THE INTESTINAL ABSORPTION AND TISSUE DISTRIBUTION OF GALLIUM AND SCANDIUM IN THE RAT: PROSPECTIVE MODELS FOR ALUMINIUM

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Aluminium has become increasingly implicated in the aetiology of several neurodegenerative diseases and the oral route of entry is probably responsible for the majority of body aluminium. Research however, has been hampered because of difficulties in analytical procedures and the lack of a suitable radioisotope of aluminium. The use of chemically similar Group 3 radioisotopes, ^{67}Ga and ^{46}Sc as analogues for aluminium has proved to be successful.

After the oral dosing of aluminium, gallium and scandium similar tissue distributions were observed with bone, spleen, heart and muscle being targeted for all three elements. In all cases reduced absorption was observed in the fed rat compared to the fasted. The *in-vitro* intestinal absorption of aluminium, gallium and scandium showed similar characteristics of passive energy-independent transport. These trends were repeated with gallium in an *in-vivo* perfusion system. The food and water additives citrate, maltol and fluoride were found to affect the bioavailability of gallium in oral dosing experiments and in *in-vitro* and *in-vivo* intestinal experiments. Both citrate and maltol enhanced the absorption of gallium, whilst fluoride inhibited the absorption of gallium.

Speciation analysis of small bowel fluids after the oral administration of gallium indicated that gallium existed in a large molecular weight complex (thought to be a protein) and as the 'free' hydrated gallium ion. In the fed rat the large molecular weight gallium binding species was more prominent than in the fasted and there was a consequent reduction in the amount of hydrated gallium. It is possible that the formation of this large molecular weight species is protective in nature preventing the effective absorption of gallium, since upon readministration no transport to the tissues was observed.

The blood bound gallium species consisted of either a gallium-transferrin complex (at a low gallium concentration) or a gallium-transferrin complex and a low molecular weight gallium binding species (at a high gallium concentration).

Key Words - Aluminium, gallium, scandium, intestinal absorption, intestinal speciation

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LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$	- 1,25-dihydroxycholecalciferol (vitamin D_3 metabolite)
Å	- angstrom
Al^{3+}	- the hexahydrated $Al(H_2O)_6^{3+}$ cation
$Al(OH)_3$	- aluminium hydroxide
ATP	- adenosine triphosphate
ATPase	- adenosine triphosphatase
cm^3	- cubic centimetres
CNS	- central nervous system
CSF	- cerebrospinal fluid
D	- dalton (≈ mass of 1 hydrogen atom)
DFO	- desferrioxamine
DNA	- deoxyribonuclease
DNP	- 2,4-dinitrophenol
ECF	- extracellular fluid
EDTA	- ethylenediaminetetraacetate
g	- gram
Ga^{3+}	- the hexahydrated $Ga(H_2O)_6^{3+}$ cation
$Ga(NO_3)_3$	- gallium nitrate
GFAAS	- graphite furnace atomic absorption spectroscopy
GI	- gastrointestinal
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
im	- intramuscular
iv	- intravenous
KD	- kilodalton
KeV	- Kiloelectron Volts
KHBB	- Krebs Henseleit bicarbonate buffer
kg	- kilogram
M	- molar
mg	- milligram
ml	- millilitre
mm	- millimetre
mM	- millimolar
M.W.	- molecular weight
n	- number of observations
ng	- nanogram
NMR	- nuclear magnetic resonance
PEG900	- polyethylene glycol (≈ M.W. = 900)
PD	- potential difference
ppm	- parts per million (µg/ml)
Sc^{3+}	- the hexahydrated $Sc(H_2O)_6^{3+}$ cation
$ScCl_3$	- scandium chloride
SDS	- sodium dodecyl sulphate
sec	- second
±SEM	- ±standard error of the mean
SI	- small intestine
S/M	- serosal to mucosal ratio
$t_{1/2}$	- half life
TPN	- total parenteral nutrition
Tris	- tris(hydroxymethyl) amino methane
µCi	- microcurie
µg	- microgram
µl	- microlitre
µM	- micromolar
UIBC	- unused iron binding capacity
w/v	- weight for volume (normally expressed as a percentage)
w/w	- weight for weight (normally expressed as a percentage)
wwt	- wet weight tissue

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CHAPTER ONE

INTRODUCTION (LITERATURE REVIEW)

CHAPTER 1 LITERATURE REVIEW.

1.1 INTRODUCTION

Aluminium is the most abundant metal and third most common element (after oxygen and silicon) in the earth's crust. It does not occur in the metallic form but is distributed widely in the earth's crust in combination with oxygen, fluorine, silicon and others. Industrially its most important souces are bauxite ($Al_2O_3.2H_2O$), cryolite (Na_3AlF_6) and spinel ($MgO.Al_2O_3$). Metallic aluminium is an important structural material in almost every aspect of industry and worldwide use and production of aluminium is in tens of millions of tons (Norseth, 1979). Bentonite, a complex aluminium silica clay is extensively used in the purification of general water supplies ($Al_2(SO_4)_3$ is also used for this purpose), sugar refining and in brewing and paper industries (Venugopal and Luckey, 1978). The salts of aluminium also find use in aspects of human and veterinary medicine. Antiseptics and astringents contain alum and aluminium acetate, whilst antacids are formulated with aluminium magnesium silicate and aluminium hydroxide. The hydroxide form is also used to lower the plasma phosphate levels of uremic patients by reducing the phosphate absorbed by the gastrointestinal tract.

