reported by Sedman (59) and Weintraub (284).
Infant formulae given to fullterm babies generally have lower
aluminium levels than those given to preterm infants, and
soy-based formulae. Preterm infant milk formulae contain
higher levels of calcium, potassium and phosphate compared to
fullterm infant feeds, in order to provide the necessary
minerals for rapid growth and bone development during the
first weeks of life. The elevated aluminium concentration in
these special preparations probably results largely from
mineral additives. The high levels of aluminium in soy-based
milk formulae are inherent and not due to contamination
during manufacture or from additives.

These milk-aluminium levels result in infant aluminium
intakes of between 10 to 400 µg/kg body weight/day. Whereas
intakes at this level might not be considered excessive in
adults, children with higher gut absorption (10-100 fold) may
be at risk. Additionally, with immature renal function, a
common problem in premature infants, aluminium excretion will
be reduced leading to excessive accumulation.

Table 1. Aluminium Content of Milk Formulae.

MILK PRODUCT		ALUMINIUM (µg/l) Range
Standard Formulae:		
Babymilk plus	(C&G)	88 – 107
Premium	(C&G)	107 – 191
Osterfeed	(Farley)	59 – 84
Ostermilk	(Farley)	191 – 370
Gold cap	(S.M.A.)	40 – 57
White cap	(S.M.A.)	35 – 63
Pre-term Formulae:		
Nenatal	(C&G)	499 – 660
Prematalac	(C&G)	168 – 192
Pregestamil	(Bristol Meyers)	1346 – 2080
Soya Protein Formulae:		
Formula S	(C&G)	419 – 1205
Wysoy	(Wyeth)	910 – 1330

Table 2.   Aluminium in Fluids for Parenteral Nutrition.

SOLUTION	ALUMINIUM ($\mu g/l$)	
	Mean	Range
Vamin 9 Glucose (KV)	86	44 - 127
Glucose 5%	2	1 - 3
Dextrose 5%	2	0 - 4
Dextrose 50% (Evans)	156	1 - 242
Addiphos (KV)	7176	7050 - 7293
Ped-el (KV)	1626	1336 - 1870
Solvito (KV)	29	9 - 69
Intralipid 10% (KV)	11	9 - 12
Vitlipid (KV)	307	307 - 307
Calcium gluconate 10% (PP)	4810	3430 - 6120
Potassium chloride 15% (PP)	212	164 - 290
Potassium phosphate 13% (McCarthy)	2130	1880 - 2390
Sodium chloride 0.9%	6	1 - 11
Water for injection	90	4 - 189

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Water for injection	90	4 - 189

High urinary aluminium levels in fullterm infants with normal renal function indicate high gut absorption (59). Freundlich (277) found elevated brain levels of aluminium in two infants after one and three months feeding on a milk formulae containing aluminium at 230 µg/l, indicating aluminium accumulation in impaired renal function.

Powdered milk feeds are usually reconstituted with boiled tap water, and the final level of aluminium in milk feed will depend on the level of aluminium in the water supply. The EEC regulations for aluminium in domestic water propose a limit of 200 µg/l. Levels of aluminium vary regionally (17, 46, 76, 85, 285, 286), depending on the nature and quantity of flocculant used during raw water treatment. Aluminium sulphate is generally used as a flocculant in water processing, but in some areas iron sulphate is used as an alternative, resulting in low aluminium levels in domestic water supplies. It is clear that if aluminium levels ca. 200 µg/l in milk formulae are cause for concern then consideration should also be given to the contribution from the mixing water. In this respect, the permitted maximum in tap water (200 µg/l) seems excessive.

Intravenous Solutions. - The aluminium content of commonly used fluids administered in our Units for parenteral nutrition is given in Table 2. Sedman (59) reported similar results for comparable materials, with a wide variation between batches. As with the milk products, high aluminium concentrations appear to be associated particularly with the

presence of calcium, potassium, and phosphate. It is these intravenous fluids which are administered to premature infants to enable rapid bone growth, and to the elderly to retard decalcification of the skeleton. Both of these groups of patients have poor renal function, and are susceptable to aluminium accumulation.

Table 3 shows the calculated aluminium intake (per kg body weight) which would result from the use of a total parenteral nutrition (TPN) formulation (aluminium levels in intravenous solutions are taken from Table 2). The nutrition program evaluated includes typical intravenous fluids administered to premature infants of 1 to 2kg body weight. The calculated aluminium intake (μg/kg/day) ranged from 24.6μg (typical case, Figure 1) to 70.5μg (worst case). This aluminium intake from TPN is potentially more serious than from oral feeds, even though total exposure is probably less than from milk feeds (<400 μg/kg/day), since ca. 80% of intravenous aluminium is retained (59). Aluminium accumulation from contaminated parenteral nutrition solution, and possibly consequential bone disease, has already been observed in adults with normal renal function (269). In premature infants the risk is likely to be significantly greater.

The major contribution to the aluminium load arises from the use of calcium and phosphate supplements, in the form of calcium gluconate and potassium acid phosphate or "Addiphos". However, calcium chloride solutions were found to be low in aluminium (<30 μg/l) compared to calcium gluconate, and

Figure 1. Aluminium in TPN Fluids Used for Preterm Infants.
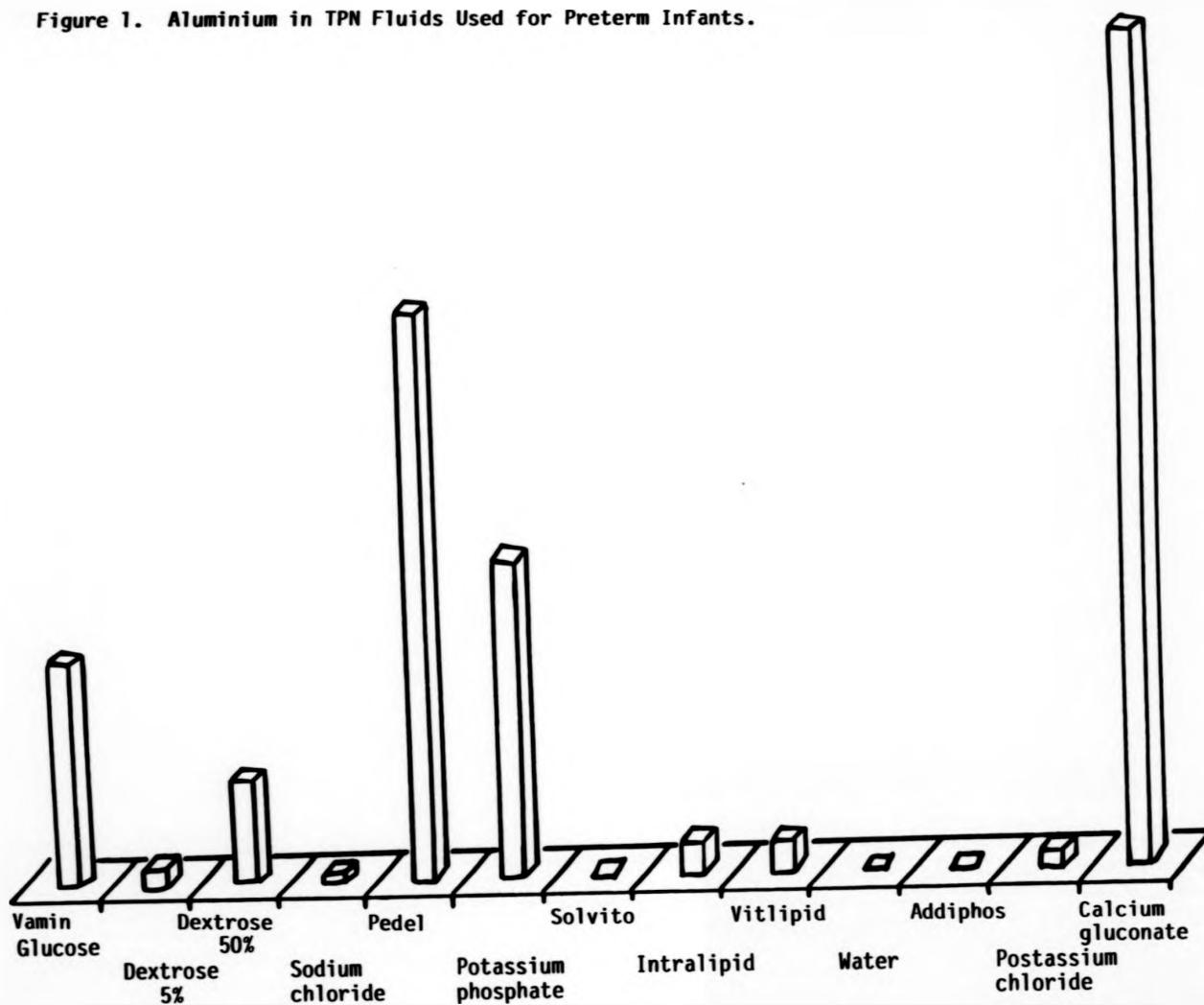
Table 3. Aluminium Intake (µg/kg/day) from Intravenious Feeds.

COMPONENT	DAILY INTAKE (ml)		Al CONTENT (µg)	
	Typical	Maximum	Typical	Maximum
Glucose (Vamin 9)	30.0	30.0	2.57	3.81
Dextrose 5%	117.0	140.0	0.20	0.56
Dextrose 50%	7.5	9.0	1.17	2.18
Addiphos	0.0	1.0	0.00	7.29
Ped-el	4.0	5.0	6.51	9.35
Solvito	0.5	0.7	0.01	0.05
Intralipid 10%	30.0	35.0	0.32	0.42
Vitlipid (Infant)	1.0	2.0	0.31	0.61
Calcium gluconate	2.0	6.0	9.63	36.72
Potassium chloride	0.7	0.9	0.15	0.26
Potassium phosphate	1.7	2.2	3.63	5.25
Sodium chloride	16.0	20.0	0.0	0.22
Sterile water	0.0	20.0	0.0	3.78
TOTAL ALUMINIUM (µg/kg/day)			24.6	70.5

therefore might be used in preference to calcium gluconate when aluminium accumulation could result. We were unable to find a low aluminium level paediatric potassium supplement, and adult preparations contain only 1% of the aluminium found in paediatric solutions. This suggests that aluminium was either excluded during manufacture, preventing contamination, or that aluminium was removed after the manufacturing process. In either case a similar course of action for paediatric solutions would not only appear possible but would also be benefical.

3. ALUMINIUM EXPOSURE FROM TPN AND MILK FORMULA FEEDS.

Having established in the first part of the study that commercial infant diets, whether milk formula feeds or TPN, contain significant levels of aluminium, we then began to study serum aluminium levels in preterm infants.

Serum aluminium levels were determined at birth in infants and then sequentially during the first weeks of life. Two groups of infants were selected, those fed commercial milk formulae, and infants on TPN. In this way we attempted to determine whether children exposed to high levels of aluminium (i.e. those fed TPN formulations) show elevated serum aluminium levels compared with infants fed lower aluminium containing diets (i.e. milk feeds), thus indicating increased levels of accumulation.

At the beginning of this study (March, 1986) we had a small
group of 11 preterm infants (Group 1), four receiving
commercial milk feeds and seven on TPN formulations.
Sequential blood samples were taken from these infants while
they remained on the Special Care Baby Unit. Four months
later (July, 1986) we received blood samples from another
group of infants (Group 2), nine patients on milk formula
feeds and seven on TPN.

Serum and red cells were separated and serum aluminium was
determined as described in Chapter 2. Red cell aluminium
levels were also determined on seven of the infants include
in Group 1. The results of red cell determinations are
discused later.

3.1 Results.

The results of the sequential serum aluminium determinations
from the preterm infants in Group 1 are given in Table 4.
Serum aluminium levels for the second group of infants are
shown in Table 5.

The results given in Table 4 (Group 1) indicate that infants
fed with TPN formulations have higher serum aluminium levels
than infants fed on milk feeds, 12.9 ± 6.1 µg/l compared with
2.5 ± 1.5 µg/l.

The results in Table 5 (Group 2) show variable aluminium
levels for infants fed both milk feed and TPN. A number of

Table 4. Sequential Plasma Aluminium Levels (µg/l) on Premature Infants (Group 1).

BABY	DIET	0	1	2	3	4	5	6	+6
CT	TPN	21	20	20	4				
BH	TPN		19	13					
WC	TPN		15	1	12				
CW	TPN			17	13	16			
AR	TPN						10		
ER	TPN						6		
MT	TPN				6				
RD	PREMIUM		1						
DD	PREMIUM			2					
GH	PREMIUM								5
AL	PREMIUM			2					

TPN: Number = 15   Mean = 12.9   S.D. = 6.3

PREMIUM: Number = 4   Mean = 2.5   S.D. = 1.7

Table 5.   Sequential Plasma Aluminium Levels (μg/l) on
Premature Infants (Group 2).

BABY	DIET	0	1	2	3	4	5	6
				AGE	IN	WEEKS		
DS	TPN	16	24*	10				
NMcC	TPN	17*	38*	7		14*	24	
MB	TPN	17						
BB	TPN	1						
VR	TPN	1						
DS	TPN	16*						
TMcG	TPN			29*			21	
LJ	PERMIUM	11*	6					
WH	PREMIUM		22*	6				
LB	PREMIUM	19*		9		2		
DB	PREMIUM	18*		4		8		
GK	PREMIUM	75*						
KW	PREMIUM	57*						
LS	PREMIUM	14*						
RW	PREMIUM			4				
BG	PREMIUM	1						

TPN: Number = 7   Mean = 11.6   S.D. = 9.3

PREMIUM: Number = 7   Mean = 4.7   S.D. = 3.2

* denotes haemolysed sample

the blood samples taken from these infants were haemolysed by
the time they reached our laboratory. Once blood has
haemolysed serum and red cells can not be separated from each
other, and so whole blood analysis was carried out on these
samples. Serum and whole blood analysis results are
comparable in normal adults, but in preterm infants red cell
aluminium levels are higher than serum aluminium levels (see
Serum and Red Cell Aluminium levels, section 4). Therefore,
serum samples which were contaminated by haemolysis have been
excluded from statisical calculations.

Serum aluminium levels of infants fed TPN were also higher
than serum aluminium levels in infants fed milk formula in
Group 2, 11.6 ± 8.6 µg/l and 4.7 ± 2.9 µg/l respectively. The
difference in serum aluminium levels was less marked in Group
2 compared to Group 1.

3.2   Discussion.

Preterm infants exposed to high levels of aluminium in TPN
formulations have higher serum aluminium levels compared with
infants fed on milk formula feeds. Elevated serum aluminium
levels in TPN feeding may result from the high level of
retention of intravenously administered aluminium, ca. 80%
(59).

Aluminium present in TPN formulations enters the circulating
blood and is rapidly transported around the body. In normal
subjects, aluminium present in serum is efficiently excreted

by the kidneys, but in premature infants with impaired renal
function aluminium excretion is reduced. In these cases
aluminium may be deposited at one of the three major storage
sites for aluminium, bone, brain or liver (56, 59, 277, 278,
284).

Aluminium exposure due to milk formula feeds is thought to be
less than exposure from TPN, due to the lower level of
gastric absorption and retention in enteral feeding. It is
generally the least premature babies with fewer clinical
complications associated with premature birth who normally
receive milk formula feeds, and the very premature babies
with low birth weights, immature gastrointestinal function,
and impaired renal function, who receive TPN; i.e. the
healthier infants are exposed to less aluminium in their diet
than infants who are already at considerable risk due to
their very premature birth.

By comparison with phosphate binding gels, the total
aluminium content of milk formulae and TPN is only small. The
fatal cases of aluminium toxicity ascribed to intravenous
fluids (56, 59) or milk feeds (277, 284) show that in the
absence of other commonly recognised risk factors cerebral
toxicity (277) and bone toxicity (269, 278) may reflect: (i)
an increased gastric absorption in preterm infants compared
with fullterm infants and adults; (ii) a greater
bioavailability of aluminium in milk formula feeds and TPN
compared with phosphate binding gels; (iii) an increased
uptake of aluminium by the immature brain due to the absence

of the blood brain barrier; and (iv) an increased deposition
of aluminium in the bone at a time of rapid growth.

Therefore, we conclude that preterm infants fed with
commercial feeds, TPN or milk formula feeds, are at risk from
aluminium exposure and accumulation because they are unable
to excrete the metal efficiently.

4. SERUM AND RED CELL ALUMINIUM LEVELS IN PRETERM INFANTS.

Blood samples taken from the first group of infants (Group 1)
that showed no signs of haemolysis were centrifuged; serum
and red cells were separated and analysed for aluminium as
described in Chapter 2.

These determinations were carried out to ascertain:

(i) Whether serum and red cell aluminium levels
correlate in preterm infants; and if so whether the
correlation is similar to that of serum and red cells in
adult renal patients, (Chapter 4);

(ii) Whether red cell aluminium levels are higher in
infants who are exposed to elevated levels of aluminium in
their diet.

4.1 Results.

Paired serum and red cell aluminium levels for TPN and milk
formula fed infants are given in Table 6. A plot of serum

Table 6.   Serum and Red Cell Aluminium Levels in Pre-term Infants.