Compounds of aluminium are also added to many food types as an agent to aid buffering, neutralising, firming and leavening processes.

Because of its ubiquitous nature in the environment exposure to aluminium is unavoidable. Aluminium has been identified in both vegetable and animal foodstuffs (Lione, 1985) and daily intake in man is thought to range from 1-100 mg/day (average 5mg/day), (Bertholf, Wills and Savory, 1988).

Aluminium has no known biochemical role in mammals, though it may be essential for the germination of plants (Lipman, 1938).

1.2. CLINICAL MANIFESTATIONS OF ALUMINIUM TOXICITY

1.2.1. DIALYSIS DEMENTIA

Haemodialysis therapy was first introduced in the early 1960's. Typical uremic symptoms, (for example neuropathy, anemia, hyperparathyroidism and bone calcification) were noted to be more frequent in dialysed patients rather than in non-dialysed uremics (Kerr et al. 1966). In 1972 distinctive neurological symptoms were observed in haemodialysis patients in Denver, Colorado (Alfrey et al. 1972) and subsequently similar cases were identified in dialysis centres worldwide (Platts et al. 1973; Rozas et al. 1978). Clinical features in the early stages of the disease included mild speech disorders, particularly following dialysis, disorientation and personality changes (Sideman & Manor, 1982). As the disease progressed typical symptoms were dyspraxia, muteness and seizures. Sufferers were observed to have characteristic EEG alterations; bursts of Delta waves followed by spike activity (Alfrey, 1986). Research showed that the above symptoms were characteristic of cases in all parts of the world (Wing, 1980; Schreeder et al. 1983). The disease was termed dialysis encephalopathy (or dialysis dementia). In the early 1970's the aetiology of dialysis encephalopathy was unknown. Possible suggestions as to the cause were deficiences in dopa and asparagine (Wardle, 1973; Gunale, 1973), although the majority of evidence centred upon an environmental toxin (Lyle, 1973). The accumulation of a trace metal contaminant was narrowed down to aluminium (Flendrig et al. 1976; Ward et al. 1978). Aluminium hydroxide is administered as a binder to counteract hyperphosphataemia and was first suggested as a possible toxin in 1970 by Berlyne et. al. Epidemiological studies in Europe also incriminated aluminium in the pathogenesis of dialysis dementia (Wing, 1980). Areas where dialysis dementia occurred had high aluminium levels (up to 1000µg/l) in the general water supplies (Ward et al. 1978). When aluminium was removed from the dialysate by haemofiltration methods the symptoms were largely eradicated from those areas (Masselot et al. 1978; Davison et al. 1982).

It was thought that reducing aluminium concentrations in dialysis fluids to an acceptable level of less than 10µg/l would altogether remove the disease. This has not been observed however. Similar symptoms to those seen in the early 1970's have been shown in patients whose dialysate fluids contain less than 1µg/l (Heaf and Nielsen, 1984). The patients had been chronic users of the aluminium containing phosphate binders. Previous evidence suggested that intestinal absorption of aluminium did not occur (Campbell et al. 1957; Browning, 1969). With the advent of more sophisticated analytical equipment however, high aluminium levels (see table 1.1) have been identified in the serum and brains of uremics who have endured long term phosphate binding treatment (Fleming et al. 1982). Encephalopathic symptoms have also been observed in non-dialysed patients who orally take aluminium containing preparations (Kaye, 1983). Chelation of aluminium by desferrioxamine (see 1.2.8) is possible in the early stages of dementia (Pogglitsch et al. 1981) and symptoms have been observed to be reversed (Bertholf et al. 1984). In severe dementia however, chelation therapy is ineffective.

It appears that the monitoring of serum aluminium levels is necessary to identify risk patients (Winney et al. 1986). The determination of serum aluminium however, is analytically difficult and there is an alarming variability in serum aluminium levels reported which must be noted with due care in the subsequent treatment of patients (Adan et al. 1986).

1.2.2 DIALYSIS OSTEOMALACIA

In 1971 prior to the discovery of dialysis dementia, Parsons et al. noted high aluminium concentrations in bone from haemodialysis patients. It was subsequently noted that patients suffering from dialysis dementia also exhibited symptoms of osteomalacia characterised by bone softening and pathological fractures (Elliott et al. 1978; Parkinson et al. 1979; Hodsman et al. 1982). It was also observed that the bone disease correlated with the appearance of dialysis encephalopathy in areas

TABLE 1.1. ALUMINIUM LEVELS IN CONTROL AND DISEASED TISSUE SAMPLES (LITERATURE REVIEW).