BABY	DIET	PLASMA ALUMINIUM ($\mu g/l$)	RED CELL ALUMINIUM ($\mu g/l$)
RD	Premium	1	14
DD	Premium	2	23
GH	Premium	5	6
AL	Premium	2	13
AR	TPN	10	53
ER	TPN	6	50
MT	TPN	6	20
Number		7	7
Mean		4.6	25.6
S.D.		3.2	18.5

Figure 2. Serum Aluminium vs. Red Cell Aluminium in Preterm Infants.
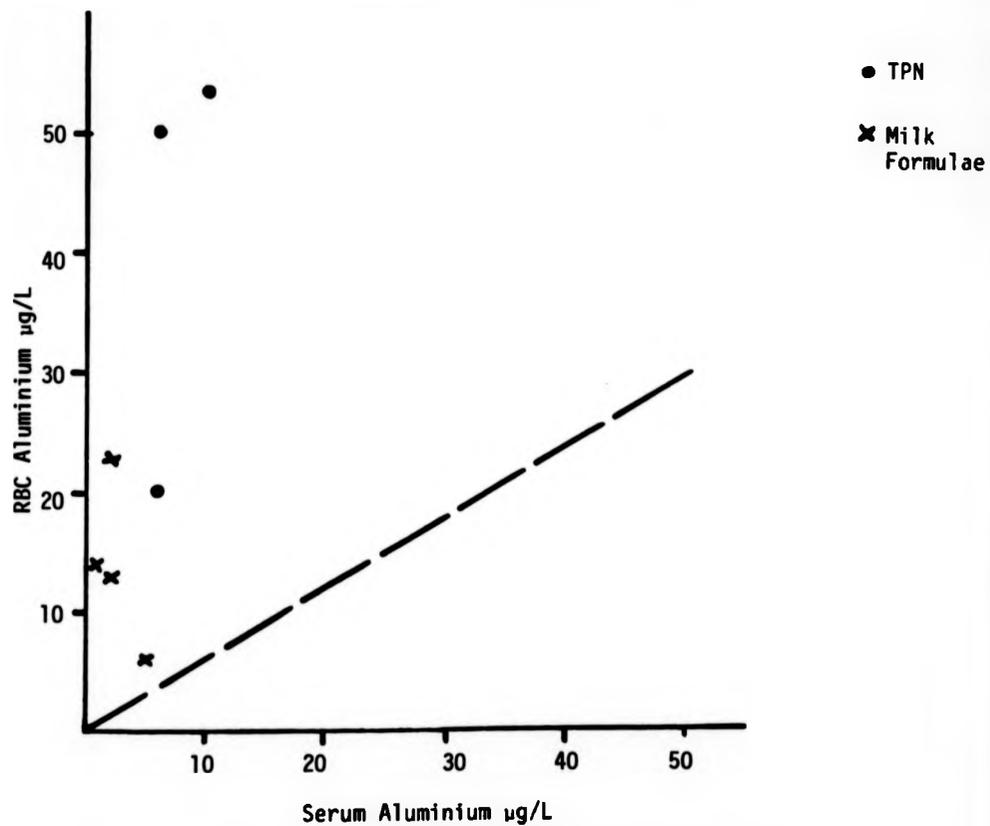
(The plotted line shows the red cell: Serum ratio in CRF patients. Chapter 4, Figure 3).

aluminium versus red cell aluminium concentration is shown in
Figure 2.

Red cell aluminium concentrations were found to be invariably
higher than serum aluminium levels in this group of infants,
25.6 ± 18.5 µg/l and 4.6 ± 3.2 µg/l for red blood cells and
serum respectively.

4.2 Discussion.

In adults with chronic renal failure, who have been exposed
to aluminium from the dialysate and from phosphate binding
gels, red cell aluminium levels are lower than serum
aluminium levels (Chapter 4). Preterm infants who are exposed
to aluminium from their diet alone, whether milk feeds or
TPN, have red cell aluminium levels elevated above serum
aluminium levels. This observation suggests either that the
red cells in preterm infants accumulate aluminium directly
from the serum (and act as a storage site in addition to
bone, brain and liver) or that aluminium is retained in the
bone marrow and becomes incorporated in the red cell at its
formation.

We have no evidence to suggest that red cells in preterm
infants actively absorb or take up aluminium. From our adult
studies we know that aluminium does not cross the red cell
membrane, because aluminium red cell concentrations decline
with a half-life approaching that of the red cell life span,
and red cell aluminium levels show no cyclical variation

during chelation therapy (Chapter 4). It is unlikely that the red blood cells behave differently in preterm infants than in adults.

Bone deposition is very rapid in neonates, to allow for rapid bone growth. Rapidly developing tissues require a good blood supply to ensure that nutrients needed for growth are transported to the deposition site as they are required. If toxic elements such as aluminium are also present in the blood they may become deposited in the bone and if not metabolised they will accumulate at the site of deposition. Bone is known to be a major site of aluminium deposition in adults (17, 32, 33, 40, 76, 85, 286) and infants (59, 269, 278). Bone marrow is the major site for red cell formation.

The circulatory blood volume of an infant is approximately 100ml blood per kg body weight. During the first three months of life the blood volume of an infant will increase to at least double its original volume at birth. This large increase in blood volume requires the rapid formation of red cells in the bone marrow and spleen. The increased requirement for red cells as the blood volume increases is coupled with the need to replace degraded red cells as they reach the end of their life span (ca. 90 days). Bone accumulates aluminium, and red cells may incorporate aluminium from the bone marrow as they are formed.

The increased red cell aluminium levels in TPN fed infants compared to milk formula fed infants suggests either that TPN

fed infants are exposed to more aluminium in their feeds, or that the aluminium speciation and method of administration of TPN formulations results in increased retention of the metal in the bone marrow with its subsequent incorporation into the red blood cells.

The highest levels of aluminium in intravenous solutions are associated with calcium gluconate, "Addiphos" and potassium phosphate (Section 2). These intravenous fluids are administered specifically to supply the necessary amounts of calcium and phosphate to ensure adequate bone growth. It seems possible that aluminium associated with phosphate in potassium phosphate solutions and aluminium contaminated calcium gluconate might be particularly incorporated into bone. Thus, the higher red cell aluminium levels in TPN fed infants compared to milk formula fed infants might be accounted for, at least in part, by the speciation of the aluminium in the source material.

5. ALUMINIUM IN BREAST MILK.

Levels of aluminium in breast milk are poorly documented. Sedman (59) reported a value of 9.9 ± 6.87 µg/l on 12 lots of breast milk expressed directly into plastic containers after careful cleansing of the skin. Freundlich (277) and Weintraub (284) give single values of 4 and 30 µg/l aluminium in breast milk respectively.

After calculating the aluminium exposure of infants on TPN
and formula feeds, we attempted to determine the level of
aluminium in breast milk in order to calculate the aluminium
exposure of breast-fed infants and infants fed breast milk
from the milk bank. This was carried out to determine whether
infants fed on breast milk were exposed to similar levels of
aluminium as infants on commercial preparations.

Blood samples were taken from a group of 18 mothers who were
breast feeding their babies, and the levels of serum
aluminium determined. Samples of breast milk were expressed
before and after feeding and analysed for aluminium. Full
analytical details are given in Chapter 2. However we had no
control over the methods used for the recovery and collection
of the breast milk in this experiment. There are serious
problems of contamination in these procedures (vide infra).

5.1 Results.

Maternal serum aluminium levels and paired breast milk levels
are shown in Table 7. Serum aluminium levels were low,
ranging between 2 to 17 µg/l (mean 6 ± 5 µg/l, within the
normal range). All mothers (except mother 22 with chronic
renal failure) had normal renal function, and would not be
expected to have elevated aluminium loads.

Aluminium concentrations in breast milk ranged from 8 to 1000
µg/l, and showed no discernable relationship to the serum
aluminium levels.

Table 7.   Aluminium (µg/l) in Paired Breast Milk and Maternal Blood.

MOTHER	SERUM ALUMINIUM (µg/l)	BREAST MILK BEFORE FEED (µg/l)	ALUMINIUM AFTER FEED (µg/l)
1	2	163	287
2	17	50	13
3	3	1000	1000
4	10	20	15
5	2	–	463
6	5	147	47
7	2	11	17
8	2	9	14
9	6	79	44
10	2	378	161
11	2	8	32
12	2	19	66
13	2	4	31
14	2	139	217
15	–	41	–
16	9	19	14
17	–	207	81
18	9	34	10
19	–	11	8
20	–	274	–
21	14	–	213
22	15	–	725
23	–	57	153
24	–	152	153

5.2 Discussion.

The wide range of aluminium concentrations in breast milk
suggests that a number of samples had become contaminated
during the collection and storage procedure. It seems
unlikely that levels of aluminium in breast milk would vary
widely when maternal serum aluminium levels are within the
normal range. A number of the samples analysed contained low
levels of aluminium and this suggests that aluminium
concentrations are normally low in breast milk. It would
appear, therefore, that the determination of aluminium in
breast milk is hindered by sample contamination during the
collection procedure.

Prior to expressing breast milk the skin needs to be
thoroughly cleaned to remove possible aluminium contamination
resulting from the use of deodrants, talcum powder and other
skin medications. These sources of aluminium contamination
are probably the major cause of sample contamination.

All milk samples were sent to the laboratory in
aluminium-free blood tubes, samples having been expressed
into intermediary containers first. Some of the samples
received had been expressed by machine following the standard
procedure for collecting milk for the Milk Bank. Components
of the breast pump may contaminate the sample as collection
is taking place. While the pump may be hygenically clean, it
is difficult to obtain equipment free from aluminium
contamination by standard sterilisation procedures.

We conclude from our preliminary study of aluminium levels in breast milk that in order to obtain contamination free samples a clearly defined cleaning procedure has to be established. This cleaning program needs to include: a standard skin cleaning procedure; the removal of aluminium contamination of pump components; and the washing of sample containers to ensure the use of contamination-free vessels throughout the collection procedure.

We conclude from the present experiments that breast milk is sometimes, and possibly always, low in aluminium if serum aluminium levels are low. However, it appears that skin contamination is a major problem of collection, and it may equally be a source of infant exposure. Breast milk, as received by infants, particularly if collected by pump for the Milk Bank, is unlikely to be less contaminated than the samples sent to us for analysis.

6. TISSUE ALUMINIUM LOADING AFTER TOTAL PARENTERAL NUTRITION.

There have been a number of reports of increased tissue aluminium accumulation in infants exposed to high levels of aluminium (56, 59, 277, 278, 284). In two fatal cases (277) brain aluminium concentrations were high, 6.4 and 47.4 $\mu g/g$, (normal value 0.1 $\mu g/g$). These children had been orally fed

on milk formulae containing ca. 230 µg/l aluminium, for one
and three months, respectively. Bone aluminium levels were
normal with no histological changes or stainable aluminium on
bone biopsy. Koch et al (278) reported a fatal case of
aluminium toxicity caused by an aluminium-phosphate binder.
Excessive amounts of aluminium were found in bone at the
mineralisation fronts. Mineralisation had stopped resulting
in pathological fractures. These reports indicate that
aluminium accumulation does occur in premature and uraemic
infants, and that the brain and bone are major sites of
accumulation. The liver is also an important tissue in
aluminium storage (263).

A premature infant (Baby 1), born at 24 weeks gestation, was
given TPN following routine hospital practice. Initially the
child thrived and generally made good progress. After 13
weeks of intravenous feeding the child had a grand mal fit
and died. We examined post mortem tissue (at the request of
Dr. N. Bishope, S.C.B.U., Hope Hospital) to determine whether
aluminium could be implicated.

Additionally, a small control study was set up, to determine
tissue aluminium loading in 12 children under 1 year of age,
who had died from non-aluminium related diseases. Brain, bone
and liver samples were collected at post mortem and analysed
for aluminium. Analytical details are given in Chapter 2.

Recently, another preterm infant (Baby 2) died after 72 days
TPN feeding. Post mortem tissue was analysed for aluminium.

6.1 Results.

Levels of aluminium found in post mortem tissue are presented in Table 8. Baby 1 had received 13 weeks of TPN and the cause of death was thought to be aluminium related. Aluminium analysis showed elevated brain and liver concentrations, 40.1 and 4.5 µg(Al)/g (on a dry weight basis). Bone aluminium was ca. 11.3 µg/g. Baby 2 had high bone and liver concentrations, 46.9 and 8.1 µg(Al)/g respectively, brain aluminium was ca. 3.8 µg/g. Normal values, from the control group, were: brain, 2.4 ± 1.6 µg/g , liver, 1.1 ± 0.7 µg/g, and bone, 8.4 ± 4.5 µg/g (all dry weight). The normal brain aluminium levels are in good agreement with Alfrey (20) who reported 2.00 ± 0.63 µg/g in controls. Thus, we concluded that one of the suspected aluminium-loaded infants had elevated brain and liver concentrations, but normal bone aluminium, and the other baby had elevated liver and bone levels but normal brain aluminium levels. Given the medical circumstances, the deaths could have been aluminium-related.

Table 8. Aluminium (μg/g) in Post Mortem Tissue.

BABY	BRAIN (Alμg/g)	BONE (Alμg/g)	LIVER (Alμg/g)
1	40.1	11.3	4.5
2	3.8	46.9	8.1
3	-	15.0	-
4	4.2	11.4	1.2
5	1.2	6.9	3.1
6	4.0	5.0	1.0
7	4.7	13.4	1.0
8	-	2.6	1.7
9	1.5	5.3	0.7
10	-	15.0	1.2
11	-	10.6	-
12	2.0	6.9	0.9
13	1.4	3.5	0.5
14	N.D.	2.4	N.D.
Number	8	12	10
Mean	2.4	8.2	1.1
S.D.	1.6	4.5	0.7

N.D. = Not Detectable

7.   CONCLUSION.

In this study we found significant aluminium contamination of infant milk formulae, and particularly in the specialised formulae used for premature infants and those on special diets. Commercial paediatric solutions for TPN also contain high levels of aluminium. In the case of preterm infants, who have increased gut absorption and poor kidney excretion, the aluminium content of these materials may lead to the retension of aluminium at toxic levels. We think that this level of exposure presents a significant risk to children with impared or immature renal function, just as high levels of aluminium in dialysate have in the past presented a fatal risk to patients with chronic renal failure. This exposure of infants to aluminium is technically avoidable.

This preliminary study also high-lights the problems of obtaining aluminium contamination-free samples for aluminium analysis in the cases of breast milk collection. This observation indicates the need for very carefully detailed collection procedures which should be carried out each time a sample is collected in order to eliminated sample contamination.

Previous recommendations (53, 54) request that manufactures of milk feeds and products given intravenously should make strenuous efforts to reduce the aluminium content of their products. We re-iterate these recommendations, since this exposure presents an avoidable risk to infants with impaired

or immature renal function. Specific recommendations as to acceptable levels of contamination of this potentially toxic metal in pharmaceutical preparations are urgently needed.

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CHAPTER 8.

BINDING AND TRANSPORT OF ALUMINIUM BY SERUM PROTEINS.

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1. INTRODUCTION.

High body burdens of aluminium are frequently found in
patients with chronic renal failure (10, 20, 95), with the
association of syndromes such as dialysis dementia (10, 14,
20, 95), fracturing osteodystropy, (17, 33) and microcytic
hypochromic anaemia (39, 94).

During haemodialysis, aluminium transfer often occurs into
the patient against a concentration gradient (11, 25, 40,
51). The difficulty in removing aluminium from patients by
conventional haemodialysis is consistent with the metal being
bound by protein, which effectively lowers the concentration
of ultrafilterable aluminium species to below that of the
dialysis solution. Reports, based on dialysis/ultrafiltration
measurements, that 60-80% of aluminium in plasma is
associated to large molecules are consistant with this view
(271, 287).

Studies of specific protein binding of aluminium (71, 243,
271, 287-291) have presented conflicting findings. Recent
reports indicate that aluminium interacts with transferrin
(71, 289-291) but it has also been suggested (71, 288) that
there is substantial binding to albumin and also to a protein
of low molecular mass. These variable findings probably
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used, and in cases where serum proteins have been separated by conventional molecular exclusion chromatography (i.e. gel filtration), the poor resolution of protein peaks may lead to ambiguous assignment of protein and metal fractions.

In this Chapter, techniques are described for studing the protein binding of aluminium, iron, and copper in serum from normal subjects and from patients with renal failure. Trace metal determinations from gel filtration, and from ion exchange chromatography, of serum samples have been coupled with protein assays and radiochemical measurement of Fe-59 labelled transferrin to elucidate the nature of the metal carrier proteins. Serum proteins were separated by high performance liquid chromatogaphy, at moderate pressure using Pharmacia Fast Protein Liquid Chromatography (FPLC™) equipment.

2.   FAST PROTEIN LIQUID CHROMATOGRAPHY.

FPLC™ is a general purpose system for protein separation which allows rapid separation and reproducable profiling of proteins in biological samples often without loss of protein activity. The rapid separation of proteins, coupled with good peak resolution, is obtained by the use of high resolution gel media.

All the chromatography equipment used in this study was manufactured by Pharmacia Ltd., and is shown in Plate 1. The

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Figure 1. Fast Protein Liquid Chromatography System.
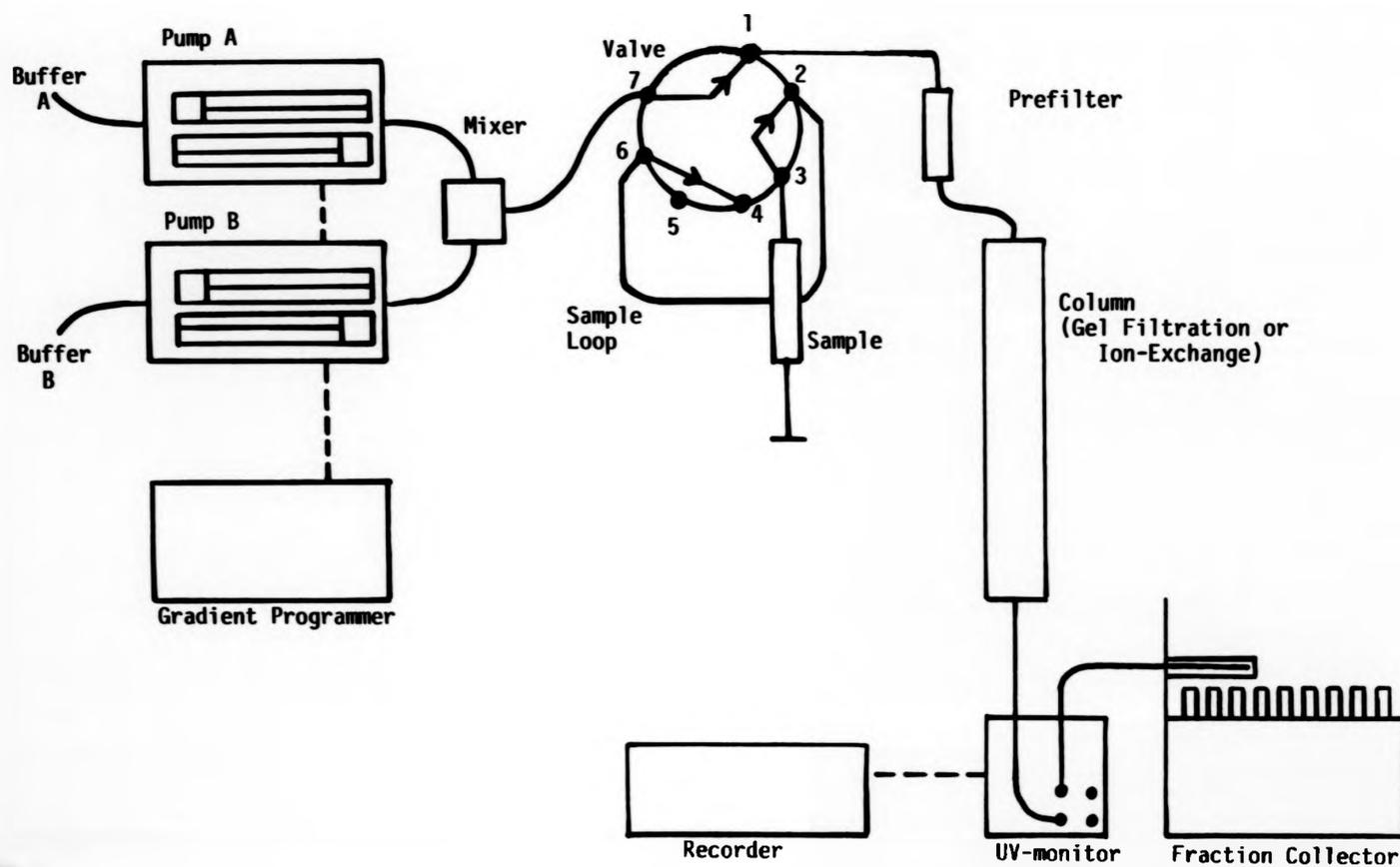
FPLC system (Figure 1) consists of a GP-250 gradient programmer containing two P-500 reciprocating pumps; each pump delivers one buffer (start buffer, A, or eluting buffer, B) into a mixing chamber. The mixed buffer then passes through a manually operated valve (V-7) for the introduction of a sample via a sample loop of specified volume, through a pre-filter and then onto the column. From the column, the eluate is monitored by ultra-violet absorbance at 280nm (at which all proteins absorb) in a 10mm path length high resolution flow cell against a reference cell containing start buffer. The output is presented on a chart recorder. Eluate fractions (100µl upwards) were collected with an automatic fraction collector (FRAC-100).

3. GEL FILTRATION.

Gel filtration separation is a simple technique which results in the separation of molecules according to their molecular size (292). The major advantages, for biological molecules, are the simplicity in interpretation, and the very gentle chemical environment of the separation.

As a sample is eluted onto a gel filtration bed, its progress down the column depends upon the bulk flow of the mobile phase, and upon the diffusion of the solute molecules both into and out of the stationary phase. Very large molecules, which are unable to enter the stationary phase, pass down the column rapidly. Smaller molecules, which can enter the gel

pores, move more slowly down the column, as they spend a portion of their time in the stationary phase. In effect, the smaller the molecule the larger the effective volume of the column, and the longer the time required to pass through the column. Therefore solute molecules are eluted in order of decreasing molecular size. This simple method of separation does not require any interaction between solute and solid phase; for optimal peak resolution it is important that minimal interaction occurs.

Any particular molecular species can be characterised by its "elution volume" (Figure 2). More specifically, the function K_d defined by the equation:

$$K_d = (V_e - V_o) / (V_t - V_o)$$

is a constant for a given protein (where V_o is the void volume of the column, V_t the total column volume and V_e the elution volume of the protein peak). Over the working range of the gel K_d is an approximately linear function of Log(molecular mass) (Figure 3).

3.1 Materials and Methods.

Gel filtration separations were carried out on a prepacked HR Superose-12™ 10/30 column, column volume 24ml. The Superose gel is an agrose based medium with cross-linkages, suitable for the effective separation in the molecular mass range 10^3-3 x 10^5 daltons.

Figure 2. Elution Characteristics for the Gel Filtration Column
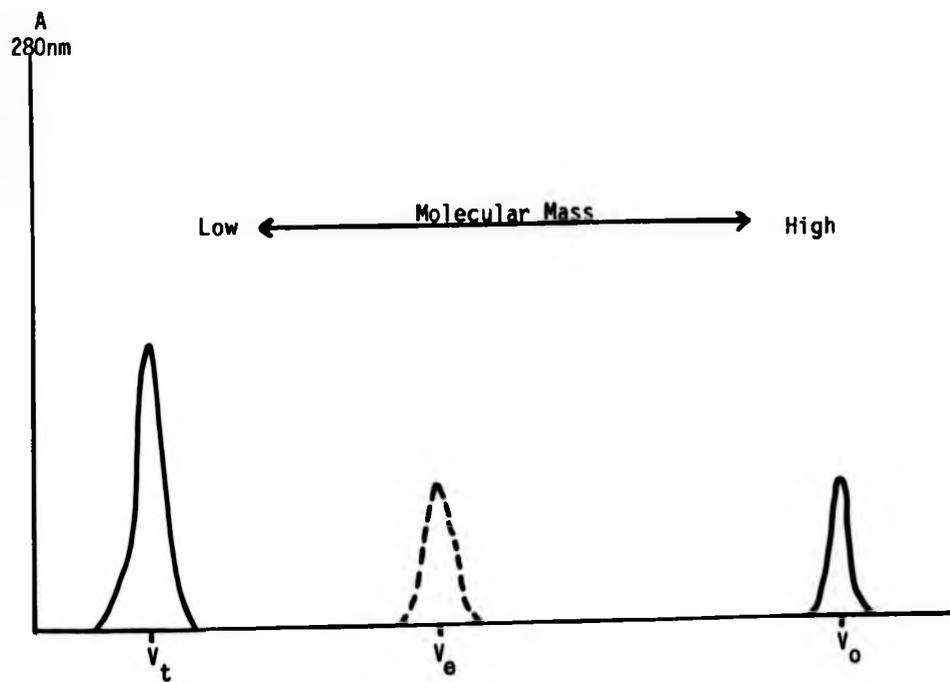
Figure 3.   Linear Calibration Graph for Superose 12 Column.
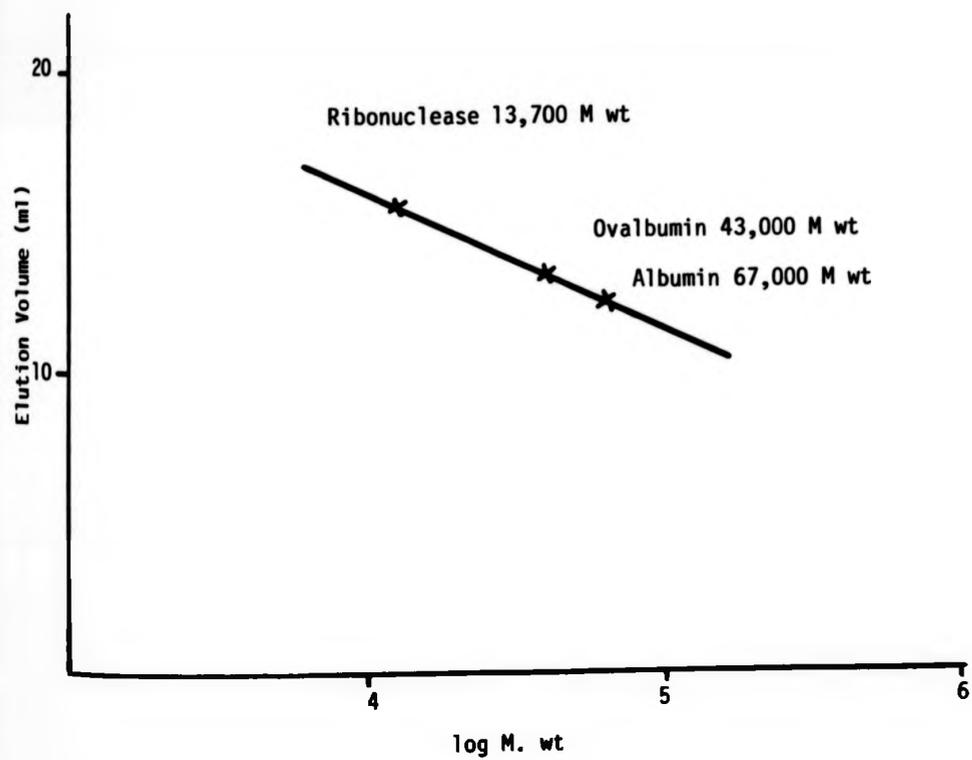
The eluting buffer used for each of the gel filtration separations was Tris(hydroxymethyl)methylamine (BDH Aristar grade), 20mM, in 100mM sodium chloride at pH 7.4. The buffer was degased under vacuum and filtered (0.22μm filter) before use.

The Superose 12 (Phamacia Ltd.) column was calibrated (Figure 3) using low molecular weight markers containing blue dextran, albumin, ovalbumin, chymotrypsinogen A and ribonuclease A, (molecular masses: 500,000; 67,000; 43,000; 25,000; 13,700 daltons respectively).

A series of experiments was carried out using gel filtration to identify whether specific serum proteins bind specific metals, e.g. aluminium, iron and copper. The first three experiments were carried out to determine the elution volumes of albumin, apo-transferrin and caeruloplasmin when eluted as single proteins solutions. The fourth experiment was designed to indicate whether or not albumin, transferrin, and caeruloplasmin interacted with each other when a combined sample of the three proteins was eluted on the column. Protein and serum samples were applied to the column after appropriate dilution with eluting buffer. The protein separations (observed by UV absorbance) were complemented by trace metal analysis of the collected fractions (0.5ml).

3.2 Experimental Details.

Experiment 1. - Albumin (200μl; 1 mg/ml) was eluted onto the

column in buffer solution. A UV-protein profile was obtained during the separation to determine the elution volume of albumin under the standard elution conditions. Fractions were not collected during this calibration run.

Experiment 2. - Apo-transferrin (200µl; 1 mg/ml) was loaded onto the column and eluted. The elution volume of this standard protein was determined and a UV-eluate profile was obtained during the experiment.

Experiment 3. - This separation was carried out to determine the elution volume of caeruloplasmin. A standard solution of caeruloplasmin (200µl; 2.5 mg/ml) was eluted onto the column and a UV-protein profile was obtained during the separation. Again, fractions were not collected during this separation.

From the UV-profile the sample loading (0.5mg caeruloplasmin) appeared to be a little high; this experiment was repeated using a 100µl sample volume and better peak resolution was attained.

Experiment 4. - A 200µl sample of caeruloplasmin (0.4 mg/ml), transferrin (0.4 mg/ml) and albumin (0.4 mg/ml) was eluted onto the column in buffer. Fractions were not collected during this separation.

Experiment 5. - Serum from a normal subject was loaded onto the column and eluted. This separation was performed so that we could obtain a UV-profile from a normal serum sample.

Normal serum diluted x4 with buffer was eluted onto the column (100µl volume). Fractions (0.5ml) were collected throughout the separation and analysed for copper and iron by GFAAS.

Experiment 6. - Serum from an aluminium over-loaded patient, serum aluminium level ca. 200 µg/l, was diluted (x4) and loaded onto the column (200µl sample). Fractions (0.5ml) were collected during the separation and subsequently analysed for aluminium, copper and iron.

This separation was repeated with serum from a child with chronic renal failure, serum aluminium level ca. 80 µg/l. Metal analyses were carried out on each of the 0.5ml fractions collected during the separation.

3.3 Results.

Experiments 1-3. - Experiments 1-3 were carried out to determine the elution volumes of albumin, caeruloplasmin and transferrin when single protein samples were eluted under standard procedures. The standard proteins were eluted from the column as single peaks (Figure 4). Caeruloplasmin (135,000 daltons) was eluted at 11.5ml, transferrin (80,000 daltons) at 12.1ml and albumin (67,000 daltons) at 12.2ml.

Experiment 4. - This experiment was carried out to determine whether the three specified proteins can be resolved

satisfactorally by gel filtration in a combined sample.
The UV-absorbance profile obtained from the separation of the
combined caeruloplasmin,transferrin and albumin solution is
shown in Figure 5. In this experiment caeruloplasmin was
eluted at 10.7ml, transferrin at 11.4ml and albumin at
12.5ml. The three protein peaks were poorly resolved
indicating that the proteins are not completly separated from
each other during the separation procedure.

Experiment 5. - The UV-absorbance profile of separated serum
proteins from a "normal" subject is given in Figure 6a.
Caeruloplasmin, transferrin and albumin proteins were eluted
at 10.4, 11.6 and 12.4ml, respectively.

Copper and iron determinations by GFAAS are given in Figure
6b and 6c. Three copper peaks are detected at elution volumes
of ca. 12.0, 15.0 and 24.0 ml. These peaks probably result
from copper bound to caeruloplasmin (and albumin), a copper
binding protein of ca. 20,000 daltons, and copper bound to
amino acids and other low molecular mass speceis. Two iron
peaks were detected at 12.0 and 24.0ml; these probably result
from iron bound to transferrin and low molecular mass species
respectively.

Experiment 6. - Figure 7a shows the UV-absorbance profile of
serum proteins from an aluminium intoxicated patient. The
elution volume for caeruloplasmin, transferrin and albumin
are 10.6, 11.5 and 12.2ml respectively.

The results of copper, iron and aluminium analyses are given in Figure 7b-d. The major copper peak corresponds to the elution volumes of caeruloplasmin and albumin, and a small copper peak is observed which suggests binding to low molecular mass molecules. Iron peaks are observed at elution volumes of 12.0 and 16.0ml; these correspond to carrier proteins of ca. 80,000 daltons (transferrin) and ca. 18,000 daltons. Aluminium analysis of each of the fractions did not indicate aluminium binding to any serum protein.

Similar separation and metal analyses results are shown for the child patient with chronic renal failure (Figure 8a-d).

All these results are summarised in Table 1.