1). UREMIA

Brain (gray matter) aluminium (mg/kg dry weight)

Control .....2.4 ±1.3 (10)[1]; 0.9± 0.9 (19)[2]
Non-dialysed uremic.....4.1 ±1.7 (3)[1]; 6.6 ± 4.2 (8)[2]
Dialysed uremic.....8.5 ±3.5 (21)[1]; 3.8 ± 1.8 (5) [2]
Dialysed uremic (demented).....24.5 ±9.9 (34)[1]; 12.4 ± 9.7 (4)[2]

Bone aluminium (mg/kg dry weight)

Control.....3.3 ±2.9 (16)[3]
Non-dialysed uremic.....27.4 ±20.9 (30)[3]
Dialysed uremic.....116 ±107 (30)[3]
Dialysed uremic (demented).....281 ±143 (38)[3]

Serum aluminium (µg/l)

Control..... 6.2 ±3.1 (31)[4]; 10.8 ±8.1 (21)[5]
Non-dialysed uremic.....19.0 ±8.0 (13)[6]; 13.4 ±6.6 (45)[4]
Dialysed uremic.....40 ±12 (24)[6];109 ±10.6 (47)[7]
Uremic on oral aluminium.....94 ±36 (24)[6]; 165 ±40 (17)[6]
Dialysed uremic (demented)..... 614.5 (8)[8]; 556±53(5)[9]

2) CHILDREN

Brain aluminium (mg/kg dry weight)

Children receiving iv feeds (aluminium concentration up to 232 ±60 ng/ml).....6.4 (1)[10]; 47 (1)[10]

Bone aluminium (mg/kg dry weight)

Non-dialysed uremic child on oral Al...156 (1)[11]

Serum aluminium (µg/l)

At birth..... ≈ 18(22)[12]
Bottle fed, 6 weeks later..... 2-10(11)[12]
Parenterally fed.....28 (9-65)(11)[12]
Non-dialysed uremic child on oral Al...334 (1)[11]

3). ALZHEIMERS DISEASE

Brain aluminium (mg/kg dry weight)

Hippocampus (control).....2.684 (30)[13]
Hippocampus (Alzheimers).....7.462 (22)[13]
Cerebral cortex (control).....2.826 (30)[13]
Cerebral cortex (Alzheimers).....9.140 (22)[13]

Serum aluminium (µg/l)

Control.....5.9 ±2.3 (8)[14]
Alzheimers.....7.1 ±5.9 (15)[14]

Cerebrospinal fluid (µg/l)

Control.....35.3 ±10.8 (9)[14]
Alzheimers.....30.7 ±7.3 (5)[14]

Notes

1). The figure in brackets indicate the number of observations per group (if given) . All results expressed with standard deviations (again if given).

2).Figures in square brackets indicate reference, [1]=Alfrey et al. 1979; [2]= Arieff et al. 1979; [3]=Alfrey, 1980; [4]=Marsden et al. (1979); [5]= Fleming et al. (1982); [6]=Boukari et. al.(1978); [7]=McKinney et. al., [8]=Elliot et. al. (1978); [9]= Pogglitsch et. al. (1981); [10]=Freudlich et. al., (1985); [11]=Griswold et al.(1983); [12]=Robinson et. al. (1987); [13]=Ward and Mason, (1986); [14]=Shore and Wyatt, (1983)

where the dialysate was contaminated with aluminium (Parkinson et al. 1979; Ward et al.1978). When aluminium was chelated from the body and excreted a spontaneous healing of the fractures occurred (Seyfert et al. 1987).

Although there is a strong body of evidence to link aluminium with renal osteomalacia, the mechanisms of aluminium toxicity in the bone mineralisation process have yet to be fully understood. There have been suggestions that aluminium interfered with vitamin D metabolites, since the pathological fractures showed an unusual poor response to therapy with vitamin D or its metabolites (Ott et al. 1982). It is possible however, that aluminium is an 'accessory' in the pathogenesis of dialysis osteomalacia rather than the 'culprit' it was originally thought to be (Quarles et al. 1986).

1.2.3. SENILE DEMENTIA OF THE ALZHEIMER TYPE (SDAT).

Alzheimers disease is a progressive and irreversible neurodegenerative disease which occurs in 3% of the population over the age of 65 (Shore & Wyatt, 1983). At present there is no satisfactory explanation for the aetiology of Alzheimers disease, although aluminium amongst other toxins is suggested as a causative agent (Crapper et al. 1973). Microscopic examination of Alzheimer brain tissue has revealed senile plaques and paired helical filaments. These structures are thought to be characteristically associated with Alzheimers disease. Aluminium has been found to accumulate in the nuclei of the neurons containing these filaments (Perl et al. 1980) whilst Candy et al. (1986) found deposits of aluminium (co-localised with silicon) in the core of senile plaques. Animals injected with aluminium developed a neurofibrillary degeneration which was similar to the degeneration observed in the brains of dementia patients. (Wisniewski et al. 1985). Crapper et al. (1978) have observed typical dementia symptoms (i.e. learning and memory deficits) in animals which had aluminium injected into the CSF. Histological evidence observed that aluminium accumulated in the nuclear chromatin.

Analysis of post mortem brain samples have shown high aluminium concentrations (see table 1.1) in both the hippocampus and cerebral cortex of dementia patients when compared to age-matched undiseased brains (Ward and Mason, 1986).

Although dialysis encephalopathy and Alzheimers disease are classified as dementias having similar clinical symptoms, pathological differences have been observed (Wisniewski et al. 1985). In dialysis dementia a whole body accumulation of aluminium is noted, whereas in Alzheimers disease only brain aluminium concentrations are elevated. In uremic patients suffering from dementia, accumulation of aluminium in the nuclear chromatin is not observed, unlike both the Alzheimer type brains and in the induced brains of animals (Crapper et al. 1980). Although it is possible that the aetiology of the dementias may be similar, the mechanisms of toxicity may differ.