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Figure 4. Elution Profiles for Standard Protein Solutions
(Experiments 1-3)

(a) Albumin

Elution volume (ml)

(b) Apo-transferrin

Elution Volume (ml)

(c) Caeruloplasmin

Elution volume (ml)
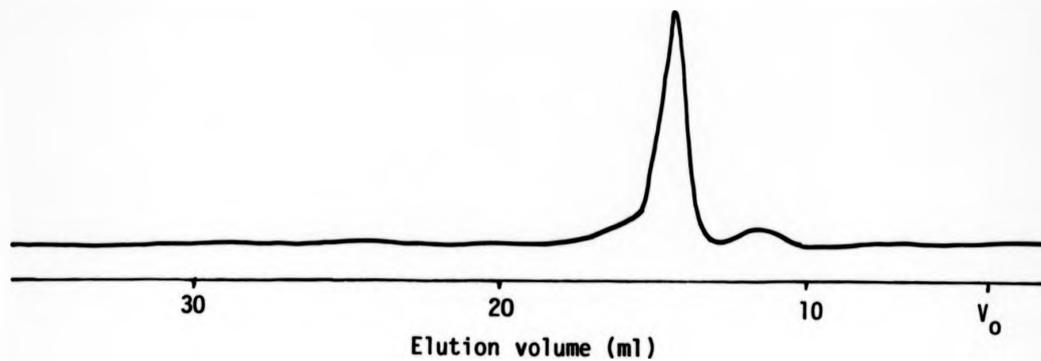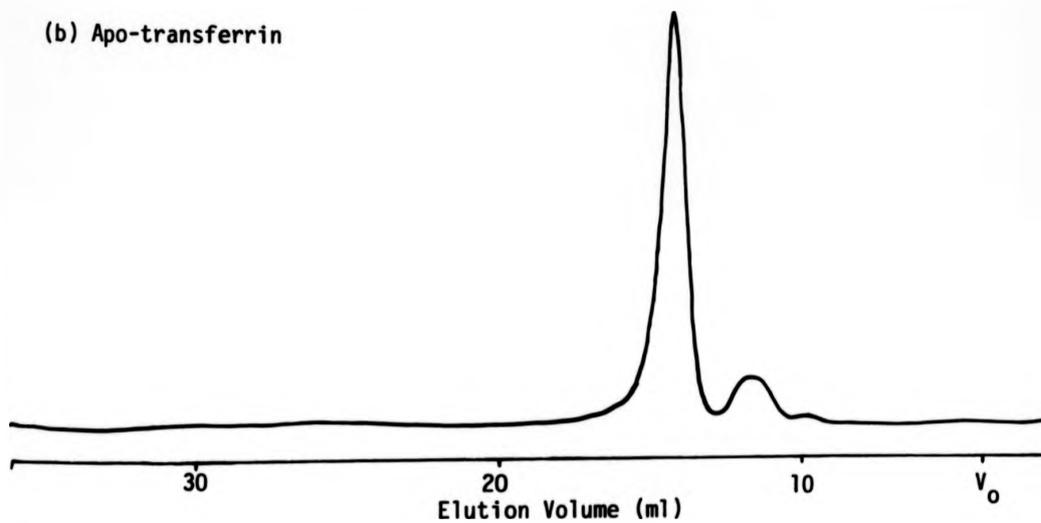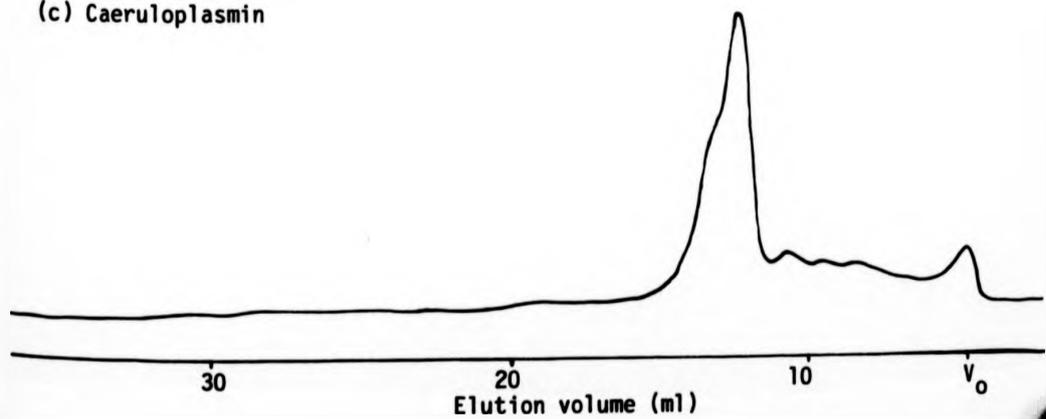
Figure 5. Transferrin and Caeruloplasmin Separation.
(Experiment 4)

UV-profile
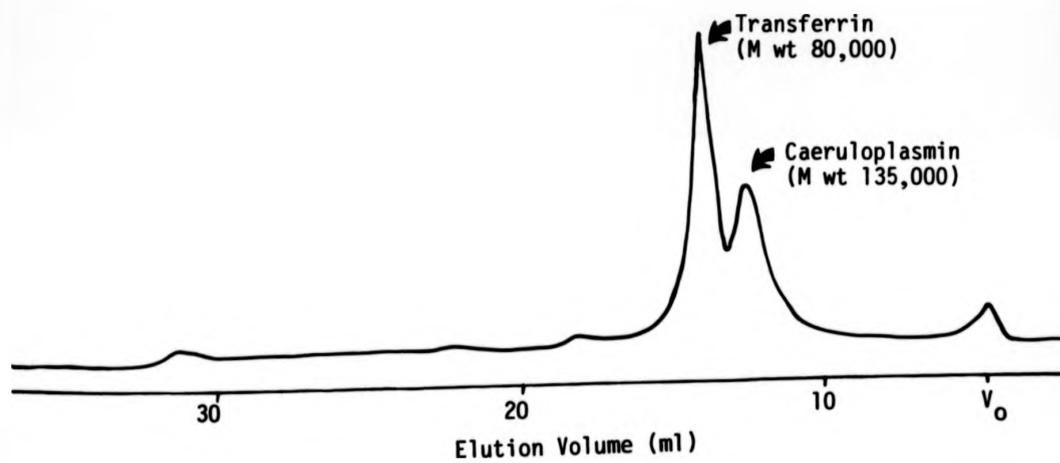
Figure 6. Separation of Normal Serum with Iron and Copper Analysis.
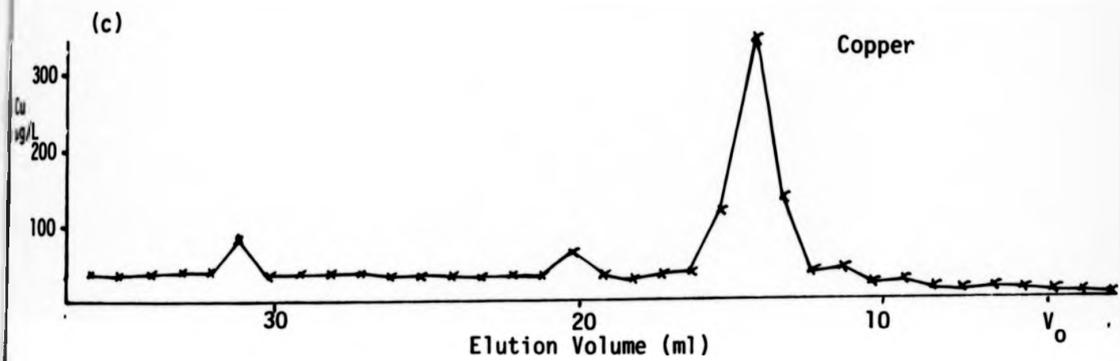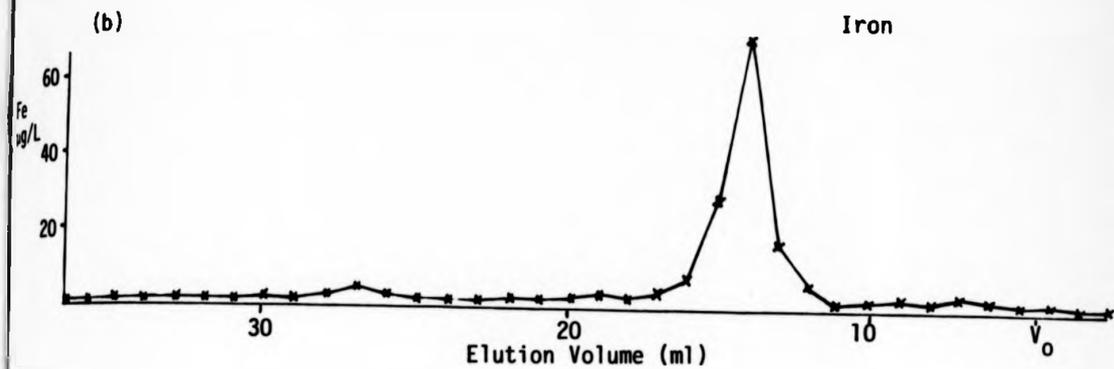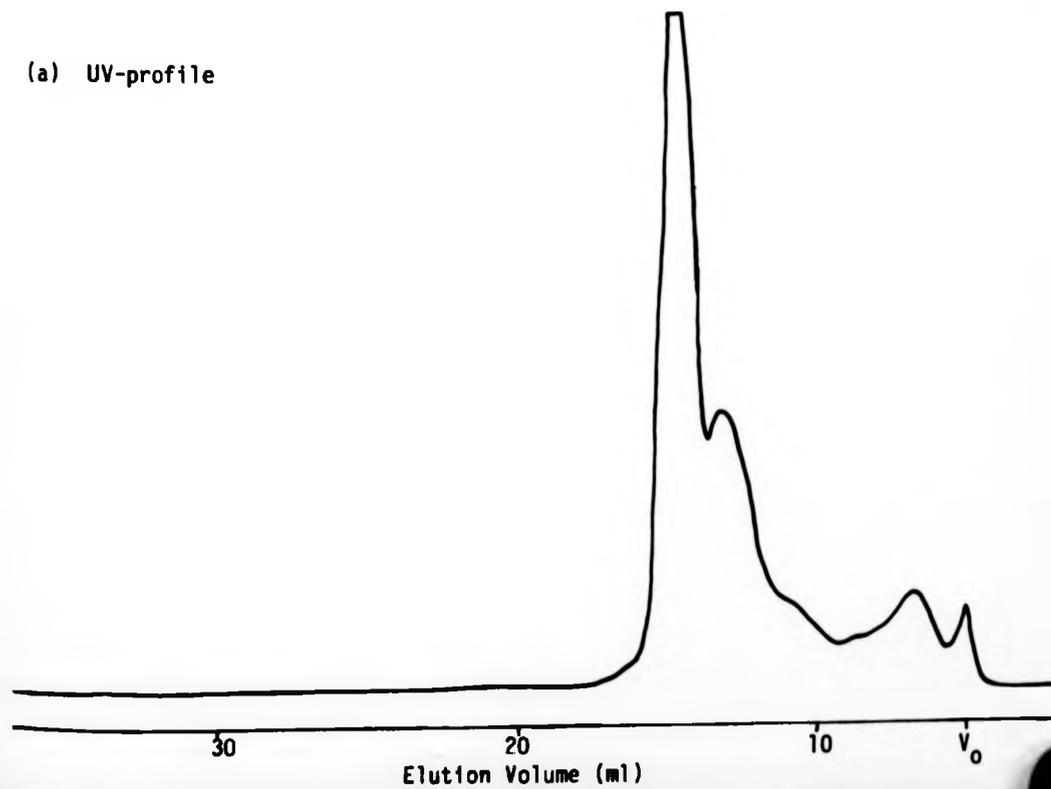
Figure 7.   Serum Separation from CRF Patient (Experiment 6)
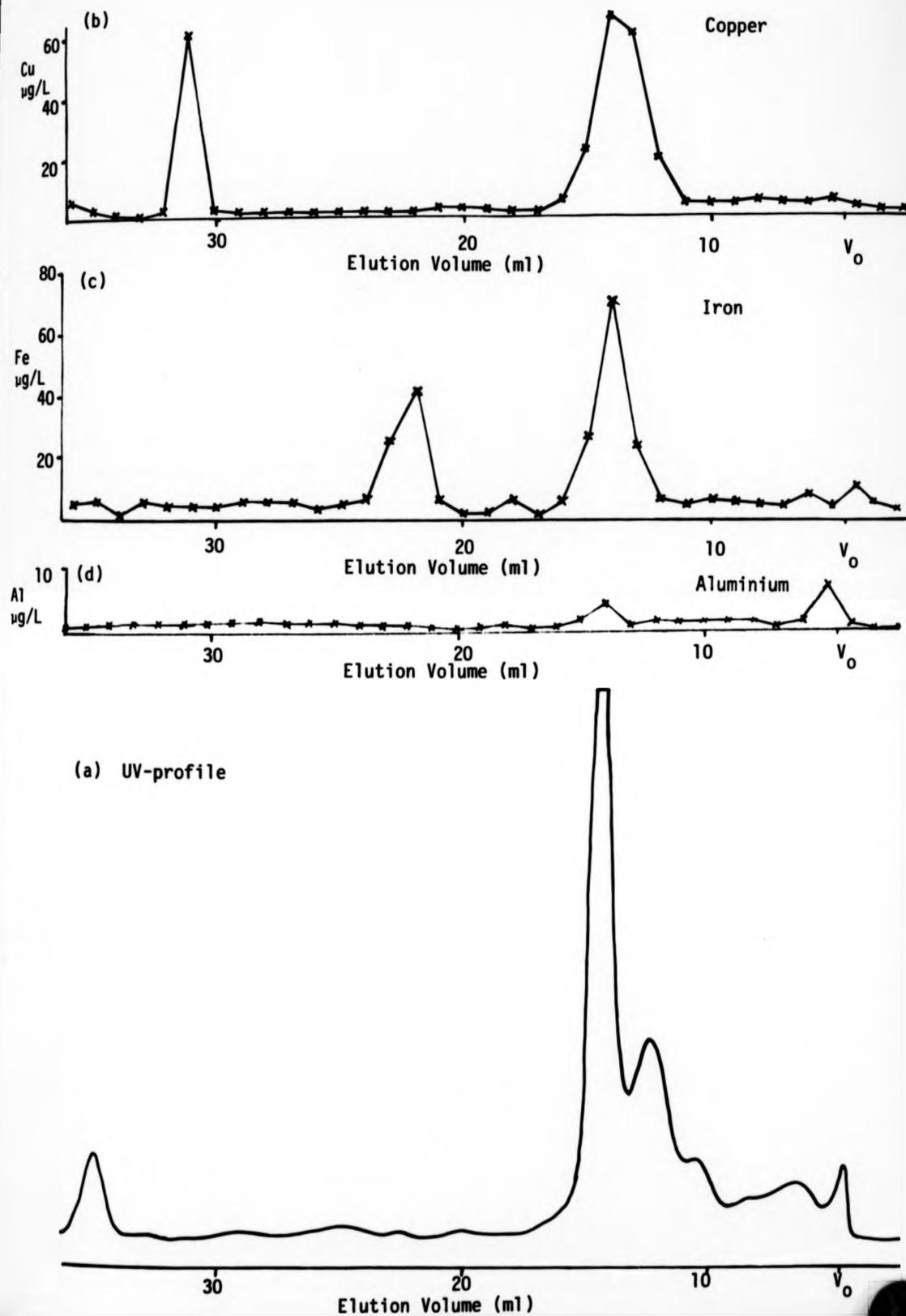
Figure 8.   Serum Separation form a CRF Child Patient (Experiment 7)
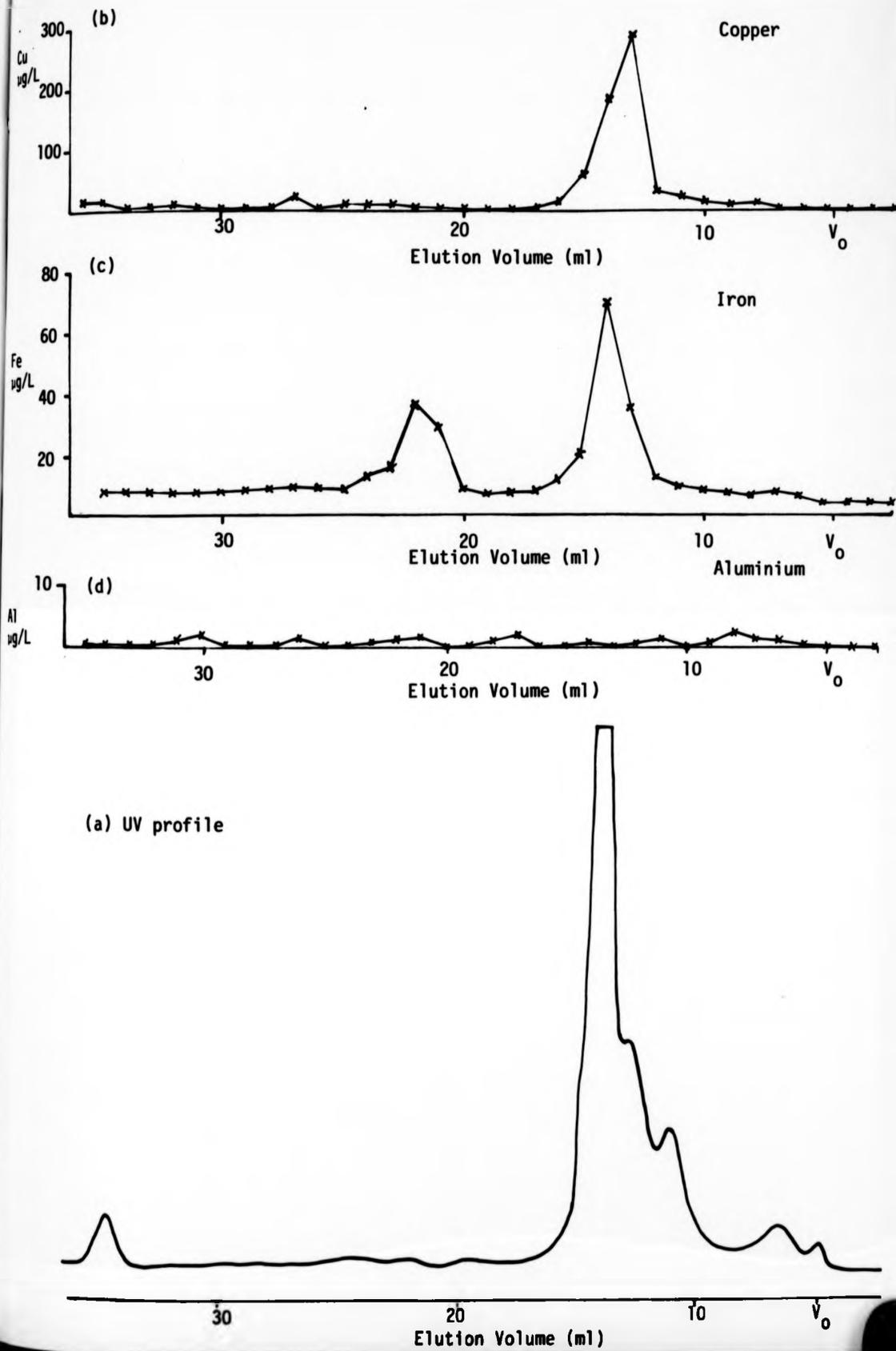
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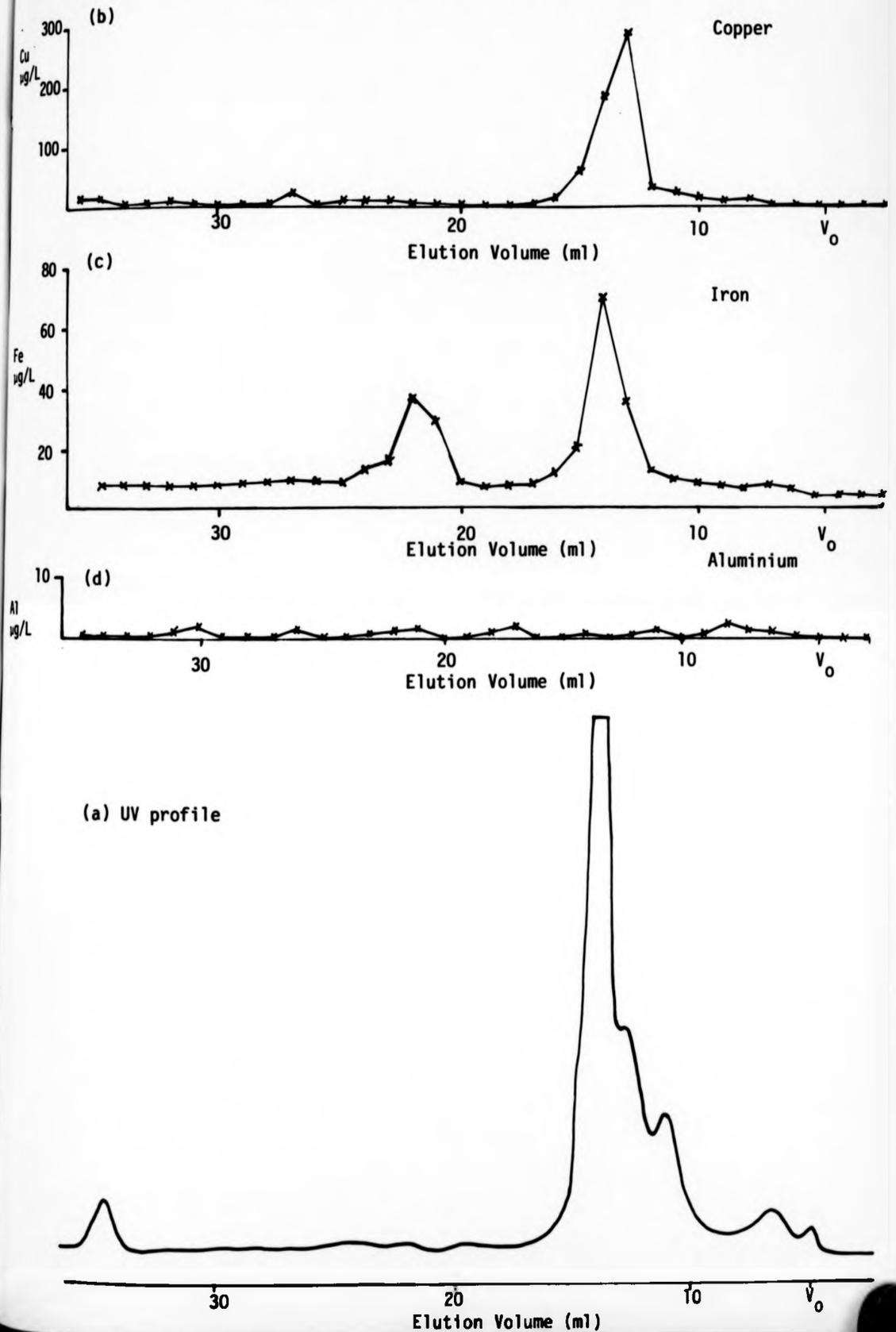
Table 1. Results of Gel Filtration Experiments.

PROTEIN (Metal)	M. MASS 10^{3}daltons	EXPT 1-3	EXPT 4	EXPT 5	EXPT 6	EXPT 7
Caeruloplasmin	135	11.5	10.7	10.4	10.6	10.3
Transferrin	80	12.1	11.4	11.6	11.5	11.3
Albumin	67	12.2	12.5	12.4	12.2	12.2
Copper	-	-	-	12.0	10.5	10.7
	-	-	-	15.0	12.2	11.7
	-	-	-	24.0	18.5	20.5
Iron	-	-	-	12.0	12.0	12.0
	-	-	-	24.0	16.0	16.0
Aluminium	-	-	-	-	N.D.	N.D.

3.4 Discussion.

This method of protein separation gave poor resolution of the three proteins of particular interest, namely caeruloplasmin, transferrin and albumin. The poor resultion could be ascribed to the large amount of albumin present in serum, which over-shadowed both the caeruloplasmin and transferrin peaks. In order to determine whether transferrin and/or albumin binds and transports aluminium in serum it is necessary to obtain good separation of these two proteins, and hence prevent the ambiguous assignment of protein and metal fractions.

Aluminium determination of protein fractions failed to detect raised concentrations of the metal in any particular fraction, despite a serum-aluminium level of 200 µg/l. It is possible that the affinity of the agarose medium is great enough to detach aluminium from the protein binding sites. However, the detection of aluminium in the fractions from gel filtration is extremely difficult because: (i) the elution procedure dilutes the sample material by a factor of ca. 100 (from 200µl to over 20ml); and (ii) the eluting buffer contains high Cl^- concentrations, which depresses the atomic absorption response of aluminium.

4. ION EXCHANGE CHROMATOGRAPHY.

Ion exchange chromatography allows the separation of charged or ionisable biological molecules from complex sample

mixtures. Proteins of similar molecular size which are not
resolved on a gel filtration column may sometimes be
separated by ion exchange chromatography (293), making use of
the zwitterionic properties.

The principle of ion exchange exploits the characteristic
charges held by the solute molecules to achieve separation.
This method of separation is capable of resolving molecules
differing by very small amounts in charge; in the case of
proteins, by differences of a single amino-acid residue.

A ion exchange medium consists of an insoluble matrix to
which charged groups have been covalently bonded. The charged
groups are associated with mobile counter-ions. These
counter-ions can be reversibly exchanged with other ions of
the same charge without altering the matrix. The separation
in ion exchange chromatography is obtained by reversible
adsorption of the sample molecules onto the exchanger in
exchange for the counter-ions. Ion exchange separations are
usually carried out in two steps (Figure 9). The first step
is sample application and adsorption in the presence of
starting buffer. Unbound substances are washed from the
exchanger bed using the equivalent of one column volume of
starting buffer. In the second stage, substances are eluted
sequentially from the column with an eluting buffer.
Separation is obtained because different molecular species
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differences in charge. The affinity of a molecule for the
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The principle of ion exchange exploits the characteristic charges held by the solute molecules to achieve separation. This method of separation is capable of resolving molecules differing by very small amounts in charge; in the case of proteins, by differences of a single amino-acid residue.

A ion exchange medium consists of an insoluble matrix to which charged groups have been covalently bonded. The charged groups are associated with mobile counter-ions. These counter-ions can be reversibly exchanged with other ions of the same charge without altering the matrix. The separation in ion exchange chromatography is obtained by reversible adsorption of the sample molecules onto the exchanger in exchange for the counter-ions. Ion exchange separations are usually carried out in two steps (Figure 9). The first step is sample application and adsorption in the presence of starting buffer. Unbound substances are washed from the exchanger bed using the equivalent of one column volume of starting buffer. In the second stage, substances are eluted sequentially from the column with an eluting buffer. Separation is obtained because different molecular species have different affinities for the exchanger because of their differences in charge. The affinity of a molecule for the exchanger can be controlled by varying the chemical

Figure 9.   Principle of Ion-Exchange Chromatography

1.	2.	3.	4.	5.
Starting Conditions	Adsorption of Sample	Start of Desorbtion	End of Desorbtion	Regeneration

O Starting Buffer Counter Ions

□

▲ Substance to be Separated

⊘

⊘ Gradient Ions

●
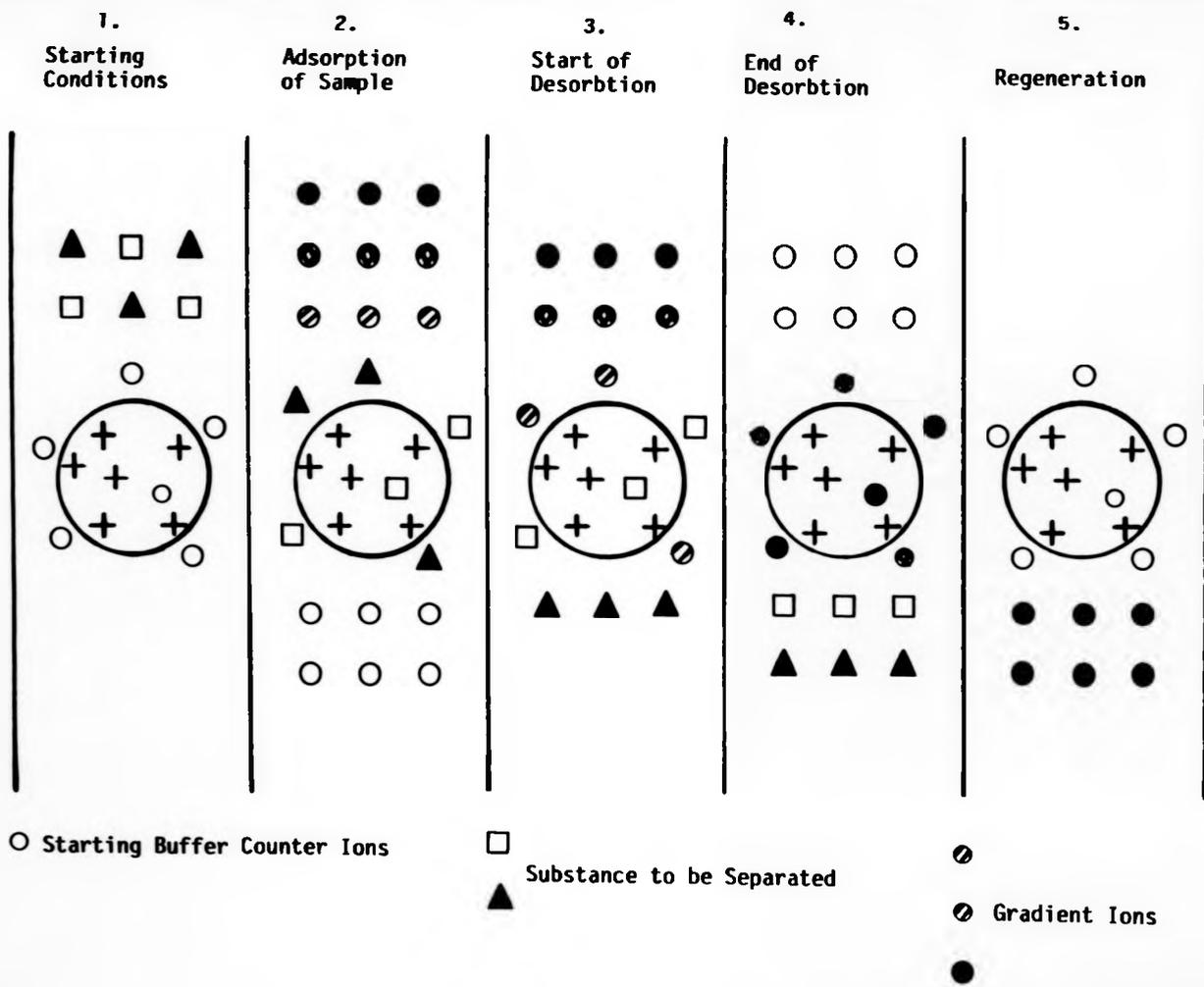
conditions e.g. pH or ionic concentration. Thus, by the use of a chemical gradient, a substance is eluted from the column when it no longer has an affinity for the column i.e when the substance is no longer charged. In the case of proteins in a pH gradient, this will occur at the isoelectric point (pI), which is also a function of the ionic strenth. Alternatively, an ionic concentration gradient may be used at a fixed pH. In general, charged protein residues at a given pH become less charged as the ionic strength is increased.

4.1 Materials and Methods.

Ion-exchange chromatography was performed on a strong anion-exchange medium, prepacked Mono-Q™ 5/5, column volume 1ml. The separation medium (Monobeads™) is comprised of uniform monodisperse spheres (diameter ca. 10μm), with good separation and flow characteristics at only moderately increased pressure above that of conventional liquid chromatography (e.g. at a flow rate of 1 ml/min, a back pressure of ca. 2 mPa is produced). The charged centres on the column are quaternary ammonium groups, stable in the pH range 2-12.

All buffers were de-gased and filtered through 0.22μm filters before use. The start buffer (A) was Tris(hydroxymethyl)methylamine (BDH Aristar grade), 20mM at pH 7.4. The eluting buffer (B) was buffer A plus 0.7M sodium acetate at pH 7.4. A linear elution gradient was produced, from 100% buffer A to 100% buffer B, over a 20ml volume. The

combined flow rate of the buffers was 1.0 ml/min. in all separations. Buffer B was passed through a chelating resin (No. Chelex 100, C-7901; Sigma Chemical Company) to remove contaminating trace metals prior to elution down the anion-exchange column.

A series of separations were carried out using the Mono Q column. The first four experiments were designed to calibrate the column, i.e. determine the elution characteristics of specific proteins, transferrin, caeruloplasmin and albumin using the specified ionic gradient. Each of the six experiments was coupled with trace metal analysis, protein assay, and liquid scintillation counting for Fe-59 where appropriate.

Samples were applied to the anion-exchange column after appropriate dilution with the start buffer. Serum samples were applied directly to the column after dilution, without futher pretreatment.

4.2 Fe-59 Transferrin.

Fe-59 (100 µCi in 1ml, as FeCl₃ in 0.1M HCl) was supplied by Amersham International plc., and iron-free bovine transferrin (No. T-5761) by Sigma Chemical Company.

Iron-free transferrin was labled with Fe-59 by a similar method to that used by Cook et al (294) to lable defibrinated serum. The labelled iron was diluted to 25ml with start

buffer plus 400nmol Na-nitrilotriacetic acid (NTA), solution
A. Solution B comprised 3 μmol/ml Fe^{3+}, 6 umol/ml NTA made up
to 25ml with start buffer, (1000 μg/l Fe^{3+} (4.25ml), 0.01M
NTA (15ml)).

Transferrin (1ml; 20 mg/ml in start buffer) was added to
solution A (0.25ml). The transferrin and Fe-59 solution (1
μCi; 37KBq) were allowed to equilibrate at 25°C for 1 hour.
Sufficient Fe^{3+} was added to saturate the iron-binding
capacity of the transferrin (0.25ml solution B). The solution
was allowed to equilibrate for a further hour to ensure that
the transferrin was fully loaded.

The labelled-transferrin (1.5ml) was eluted with start buffer
through a PD-10 de-salting column (Sephadex G-25, Pharmacia
Ltd.), to remove any unbound iron. The high molecular weight
fraction (ca. 2.1ml) containing the labelled-transferrin was
collected and stored at 4°C.

4.3   Liquid Scintillation Counting.

Labelled transferrin was measured by liquid scintillation
counting (295) using a Searle Mark III Liquid Scintillation
Counter (Model 6880). This counter uses two photomultiplier
tubes for counting, recording a count only when a
scintillation is detected in both tubes within a specified
time interval. This technique is called coincidence counting
and discriminates against random noise pulses from the two
photomultiplier tubes. Background beta-radiation measured ca.

25 d.p.m. (0.38Bq), giving a minimum countable activity of ca. 40Bq (to an accuracy of $\pm$ 1%).

Standard preparation. - Solution A; containing 1 μCi/ml Fe-59 (37 KBq/ml) was diluted (x10) with solution B (Section 3.2) to give a solution containing 3700 Bq/ml. Aliquots of the diluted solution were placed into five polypropylene vials; 0, 10, 50, 200 and 500μl (volumes were made up to 500μl with solution B), and the scintillation solution,"Lumagel" (5.0ml) was added to each tube. These standards were equivalent to 0, 6.7, 33.6, 134.6 and 336.4Bq respectively.

Sample preparation. - The aim of the preparation was to obtain a good mix between the aqueous phase (sample) and the organic phase (scintillation solution). Aqueous samples (100μl) obtained by FPLC separation were added to "Lumagel" (5.0ml) in a polypropylene vial and the mixture shaken to produce a homogenous solution. Each sample was counted for 10 minutes and calibrated against the set of Fe-59 standards.

4.4 Experimental.

Experiment 1. - Albumin is one of the two major serum proteins, and in cases where this protein is of no interest it is advantageous to remove the albumin in a preliminary step. It has been suggested that albumin may interact with aluminium (71, 288); thus the protein is of specific interest to us and serum samples were separated with their associated albumin. This experiment was set up to determine the point at

which albumin is eluted from the column.

Albumin (200µl; 1 mg/ml) was injected onto the column and eluted. In this experiment no fractions were collected, but a UV-protein profile was obtained.

Experiment 2. - This experiment was performed using Fe-59 labelled transferrin. A sample of transferrin was injected onto the column to determine at what acetate concentration the protein was eluted from the column. Previously labelled Fe-59 transferrin (200µl; ca. 10 mg/ml) was loaded onto the column and eluted; 0.5ml fractions were collected into polypropylene vials (6ml). Fraction numbers 10-30 were counted by liquid scintillation.