Other neurodegenerative disorders possibly associated with aluminium neurotoxicity are Down's syndrome with Alzheimers disease, the Guam parkinsonian dementia complex (PD) and the Guam amyotrophic lateral sclerosis (ALS)(Ganrot, 1986). Neurofibrillary tangles similar to those identified in Alzheimers disease have been observed in the above syndromes (Perl et al. 1982; Garruto et al. 1986) whilst 'premature' senile plaques have been identified in uremic brains (Edwardson, 1988). Although not positively correlated with these rarer neurodegenerative disorders, aluminium has been implicated as a possible environmental toxicant.

It is possible that aluminium plays a role in the pathogenesis of these disorders, collective evidence however, points to a variety of environmental and genetic reasons for the onset of dementia (Wurtman, 1985).

1.2.4. CHILDREN AND ALUMINIUM TOXICITY.

Children with chronic renal failure have been shown to be at a greater risk of aluminium toxicity as a result of the ingestion of large doses of phosphate binding aluminium hydroxide (Griswold et al. 1983). Features of the progressive

encephalopathy are a delay in development, poor nutritional status, seizures and microencephalopathy (Santos et. al. 1986). In addition to the high levels of aluminium absorbed via the gastrointestinal tract as a result of oral aluminium compounds, infant milk formulaes and intravenous fluids used for total parenteral nutrition have been reported to contain widely variable aluminium concentrations (Robinson et al. 1987). Elevated serum and bone aluminium concentrations (see also table 1.1) have been observed in preterm infants undergoing intravenous therapy (Sedman et al. 1985).

There have been two cases of fatal aluminium toxicity in preterm infants (Freudlich et al. 1985). Autopsies of brain tissue indicated there to be high aluminium levels (see table 1.1) in these children. The source of aluminium was attributed to the powdered milk formulae (Al-230μg/l of made-up milk). It appears that infants are susceptible to aluminium toxicity as a result of impaired gastrointestinal function in the first few months of life in combination with impaired renal function (Hewitt et al. 1987).

In this particular group of risk patients there is an obvious need for a continual monitoring of aluminium serum levels, content of milk powders and intravenous fluids in order to minimalise the chance of toxic symptoms developing (Weintraub et al. 1986).

1.2.5. ALUMINIUM INDUCED ANAEMIA

Anaemia is a characteristic complication of chronic renal failure and involves decreased production and increased destruction of red blood cells. Aluminium has been implicated as a causative agent in the pathogenesis of renal anaemia. This possible causal relationship was first described by Elliot et al. (1978). They noted that the decrease in red blood cell count preceeded the onset of encephalopathic symptoms in uremic patients. A microcytic anaemia was also shown to develop in patients who later showed clinical signs of aluminium intoxication (Short et al. 1980).

With the removal of aluminium from dialysate fluid by reverse osmosis both the red cell size and haemoglobin levels returned to normal. Although it could be argued that reverse osmosis may remove a number of potential toxins, plasma aluminium levels decreased in conjunction with the increased haemoglobin level (Short et al. 1980). In addition to the removal of anaemic problems there have been concomitant improvements in osteomalacia (see 1.2.2.) when the water supplies were deionised to remove aluminium (O'Hare and Murnaghan, 1982).

Although the evidence to suggest the link between aluminium intoxication and anaemia is well documented the actual pathogenic mechanisms involved have yet to be clarified. Several theories have been postulated involving rate limiting enzymes in haem synthesis, aluminium interactions with iron and transferrin (Kaiser and Schwartz, 1985) and a possible catalysis of erythrocyte membrane lipid peroxidation causing fragility of the red cell membrane (Hewitt, Day and Ackrill, 1987).

1.2.6. TOTAL PARENTERAL NUTRITION (TPN)

Total parenteral nutrition (TPN) has been successfully employed as a method of nutrient intake since the late 1960's (Wilmore and Dudrick, 1968). The technique involves administration of nutrients through a large vein and is used to sustain patients who have a malfunction of the gastrointestinal tract. Chronic TPN has yielded deficiencies in micronutrients, whilst metabolic bone disease in conjuction with TPN has also been observed (Klein et al. 1980; Ott et al. 1983). Characteristic symptoms typical of aluminium induced osteomalacia (see section 1.2.2) have similarily been identified in patients undergoing chronic TPN (Sherrard et al. 1983). Aluminium was suggested as a possible causative agent. Again it appears that TPN fluids must be carefully monitored for aluminium content in order to prevent any possible intoxication occurring (Hewitt et al. 1987).

1.2.7. BIOCHEMICAL AND MOLECULAR EFFECTS OF ALUMINIUM

In the physiological pH range of between 5 and 8 and below concentrations of 10^{-5}M the prevalent aluminium species consist of the positively charged $Al(H_2O)_4(OH)_2^+$ cation, the neutrally charged $Al(H_2O)_3(OH)_3$ and the anion $Al(H_2O)_2(OH)_4^-$ (see also figure 1.1). Toxicity of aluminium occurs at micromolar levels and it is the above mononuclear species which are toxic.

Aluminium has been observed to inhibit hexokinase activity (Womack and Colowick, 1979) as a result of aluminium contaminated ATP preparations, aswell as inhibiting glycerokinase activity in combination with ATP (Viola and Clevand, 1979). Aluminium has a high affinity for the nuclear fractions of cells. In encephalopathic rats 80% of aluminium in the brain was found to be bound to the chromatin fractions (McLachlin and DeBonic, 1980). Studies have also identified physical changes in DNA upon aluminium binding (Eichom et al. 1980). Aluminium has been demonstrated to impair transcription and translation of genetic information (Sarkander et al. 1983).