Experiment 3. - This experiment was performed on a standard caeruloplasmin solution (No C-4770, Sigma Chemical Company). Caeruloplasmin (100µl; 5 mg/ml) was loaded onto the column and eluted to determine at what acetate concentration the protein was eluted. A UV-eluate profile was obtained for this separation.

Experiment 4. - Experiments 1-3 indicated at which point each of the specified proteins were eluted on the ionic gradient. Each of the first three experiments had been carried out on standard protein solutions containing single proteins. This separation was carried out to determine whether in a combined sample of transferrin and caeruloplasmin each of the protein peaks was resolved and hence whether these two proteins can

be separated. Iron and copper determinations were carried out on each of the 0.5ml fractions collected throughout the separation. A caeruloplasmin assay was performed on 100µl aliquots from fraction numbers 40-47, and liquid scintillation counting was carried out on 100µl aliquots of fraction numbers 18-27.

Experiment 5. - Serum from a normal subject, diluted x4 with start buffer was loaded onto the column using first 100µl and then 200µl sample volumes.

Experiment 6. - Serum from a renal patient known to have a very high serum aluminium level (280 µg/l) was loaded onto the column. Diluted serum (x4; 200µl sample) was eluted and 0.5ml fractions were collected during the separation. Each fraction was analysed for aluminium, iron and copper. Aliquots (200µl) of fraction numbers 37-44 were assayed for caeruloplasmin. This separation was performed three times.

Experiment 7. - Experiment 6 was repeated with serum from another CRF patient with high serum aluminium levels. Fractions were collected and analysed for aluminium, iron and copper.

4.5 Results.

Experiments 1-3. - Each of the standard proteins was eluted as single peaks from the column. Transferrin had an elution volume of 9.5ml, at 0.19M sodium acetate; Albumin eluted at

0.36M sodium acetate, 16.0ml; and Caeruloplasmin (major peak)
at an elution volume of 19.0ml, 0.55M sodium acetate (Figure
10a-c).

Liquid scintillation counting on fraction numbers 10-30
(Experiment 2) gave a peak maximum (c.p.m.) for fraction 21,
elution volume 9.5ml (Figure 10d). This result suggests that
labelled Fe-59 remains bound to the transferrin protein
during the ion-exchange separation.

Experiment 4. - The UV-absorbance profile obtained for this
separation is shown in Figure 11a. Large absorbance peaks are
observed at ca. 9.5ml and 19.5ml (at 0.19 and 0.54M sodium
acetate); these peaks correspond to transferrin and
caeruloplasmin respectively.

The results of iron and copper determinations by GFAAS are
shown in Figure 11b-c. The double iron peak corresponds to
the transferrin protein (at 0.18 and 0.19M sodium acetate).
Two copper peaks are detected by GFAAS, one peak corresponds
to caeruloplasmin (0.54M sodium acetate), the other copper
peak probably results from the presence of albumin.

The results of the caeruloplasmin assay are given in Figure
11d. Maximum oxidase activity is seen in fraction 41, at
0.54M sodium acetate.

Liquid scintillation counting shows maximum activity in
fractions 20 and 22. This activity corresponds to the two

transferrin peaks observed at 0.18 and 0.19M sodium acetate (Figure 11e).

Experiment 5. - The UV-absorbance profile obtained from the separation of "normal" serum is shown in Figure 12. Transferrin and albumin peaks are clearly shown at ca. 0.18 (and 0.19) and 0.37M sodium acetate respectively. A small peak is seen at 0.54M sodium acetate corresponding to caeruloplasmin.

Experiment 6. - Figure 13a shows the UV-absorbance resulting from the separation of serum from an iron and aluminium overloaded patient.

The results of copper, iron and aluminium determination by GFASS are shown in Figure 13b-d.

Copper peaks are seen at ca. 0.17, 0.37 and 0.54M sodium acetate. This suggests that copper is associated with three serum proteins, caeruloplasmin, albumin and transferrin (Figure 13b).

A double iron peak is detected at elution volumes 9.0 and 9.5ml; this corresponds to the transferrin protein at 0.17 and 0.18M sodium acetate (Figure 13c).

Aluminium analysis shows a single aluminium peak at ca. 0.17M sodium acetate. This suggests that aluminium is associated with only one protein in serum, transferrin (Figure 13d).

The caeruloplasmin assay indicates maximium oxidase activity at 0.54M sodium acetate, corresponding to the largest copper peak and the expected ionic concentration for the elution of the caeruloplasmin protein (Figure 13e).

Experiment 7. - The UV-absorbance profile from this separation is shown in Figure 14a. Copper, iron and aluminium determinations by GFAAS are also shown in Figure 14b-d.

The copper peaks corresponded with albumin and caeruloplasmin, and a very large iron peak was detected which corresponds to transferrin.

Aluminium analysis indicated a single peak associated with transferrin.

All the results from the anion-separations are summarised in Table 2.

Figure 10. UV-Profiles for Standard Proteins (a) Albumin, (b) Transferrin, (c) Caeruloplasmin.

Start Buffer : 20 mM TRIS/HCl, pH 7.4

Eluting Buffer : 20 mM TRIS/HCl, 0.7M NaOAc pH 7.4
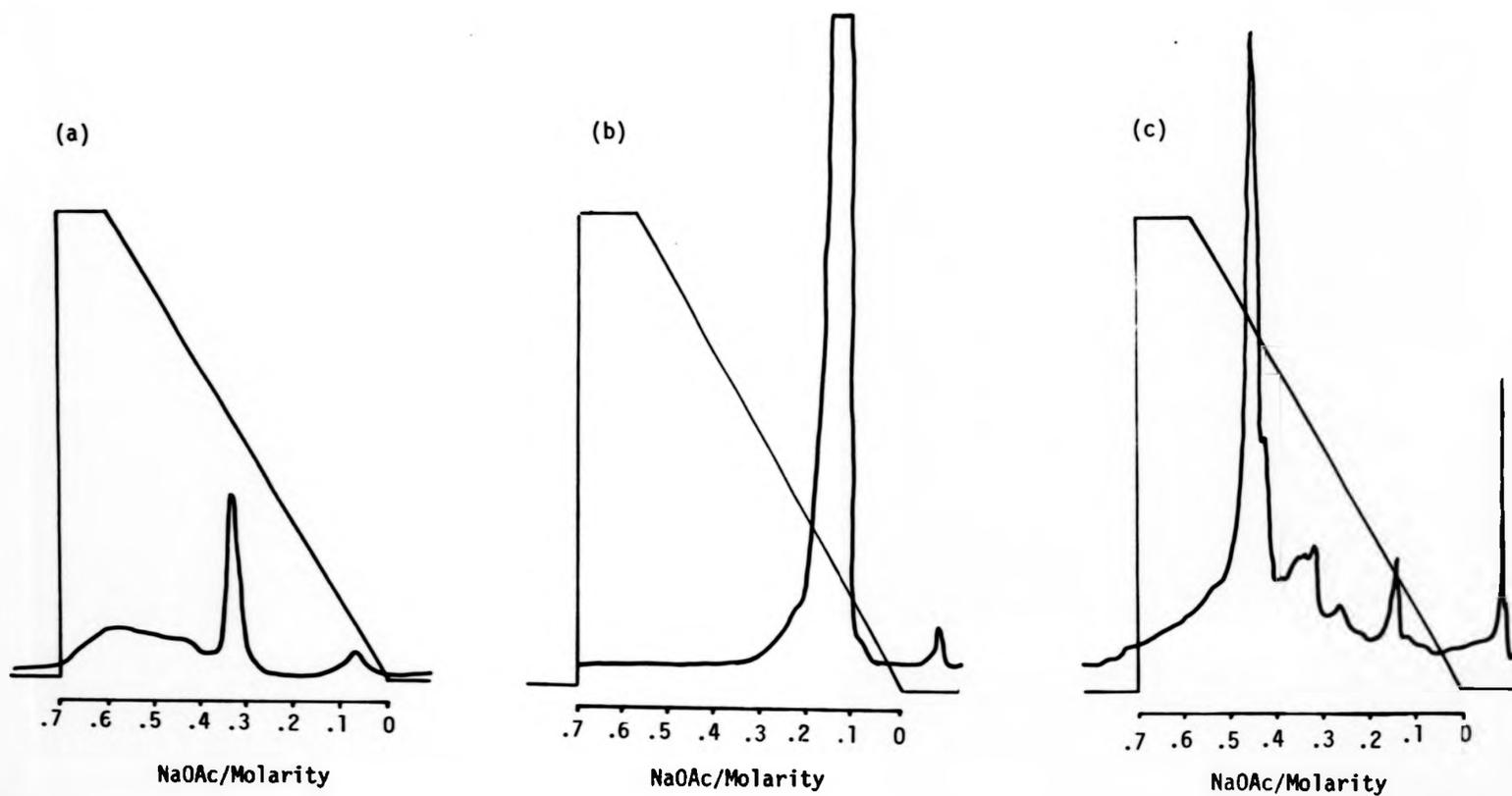
Figure 10(d)   Liquid Scintillation Counting on Fe-59 Labelled Transferrin (Experiment 2).
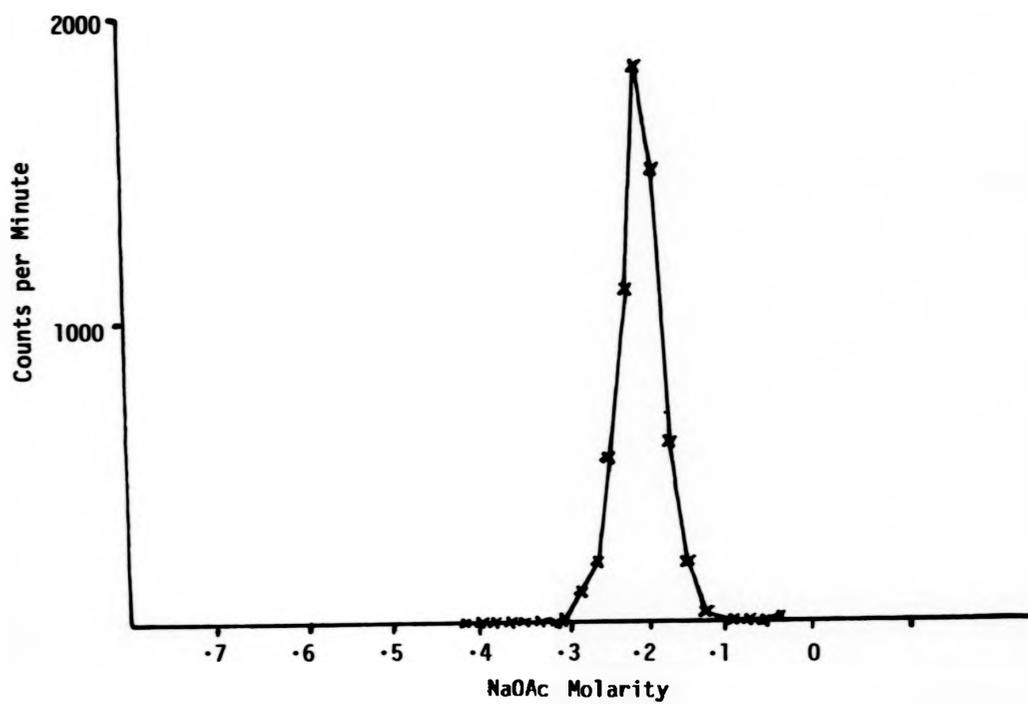
Figure 11. Separation of Transferrin and Caeruloplasmin Proteins (Experiment 4)
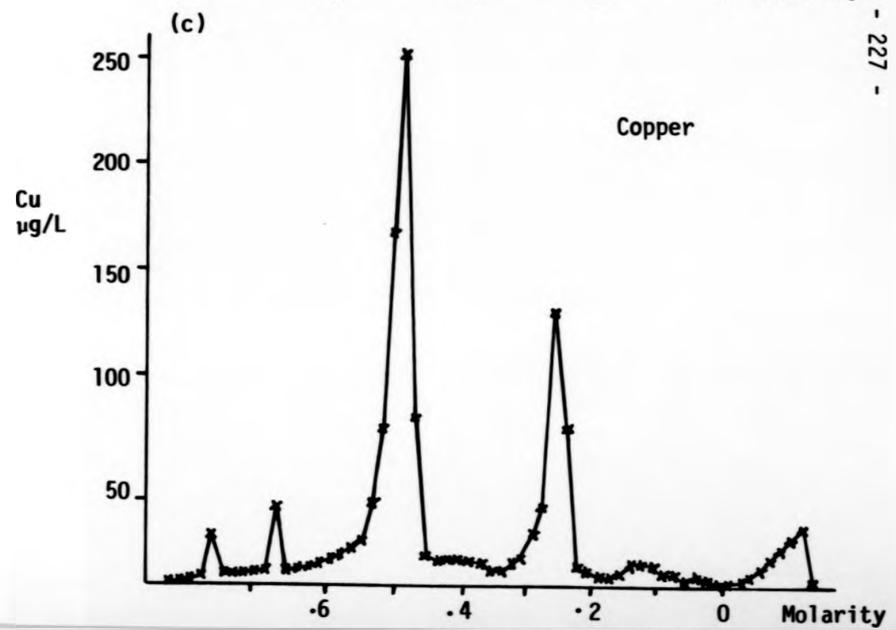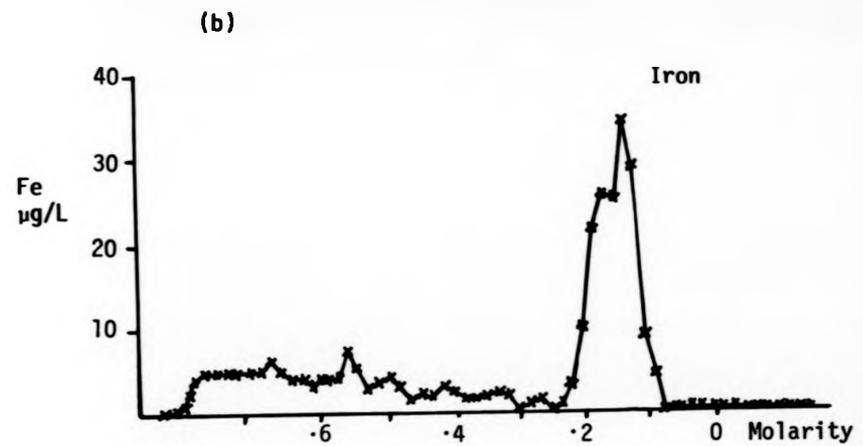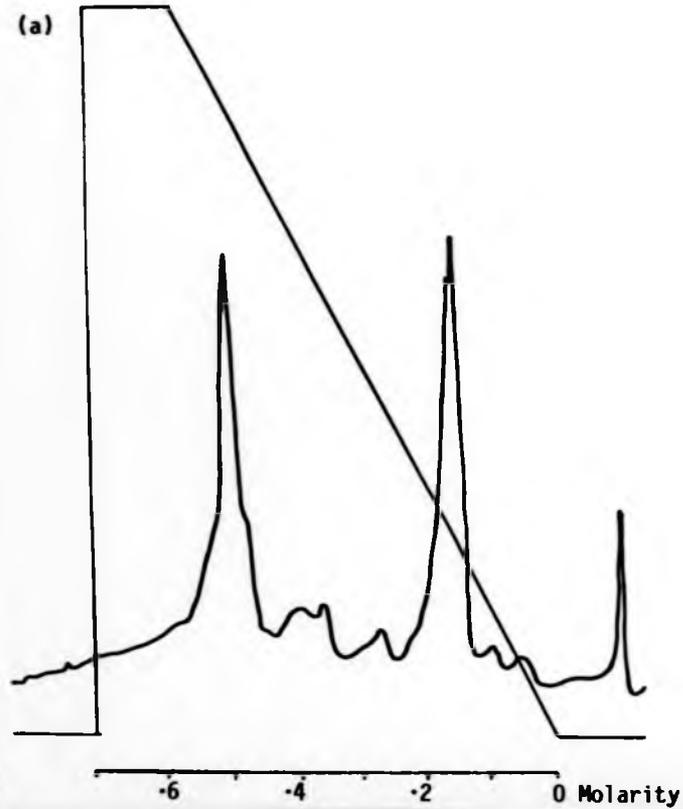
Figure 12.   Ion-Exchange Separation of Human Serum (Experiment 5)

NaOAc Molarity
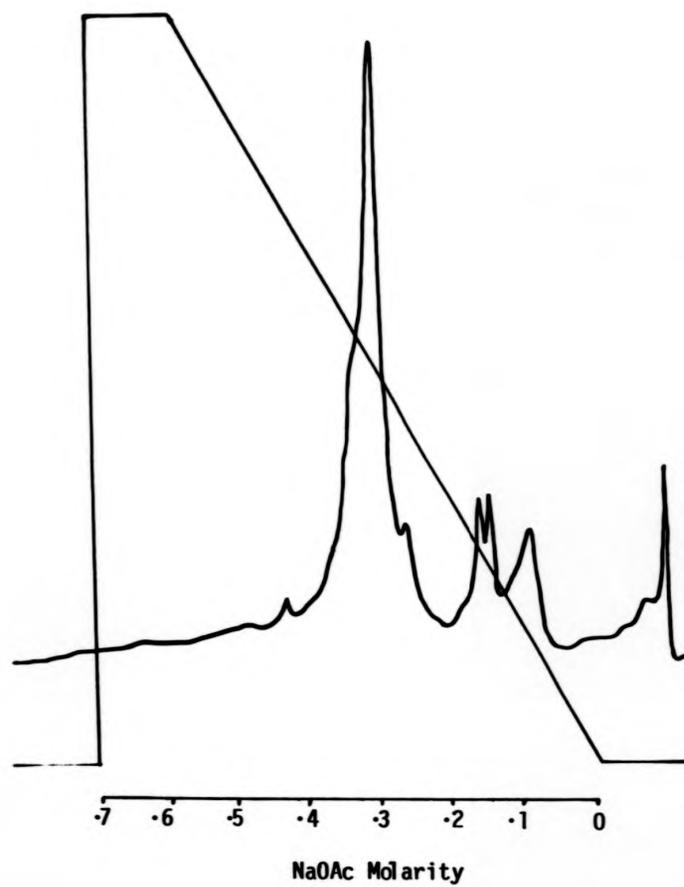
Figure 13.   Separation of Serum From an Aluminium Overloaded Patient (Experiment 6)
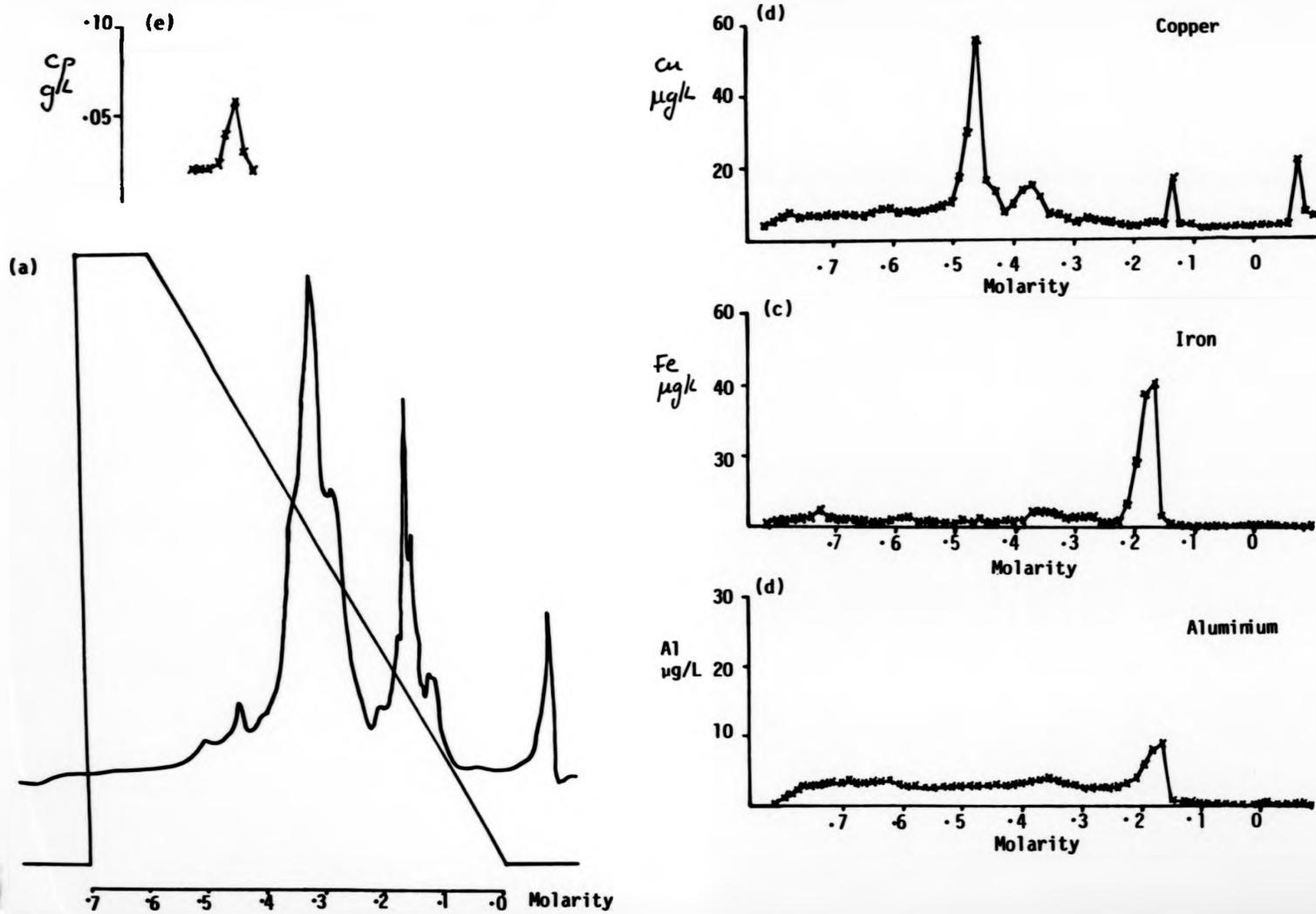
Figure 14. Separation of Serum From an Iron and Aluminium Overloaded Patient (Experiment 7)
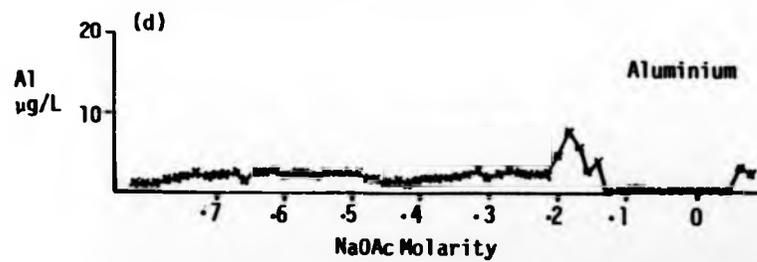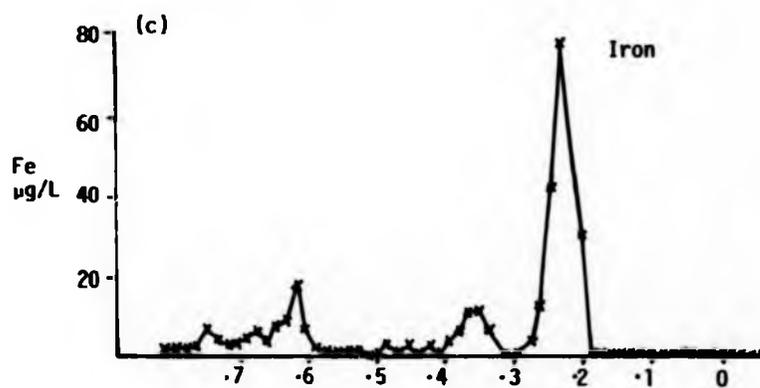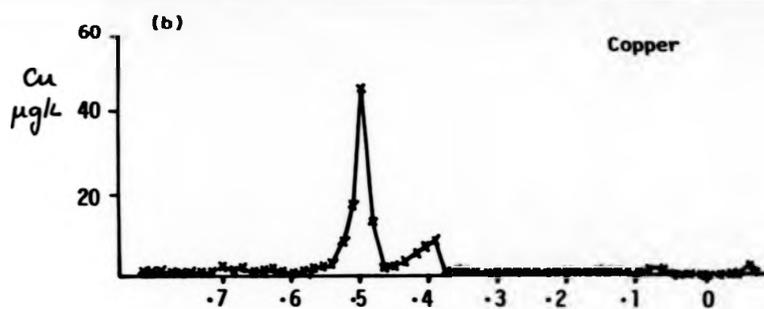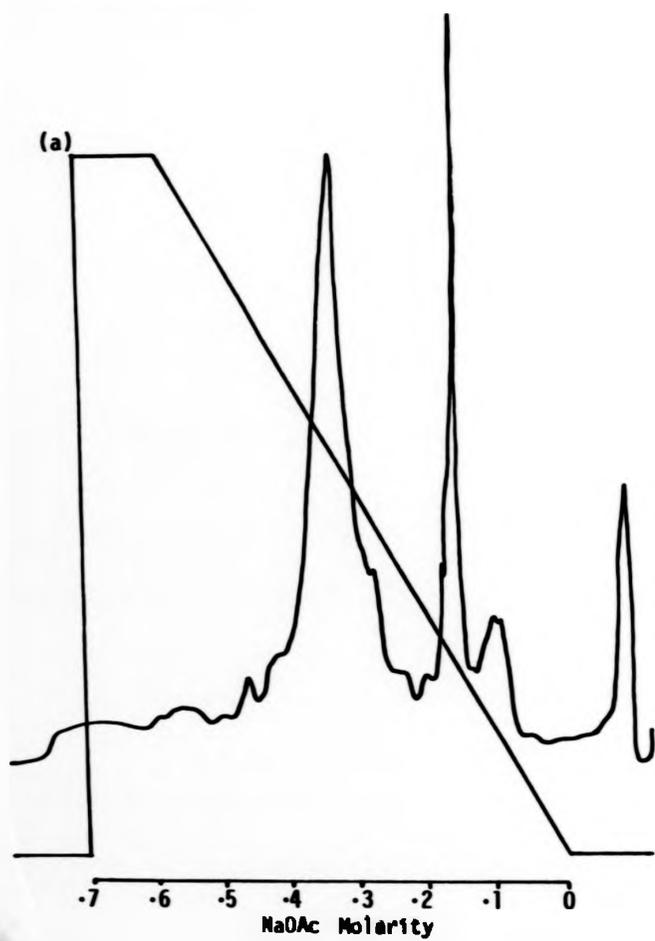
Table 2.   Results of Anion-exchange Separations.

PROTEIN		ELUTION IONIC STRENGTH (NaOAc/$\underline{M}$)			
		EXPT 1-3	EXPT 4	EXPT 5	EXPT 6
Transferrin	(U-V)	0.19 –	0.19 –	0.18 0.19	0.17 0.18
Albumin	(U-V)	0.36	–	0.37	0.37
Caeruloplasmin	(U-V)	0.55	0.54	0.54	0.52
Caeruloplasmin	(Assay)	–	0.54	–	0.54
Fe-59	(LS)	0.19 –	0.18 0.19	– –	– –
Copper	(AAS)	– – –	0.30 0.54 –	– – –	0.17 0.37 0.54
Iron	(AAS)	– –	0.18 0.19	– –	0.17 0.18
Aluminium	(AAS)	–	–	–	0.17

4.6   Discussion.

Good resolution of serum proteins (caeruloplasmin,
transferrin and albumin) was obtained by anion-exchange
chromatography using the described method (Experiment 6).
From trace metal determinations we found that copper and iron
remained attached to their carrier proteins (caeruloplasmin
and albumin, and transferrin, respectively), which suggests
that the adopted chemical gradient separated the serum
proteins without altering the metal binding sites. Aluminium
also remained protein bound during ion-exchange separation. A
single aluminium peak was detected, which corresponded to the
iron peak, and was assigned to transferrin.

The presence of oxidase activity in the caeruloplasmin
fractions (Experiment 4 and 6) indicates that protein
activity is not destroyed during the separation procedure.

5.   CONCLUSIONS.

Anion-exchange chromatography indicates that transferrin is
the major (and probably the only) aluminium carrier-protein
in serum. This finding is consistent with that of Cochran et
al (289), who reported significant binding of aluminium by
human transferrin, after replicate gel filtration
separations. Rahman et al (290) also found that aluminium was
associated with transferrin using affinity chromatography.
These findings are in contrast to Trapp (71) who found

aluminium binding to both transferrin and albumin and King et al (288) who reported five major aluminium peaks after gel filtration, four associated with protein peaks and one associated with low molecular weight species.

Our findings strongly suggest that transferrin is the sole protein carrier of aluminium in plasma. The association constant (K_a) for the reaction:

$$Al^{3+} + \text{Apo-transferrin} \rightleftharpoons Al^{3+}\text{-transferrin}$$

is not known, but is thought to be within a few orders of magnitude of K_a for Fe^{3+}-transferrin, ca 10^{24} M^{-1} (262),under physiological conditions. Cochran et al (296) have shown a high affinity binding of aluminium at both of the binding sites on the transferrin molecule, indicating the role of transferrin as a specific carrier for aluminium. Thus if binding sites are available, as in the case of partially iron-loaded transferrin, aluminium entering a patient during dialysis (11, 13, 40, 51) or after gastrointestinal absorption (22, 74, 77) may compete with Fe(III) for the transferrin binding sites, and thus inhibit the incorporation of Fe(III) into transferrin. Any mechanism which interacts with the binding of iron to transferrin also inhibits the rate at which iron is transported around the body to its deposition sites. The inhibition of iron transport often results in iron-deficient anaemia (92, 297), and this point is discussed in the final chapter.

Once aluminium becomes bound to transferrin, removal by dialysis is greatly inhibited (11, 51), even when low aluminium level dialysate is used (43, 51).

Although it is not known what happens to transferrin-bound aluminium once it has formed, raised tissue aluminium levels in brain, bone and liver indicate that aluminium is deposited at these sites (57, 77, 298, 299). The deposition of aluminium at sites such as bone and liver suggests that aluminium may interfer with iron and/or other trace element metabolism (this point is discussed in the final chapter). Alternatively, transferrin may function as a detoxification mechanism, to reduce the deposition of aluminium in tissues.

The in vivo interference of aluminium with other biochemical processes in man is still poorly understood. The concept of an analogous behaviour of aluminium and other metal ions (particularly Fe(III)) may help to establish the mechanism of aluminium toxicity. This point is taken further in the final chapter.

CHAPTER 9.

THE INTERACTION OF ALUMINIUM WITH IRON AND COPPER METABOLISM.

THE INTERACTION OF ALUMINIUM WITH IRON AND COPPER METABOLISM.

The objective of this chapter is to summarise the main
conclusions and findings of the preceding experimental
chapters and to discuss some of the questions raised
concerning aluminium biochemistry. It is apparent that the
physiological behaviour of aluminium is linked to that of
iron and copper, and these links are also explored in this
chapter.

Similarities in the behaviour of aluminium and iron have been
documented at many points in this thesis. Furthermore, the
biochemistry of iron is relatively well understood.
Therefore, it seems appropriate to take the biochemistry of
iron and its metabolic pathways as a starting point for a
discusion of the behaviour of aluminium, remembering that
aluminium is unlikely to follow iron in pathways where
reduction to Fe(II) is a necessary mechanistic step.

Figure 1 shows the basic details of iron biochemistry,
transport and storage. The interaction of aluminium
biochemistry with the iron metabolic pathways is shown
schematically in Figure 1a.

1. Aluminium Overload. - Aluminium overload results in
chronic renal failure, prior to dialysis treatment, due to
the administration of aluminium containing-phosphate binders.
Aluminium is absorbed from the gastrointestinal tract and