Intracellular calcium is maintained within a narrow range of 0.1-1μM (Sulhake et al 1980). It is possible that its role in the secondary messenger system may be impaired by aluminium. In usual physiological circumstances the intracellular calcium binding protein calmodulin stimulates the activity of Ca^{2+}-Mg ATPase (Wang and Waisman, 1979; Siegal and Haug, 1983). In the presence of microlevels of aluminium however, the calmodulin stimulated activity of ATPase significantly decreased. NMR studies have also shown aluminium to bind to (leu)-enkephalin, an endogenous CNS peptide (Mazarguil et al. 1982).

More recently Jeffrey et al. (1987) have described the *in-vivo* interactions of aluminium with cytochrome P450 and metallothionein. Aluminium was observed to inhibit cytochrome P450 in a dose dependent manner, and was shown also to bind to metallothionein. Metals normally associated with metallothionein (zinc and copper) were lowered in liver and kidney when aluminium was administered.

1.2.8. DETOXIFICATION OF ALUMINIUM

In ordinary non clinical situations removal of aluminium is via the kidneys. In overload situations however, aluminium becomes strongly protein-bound in the blood (Bertholf et al. 1985). Large stores are also found in bone and nervous tissue (Skalsky and Carchman, 1983). Apart from lowering aluminium concentrations in the dialysate fluid itself (Rapaport et al. 1987) chelating agents are available which mobilise aluminium and facilitate excretion. Desferrioxamine (DFO) is a by-product of the mould *streptomyces* and is a hexodentate chelator with a high specificity for ferric and Al^{3+} cations (Swartz, 1985). Unlike other chelators such as ethylenediaminetetraacetate (EDTA) it has a low specificity for physiological cations such as Mg^{2+} and Ca^{2+}. DFO causes an increase in plasma aluminium reaching a maximum 24-48 hours after administration (Milliner et al. 1984). There is also an increase in the proportions of unbound ultrafiltratable aluminium from 20 to 30% as complexed aluminium is mobililzed. DFO therefore appears to alter the dialyzability of aluminium facilitating its removal by haemodialysis. In long term dialysis patients chronic administration of DFO decreased both bone and plasma aluminium levels (Ott et al. 1984; Bertholf et al. 1984). Symptoms of dialysis encephalopathy, osteomalacia and microcytic anaemia have also been reversed following DFO therapy (Bertholf et al. 1984; Ackrill et al. 1980, 1982; Touam et al. 1983).

1.3. EXPOSURE ROUTES TO ALUMINIUM.

1.3.1. SKIN.

The compounds of aluminium are used extensively in the preparation of cosmetics and antiperspirants. There is however, no evidence of aluminium being absorbed through the skin. Although certain salts can penetrate the stratum corneum they are all impermeable to the epidermal layer (Skalsky and Carchman, 1983).

1.3.2 LUNGS.

As aluminium is ubiquitous in the environment, the inhilation of dust containing aluminium is unavoidable. There is limited evidence to show that aluminium can be absorbed from the lungs in a normal situation, although in an aluminium saturated environment toxicity has been observed. The first case of aluminium poisoning was reported by Spofforth (1921), where an industrial metal worker showed signs of memory loss, tremor and incoordination. Similar symptoms were described by McLaughlin (1962) in an aluminium powder factory worker who suffered extensive pulmonary fibrosis in conjuction with a progressive encephalopathy. The inhilation of dust contaminated with aluminium is now a recognised cause of pulmonary fibrosis in industry.

1.3.3. KIDNEY DIALYSATE.

Epidemiological studies have correlated the incidence of dialysis dementia with a high concentration of aluminium in the general water supplies (Rozas et al. 1978; Ward et al. 1978; Elliot et al. 1978; Wing et al. 1980; Davison et al. 1982). In the UK a total of 100000 tonnes of alum (aluminium sulphate) is added to the water supplies as a clarifying agent. The aluminium content of water varies throughout the country from 10µg/l to 3500µg/l (Davison et al. 1982). Standards have been se to limit the amount of aluminium in the water to 30µg/l (European Communities Resolution, 1986). Ward et al. (1978) were amongst the first to purify dialysate water, lowering the aluminium content from over 1000µg/l to less than 20µg/l by deionising and reverse osmosis techniques. These techniques have been successfully used to abate the symptoms of early dialysis encephalopathy (Pogglitsch et al. 1981). The continued use of the techniques to remove aluminium from dialysis water and the careful monitoring of plasma and dialysate aluminium has effectively removed the threat of aluminium toxicity from this particular route of entry (O'Hara et al. 1987).

1.3.4. GASTROINTESTINAL

Berlyne et al. (1970) were the first to warn that oral aluminium may lead to toxicity. Previous to these observations it was thought that aluminium was excreted in the faeces as an insoluble phosphate complex and not available for absorption (Campbell et al. (1957).

These early studies used analytical techniques which suffered from a general lack of sensitivity. More recent developments in analytical techniques such as graphite furnace atomic absorpton spectroscopy (GFAAS), inductively coupled plasma mass spectroscopy (ICP-MS) and neutron activation analysis (NAA) have improved to detection limits of aluminium to picomole levels (LeGendre et al. 1976; Ward and Mason, 1986).

In normal individuals Gorsky et al. (1979) found only 1-2% of an administered dose to permeate the gastrointestinal tract, the majority of absorbed aluminium being excreted within 24 hours. Studies of exposure to aluminium by humans (Sorenson et al. 1974; Kruegar et al. 1984) have indicated that the daily intake is approximately 5mg (range 1-100mg). Metabolic balance studies by Greger and Baier (1983), have indicated that there was no retention of aluminium when the dietary intake was below 5 mg/day. Risks of aluminium toxicity in a normal environment therefore, appear to be limited. When the daily intake of aluminium reaches 125mg/day however, accumulation occurs. In uremics, urine aluminium content can be elevated to approximately 500µg/day, more than 10 times the aluminium which is excreted by control subjects (Kaehny et al. 1977; Recker et al. 1977; Gorsky et al., 1979). In chronic renal failure total body aluminium can markedly increase. Some patients undergoing dialysis with aluminium free dialysate have been reported to exhibit characteristic toxicity symptoms (see section 1.2.1) with high aluminium concentrations in the main tissue reservoirs, bone, heart, muscle and spleen (Fleming et al. 1982; Kaye, 1983; Sedman et al., 1984). It is thought that this toxicity is due to the intestinal absorption of oral aluminium formulations

administered to bind excess gut phosphates thus preventing hyperphosphataemia. Aluminium is also used as a constituent of antacids in the treatment of ulcers and other intestinal disorders; it is possible that continued use may lead to enhance aluminium absorption and possible neurotoxicity.

Since the removal of aluminium from the dialysate by haemofiltration methods, the absorption of aluminium from the gastrointestinal tract has been recognised and current interest is focused on determining levels and mechanisms of uptake.

1.4. ALUMINIUM AND THE GASTROINTESTINAL TRACT

1.4.1. THE GASTROINTESTINAL (GI) TRACT.

The previous section outlined the GI tract as an important route for aluminium entry. A knowledge of the physiological and morphological features of the GI tract, in particular the small intestine (SI) is needed in the understanding of absorptive processes.

The GI tract can be viewed as a cylindrical tract whose primary function is to supply the body with water, electrolytes and nutrients. The luminal surface of the tract, especially in the absorptive regions of the SI is highly convoluted to increase the surface area available for uptake. This area is further increased by villi, which project into the lumen with a density of between 10 and 40 per mm^2, (Moog, 1981). Each villus contains a network of blood and lymph vessels, present for the transport of nutrients, etc, after digestion. The villi are covered by an epithelial layer one cell thick.

Epithelial cells are of two types, goblet cells which secrete a protecting mucus fluid, and absorptive epithelial. It is the latter cells which carry out the functions of digestion and absorption. Typical absorptive cells, present at the tips of villi, have a characteristic tall columnar shape and a prominant brush border of microvilli. It is only in the upper luminal part of the cell that neighbouring cells are opposed and

connected. This connection, present immediately below the brush border is known as the junctional complex (Farquhar and Palade, 1963), and its main component is the tight junction (zona occludens). Tight junctions show degrees of 'leakyness' and in paracellular transport their ability to show varying degrees of openess is an important factor in the uptake of certain ions (Diamond, 1977).

The most distinctive feature of the absorptive cell is the striated microvillus border (brush border membrane, BBM), first resolved by electron microscopy in 1950 by Granger and Baker. In a mm^2 of human jejunum the microvilli are packed at a density of approximately 200000. With the resolution of the BBM, the human intestine is now known to have a surface area of $300M^2$ compared to the previously accepted $15M^2$ (Moog, 1981).

The actual site of absorption of water, electrolytes and nutrients is the membrane of the microvillus. Incorporated into the membrane are an array of enzymes and transport systems. Their purpose is to digest and transport the foodstuffs into the epithelial cell layer. These molecules have been observed by Yamada (1955) as delicate filaments radiating from the microvilli. Bennett (1963) found this coating to be polysaccharide rich, widespread and present on many cell surfaces. The general term 'glycocalyx' was proposed for this enteric surface coating present on the BBM of absorptive epithelial cells.

1.4.2. PROPOSED MECHANISMS FOR THE GI UPTAKE OF ALUMINIUM.

The sites and specific mechanisms for the absorption of aluminium have yet to be fully clarified. Preliminary work in experimental animals has indicated that the absorption of aluminium from rat jejunum may involve an energy-dependent, carrier-mediated mechanism (Feinroth et al. 1982). As a result of clinical observations by Clarkson et al., (1972) and Cam et al. (1976), Skalsky and Carchman (1983) have proposed a constant low level of aluminium absorption when the oral dose is less than 125mg/day. This may be indicative of active

transport occurring as urinary excretion of aluminium was constant over this low dose range. As the oral dose was increased over 125 mg/day urinary excretion of aluminium increased linearly with the concomitant increase in aluminium ingested. Over the threshold level of 125mg/day, Skalsky and Carchman suggested that aluminium in the GI tract was transported via passive routes to become the dominant mechanism. Although this two phase mechanism is an attractive hypothesis, its proof awaits the development of further data, as it was based only upon a compilation of human balance studies and not as the result of an *in situ* physiological experiment.