Figure 1.   Essentials of Iron Biochemistry
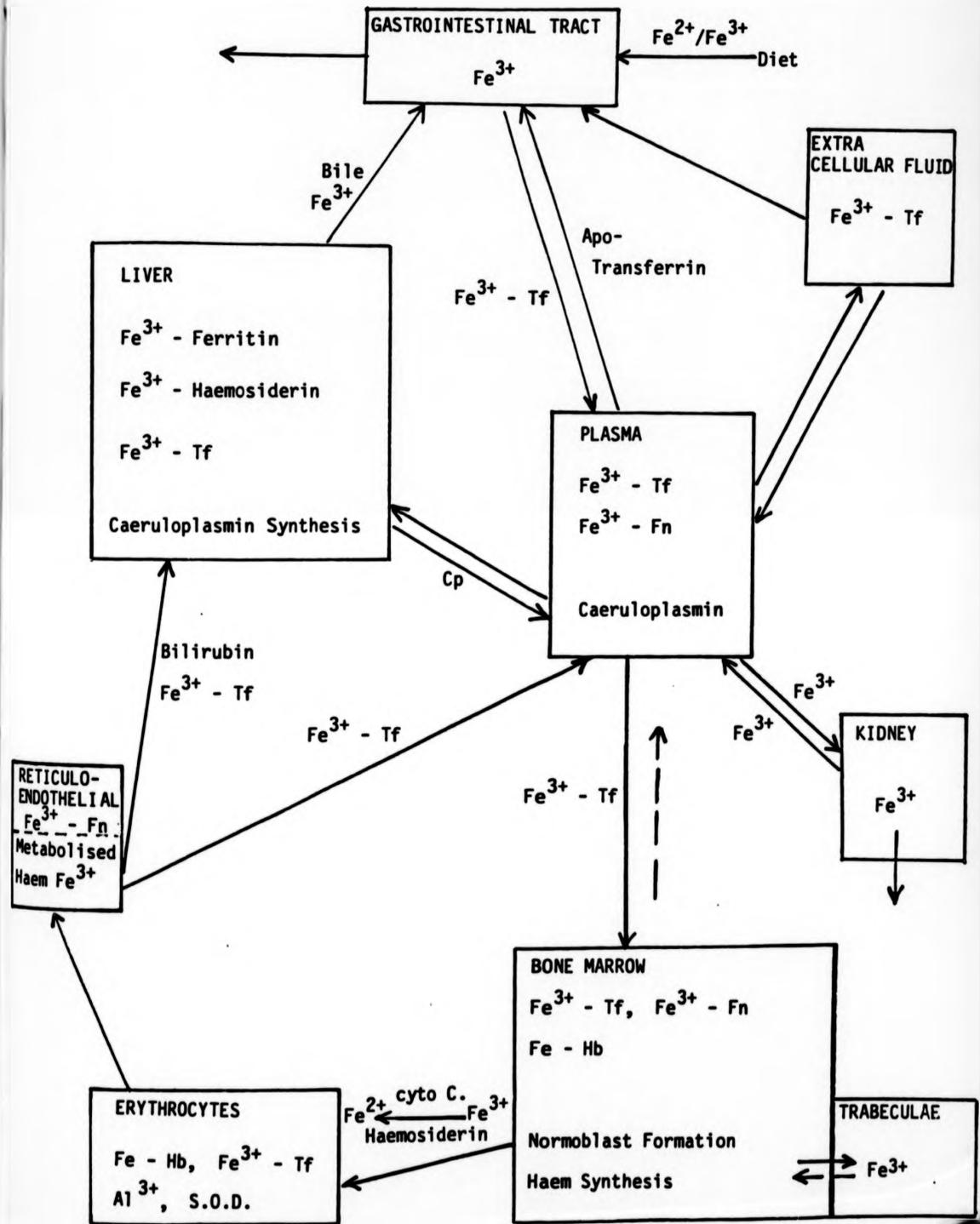
Figure 1a. The Interaction of Aluminium with Iron and Copper Biochemistry
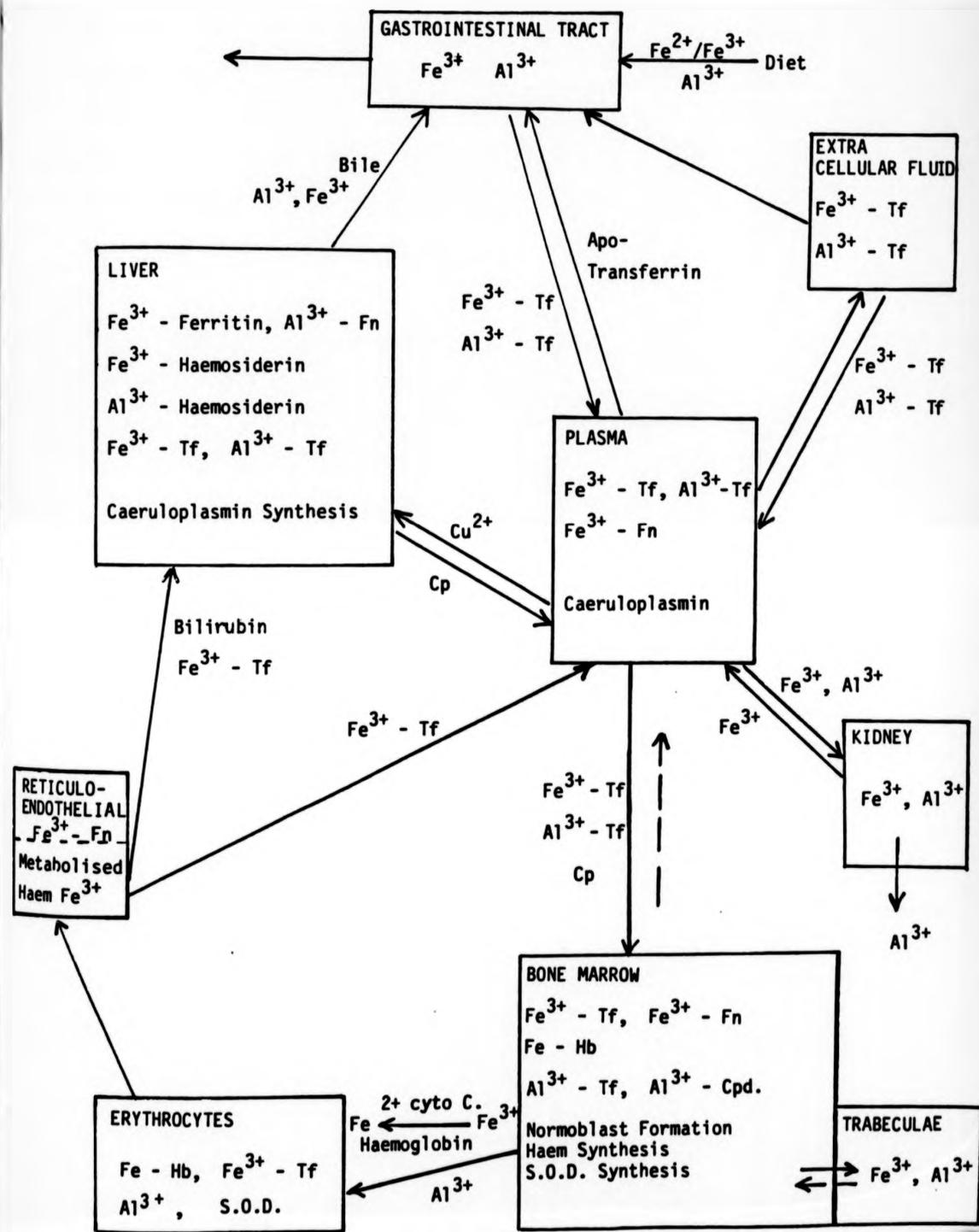
accumulates within the body. Iron absorption through the gut mucosa results from the active uptake of iron by the gut epithelial cells in response to physiological losses and the internal iron status. In patients with high iron stores, whose daily iron absorption is low, aluminium absorption is also low (61). This suggests a "common pathway" for the active absorption of iron and aluminium.

2. Aluminium in Dialysate. - When the level of aluminium in dialysate is high, aluminium crosses the dialyser membrane against the concentration gradient, resulting in an increase in serum aluminium level post dialysis.

If the level of aluminium in dialysate is low, < 14 µg/l, aluminium is dialysed out of the patient during dialysis (51), i.e. under these conditions the dialyser membrane is acting in a similar way to a normal kidney and removes absorbed aluminium from the patient.

3. Aluminium Transport. - Once absorbed, aluminium is transported around the body bound to transferrin, the major iron transport protein. This binding of aluminium to transferrin competes with the iron binding, and thus may reduce the rate at which iron is transported around the body. Factors which inhibit iron metabolism induce iron deficient (or hypochromic normocytic) anaemia.

4. Overload. - In cases of overload, aluminium accumulates in the bone, liver, and brain. The liver and bone marrow are

the main storage sites of iron in normal iron status, and in overload iron is also stored in the trabecular bone. Thus, in overload aluminium uses the same storage sites usually occupied by iron.

5.   Chelation Therapy. - During chelation therapy with desferrioxamine, increased levels of aluminium are observed in the blood plasma, waste dialysate fluid and, in patients who have residual renal function, in the urine.

The increased levels of aluminium in urine, and hence increased removal of aluminium, is paralleled by the simultaneous removal of iron in the urine, suggesting that both aluminium and iron have been mobilised from the same storage sites. During chelation therapy for iron overload, the removal of iron in the urine originates from extracellular sites, from plasma, iron deposited in bone, or the release of iron in chelatable form as it is mobilised for red cell production, i.e. by the release of Fe^{3+} from transferrin to haem.

The release of Fe^{3+} from transferrin is pH dependent (Figure 2). Iron remains bound to the binding sites of transferrin at normal physiological conditions (pH 7.4), but is released from transferrin within the reticuloendothelial (RE) cell at low pH (pH 5.0). "Free" Fe^{3+} within the RE cell induces and catalyses lipid peroxidation within the cell. Iron readily undergoes redox reactions: Fe^{3+} may be reduced to Fe^{2+} by cytochrome c and become associated to protoporphyrin in

Figure 2.   The release of Iron From Transferrin.

Apo-transferrin released
pH 7.4

Iron loaded transferrin at a
receptor site. (pH 7.4)

pH increases
of 7.4

pH lowered
to ca. 5

Endosome
returns to
cell membrane

Fe^{3+} is released
at pH 5.0

At pH 5 Apo-transferrin
remains attached to the receptor

'Free' Fe^{3+}

Fe^{3+} catalyses lipid peroxidation

$Fe^{3+} \xrightarrow{\text{cyto.C}} Fe^{2+}$ + Protoporphyrin Haem

$Fe^{3+} \longrightarrow Fe^{2+}$ + Apo-Ferritin   Ferritin
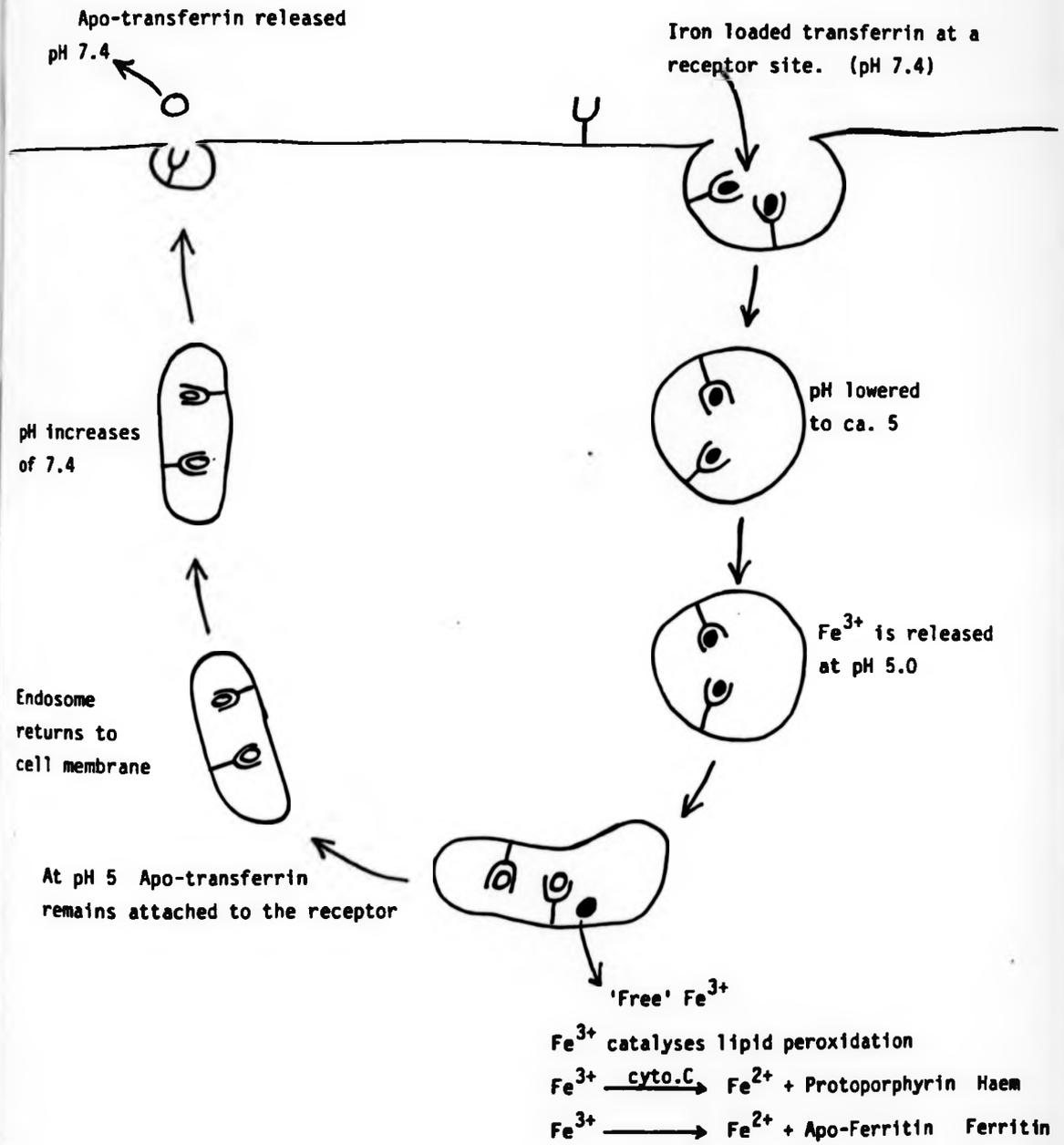
haem synthesis; or Fe^{3+} may be reduced during incorporation into ferritin (where it is re-oxidised).

Aluminium bound to transferrin may be released from the carrier protein in a similar way to iron. After release, "free" aluminium is able to catalyse the lipid peroxidation induced by Fe^{3+} within the RE cell. Aluminium is unable to undergo redox reactions, suggesting that Al^{3+} may block redox dependent iron metabolic pathways. Aluminium released from transferrin, which cannot be metabolised, accumulates at the site of deposition e.g. bone and liver.

6.   Removal in Faeces. - The rate of aluminium and iron removal is increased in faeces during chelation therapy. Iron removal results from hepatic storage (245, 258), i.e. intracellular storage within the hepatic parenchymal cells. Increased levels of aluminium and iron appear in the faeces at the same rate, suggesting that aluminium and iron are stored in the same site, i.e. in ferritin. The currently accepted theory for the incorporation of iron into ferritin is that iron enters ferritin as Fe^{2+}, being oxidised to Fe^{3+} when it reaches the centre of the ferritin core, but the actual point of oxidation is uncertain. Hence, if oxidation occurs at the outer surface of ferritin, aluminium may enter the ferritin structure alongside iron. If oxidation occurs after Fe^{2+} has entered the ferritin structure, then the passage of aluminium into ferritin seems unlikely.

In cases of iron overload, iron is stored in ferritin and

haemosiderin. Haemosiderin is a metabolite of ferritin. As the ferritin structure begins to break up, the outer shell ruptures and the inner Fe_2O_3 is exposed. Al^{3+} could become incorporated into the Fe_2O_3 core and stored within the haemosiderin structure in the liver.

During chelation therapy the major route of removal of both aluminium and iron is via the faeces.

7. Removal in Urine. - In patients with residual renal function, the clearance of aluminum by the kidney is increased during chelation therapy, presumably due to the formation of the low molecular weight Al-DFO complex. Hence, patients suffering from aluminium overload prior to commencing dialysis, who have some residual renal function, may be successfully treated with chelation therapy.

8. Inhibition of Ferrioxidase Activity. - Aluminium biochemistry also interacts with copper metabolism. In chronic renal failure, and particularly during DFO treatment, serum copper levels are increased. This increase in serum copper is due, at least in part, to increased level of caeruloplasmin in the plasma. Caeruloplasmin synthesis is increased in response to the so called "acute phase" reaction which results from inflamation, infection and during dialysis treatment. Caeruloplasmin plays a central role in regulating the Fe(II) to Fe(III) cycles, i.e. caeruloplasmin has ferrioxidase activity and in turn affects the storage, transport and biosynthesis of iron, and more specifically

affects the availability of iron for its incorporation into
haem. Levels of caeruloplasmin may be inceased in the plasma
in order to ensure a sufficient supply of Fe^{3+}, as
transferrin, to the haem pathway. Trivalent cations, and
particularly Al^{3+}, are known to inhibit the ferrioxidase
activity of caeruloplasmin (104). This inhibition of
ferrioxidase activity, in addition to the binding of
aluminium to transferrin instead of iron, is likely to
disrupt iron metabolism and haem synthesis, resulting in
anaemia.

9.   Removal from Bone. - In bone, high levels of aluminium
are found in aluminium intoxication. The anaemia which
results from aluminium overload is probably due to the toxic
effects of aluminium found in the bone marrow, the site of
red cell production and haem synthesis. During chelation
therapy the level of aluminium and iron in the bone marrow
and trabeculae is reduced, as a result of the formation and
mobilisation of Fe^{3+}- and Al^{3+}-DFO complexes.

The level of copper in bone is reduced during chelation
therapy; this is paralleled by an increase in copper
concentration in RBC. Approximately 60% of copper within the
erythrocyte is associated with super oxide dismutase, an
enzyme synthesised in the bone marrow. The increase in RBC
copper concentration may be due to an increase in either
caeruloplasmin or perhaps more likely super oxide dismutase
level. In either case the overall effect would be the same;
the removal of toxic oxygen radicals which stimulate the

peroxidation erythrocyte membranes. Aluminium in bone may block or inhibit the normal copper metabolism from bone to RBC, resulting in bone copper accumulation during aluminium intoxication. The removal of aluminium from bone during chelation therapy results in an improvement in bone mineralisation and turnover, and this may result in an improvement in bone copper turnover.

10. <u>Anaemia.</u> - During chelation therapy the anaemic state of patients improves. In addition to the interaction of aluminium with iron metabolism and the inhibition of ferrioxidase activity, aluminium in the bone is thought to block haem synthesis, by preventing the formation of delta-aminolevulinic acid, the enzyme which catalyses the second step in haem synthesis (103).

11. <u>Lipid Peroxidation.</u> - Aluminium is present within the erythrocyte. During chelation the level of aluminium in the erythrocyte is gradually reduced. Erythrocyte aluminium levels do not show the short term cyclic variation which is seen in plasma aluminium levels during DFO therapy, i.e. DFO does not pass from the plasma to the erythrocyte through the cell membrane. The slow reduction in erythrocyte aluminium suggests that aluminium becomes incorporated into the RBC during its formation in the bone marrow. Aluminium is removed from the bone marrow and mineralising fronts by DFO complexation, hence less aluminium becomes incorporated into the erythrocyte during synthesis.

"Free" Al^{3+} accelerates the membrane peroxidation seen within
the erythrocyte and caused by the presence of hydrogen
peroxide and "free" Fe^{3+} (300). Thus, a reduction in the
level of aluminium in the erythrocyte would tend to decrease
the level of membrane peroxidation, increasing the life-span
of the erythrocyte.

12.   Further Research. - Several questions remain to be
answered:-

How is aluminium incorporated into ferritin?

Is aluminium able to bind to both binding sites in the
transferrin protein?

Does Al^{3+} bind to the protoprophyrin ring preventing the
formation of haem in the erythrocyte, and the subsequent
binding and transport of oxygen around the body?

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