Van der Voet and de Wolff (1984), have developed an *in vivo* method of studying the intestinal absorption of aluminium where the small intestine was perfused in combination with a cannulated portal vein for serial blood sampling. The uptake pattern of aluminium in the small intestine (i.e. aluminium loss from the perfusate) did not wholly correspond with the appearance of aluminium in the portal blood. Although significant quantitative differences were found between uptake and transfer values (mg vs µg aluminium/unit time) this may simply reflect non-specif tissue binding which is not related to transport. The results of Van der Voet and de Wolff (1986) however, are in agreement with those of Feinroth et al. (1982), suggesting a two-step mechanism (mucosal uptake-serosal transfer) for intestinal aluminium absorption. Further studies by de Wolff (1987) have observed aluminium uptake to be concentration and pH dependent.

The actual site of aluminium absorption is a problem which has yet to be resolved. The solubility of $Al(OH)_3$ gel was found to be 1000 times more soluble at pH 4.2 than in the range 6.2-8.0 (Kaehny et al. 1977). This is due to the amphoteric nature of aluminium compounds in solution. Since aluminium would need to be in a soluble form for transport, Kaehny et al. implied that uptake might occur in the acid milieu of the stomach or proximal duodenum. Reduced levels of uptake would occur in the more distal regions of the small intestine as the pH reached neutrality and the aluminium compounds became more insoluble.

1.4.3. FACTORS AFFECTING THE GI ABSORPTION OF ALUMINIUM.

Renal tissue autopsy samples have been found to exceed the concentrations of aluminium which are normally absorbed by the GI tract. In renal failure it has been suggested that mechanisms exist to augment the uptake of aluminium by the intestine (Cannata et al. 1984).

Studies by Mayor et al. (1977) have shown parathyroid hormone (PTH) to increase tissue levels of aluminium after oral dosing. The effect of PTH on the tissue levels of aluminium may be controlled by increased circulating levels of active vitamin D metabolites. Drueke et al. (1985) found the oral administration of 1,25 $(OH)_2D_3$ to increase plasma aluminium levels in rats. It was suggested that aluminium uptake increases concomitantly with that of calcium. Feinroth et al. (1982) however, found aluminium transport to be lowered when buffer Ca^{2+} concentrations were raised. They proposed that Al^{3+} and Ca^{2+} were competing for the same transport mechanism. Cannata et al. (1984) suggested that aluminium uptake is mediated by a 'common pathway' of metal absorption which also may account for the uptake of iron, lead, cadmium and cobalt.

Slanina et al. (1984, 1986), have shown the addition of citric acid to oral aluminium increases aluminium levels in both bone and cerebral cortex in rats. In human studies the addition of citric acid to $Al(OH)_3$ significantly increases blood aluminium from 4µg/ml to 230µg/ml. This may be important as tablets (including aluminium binders) are normally taken with a beverage which may be a citrus based drink. The aluminium complex expected to cross the GI barrier would be the non-ionized aluminium-citrate species formed in the acidic regions of the stomach (Martin, 1986).

1.5 PROBLEMS ASSOCIATED WITH ALUMINIUM STUDIES

The elucidation of aspects of aluminium uptake and subsequent metabolism have been severely hampered by the lack of a suitable radioisotope. Clinically there have also been problems in the measurement of serum aluminium levels, an essential aspect in the diagnosis and treatment of patients with symptoms of aluminium toxicity (Adan et al. 1986). Aliquots of serum sent to different laboratories reported ranges from 2µg/l to 150µg/l.

A variety of techniques are employed to measure aluminium including atomic absorption (both flame and flameless), emission spectroscopy and neutron activation analysis. Unfortunately within these techniques there is a wide variation in the measurement of aluminium in serum samples (Cornelius and Schutyser, 1984; Berlyne and Adler, 1985).

A major problem in all measuring techniques is that of contamination. Aluminium is a constituent of laboratory glass and plastic ware and is also found in ultrapure acids. Aluminium is also known to adsorb onto the sides of glass and plastic containers, the losses of aluminium depending upon sample composition and the material of the container (Cornelius and Schutyser, 1984).

Graphite furnace atomic absorption spectroscopy (GFAAS) has become the most common method for measuring aluminium (Alfrey, 1983). Although it can provide routine aluminium measurements to below 1µg/l, (Cornelius and Schutyser, 1984) there are still numerous problems involved. Chemical interferences are unavoidable and care must be taken to ensure that samples and calibration standards are of a similar chemical composition (Berlyne and Adler, 1985). The acid digest system (Birch and Jenner, 1973) commonly used to solubilize tissues must be avoided because of the interference hydrochloric acid causes to the GFAAS system leading to a loss of signal (LeGendre et al. 1976). Other methods of sample preparation include a complex chelation by EDTA, analysis being performed on the resulting supernatant (Alfrey, 1983).

Although the clinical monitoring of aluminium is essential for the assessment of toxicity (Adan et al. 1986), it is obvious that research into mechanisms of absorption and metabolism would benefit from the use of a suitable analogue.

1.6. THE USE OF GALLIUM AND SCANDIUM AS MODELS FOR STUDYING ALUMINIUM UPTAKE AND DISTRIBUTION.

The problems of measuring aluminium in biological fluids has been documented in the previous section (1.5). The radioisotopes of other Group 3 members ^{67}Gallium (half-life 78 hours) and ^{46}Scandium (half-life 84 days) are commercially available; it is proposed because of their close chemical similarities (see sections 1.7 and 1.8) and femtomole levels of detection by γ-counting to use them as models in the study of aluminium uptake and distribution in the rat.

The advantages of using radioisotopes in biological studies are numerous. The major advantage is a complete lack of sample preparation in counting γ-emissions. The complex nature of sample preparation in the measurement of aluminium is probably the major factor in the huge variation in plasma aluminium levels observed (Cornelius and Schulyser, 1984; Adan et al. 1986). The use of radioisotopes also enables the differentiation between endogenous levels of trace metals and orally introduced exogenous levels. Other techniques of measurement such as GFAAS must include these 'background' levels although they could be subtracted after the measurement of suitable control values.

1.7. SOLUTION (HYDROLYSIS) CHEMISTRY OF ALUMINIUM, GALLIUM AND SCANDIUM.

All three trivalent elements exhibit similar hydrolysis chemistry in solution. They are primarily unhydrolysed below pH 2.5-3 to give the hexahydrated species (Baes and

Mesmer, 1976). From acidity to alkalinity all three elements follow a similar series of hydrolysis products (see equation 1.1).

(equation 1.1)

$$[Al(H_2O)_6]^{3+} \underset{H_3O^+}{\overset{OH^-}{\rightleftharpoons}} [Al(H_2O)_5(OH)]^{2+} \underset{H_3O^+}{\overset{OH^-}{\rightleftharpoons}} [Al(H_2O)_4(OH)_2]^{+}$$

$$\underset{H_3O^+}{\overset{OH^-}{\rightleftharpoons}} [Al(H_2O)_3(OH)_3]^{0} \underset{H_3O^+}{\overset{OH^-}{\rightleftharpoons}} [Al(H_2O)_2(OH)_4]^{-}$$

The mononuclear species $AlOH^{2+}$, $ScOH^{2+}$, $GaOH^{2+}$, $Al(OH)_4^-$, $Sc(OH)_4^-$ and $Ga(OH)_4^-$ are the most well established hydrolysis products although intermediary species have also been identified. The above species are formed rapidly and reversibly depending upon pH conditions. All three elements form polymeric species under conditions of supersaturation and standing at room temperature (see figure 1.1.). The species formed however, are not similar for the three elements. A summary of the hydrolysis products at 10^{-1}M and 10^{-5}M for aluminium, gallium and scandium is shown in figure 1.1.

1.8. SPECIATION CHEMISTRY OF ALUMINIUM, GALLIUM AND SCANDIUM.

Aluminium, gallium and scandium all have a common oxidation state of 3^+. Because the ions are highly charged their ionic radii is important when considering speciation chemistry (Al^{3+} - 0.54Å, Ga^{3+} - 0.62Å, Sc^{3+} - 0.82Å). In order to counteract the trivalent charge of the ion, it is thought that the ions will bind to anionic oxygen donor ligands, for example carboxylates, phosphates, nucleotides and polynucleotides (Martin, 1986). All three elements are shown to exhibit these

FIGURE 1.1. DISTRIBUTION OF HYDROLYSIS PRODUCTS WITH VARYING pH AND CONCENTRATION (REPRODUCED FROM BAES AND MESMER, 1976)

Figure 1.1A. Aluminium

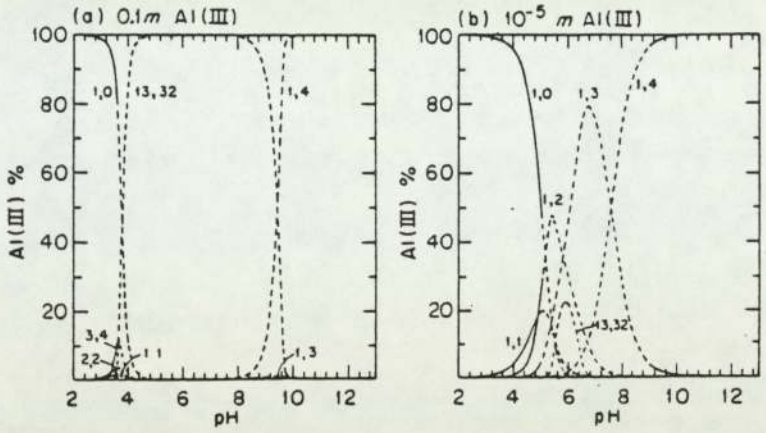


Figure 1.1B. Scandium

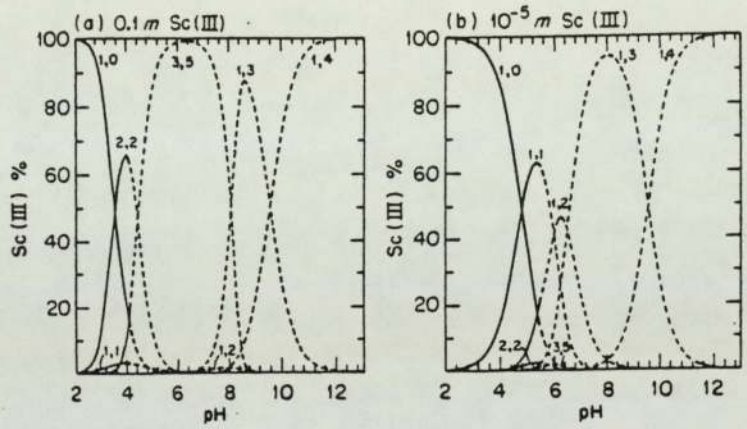
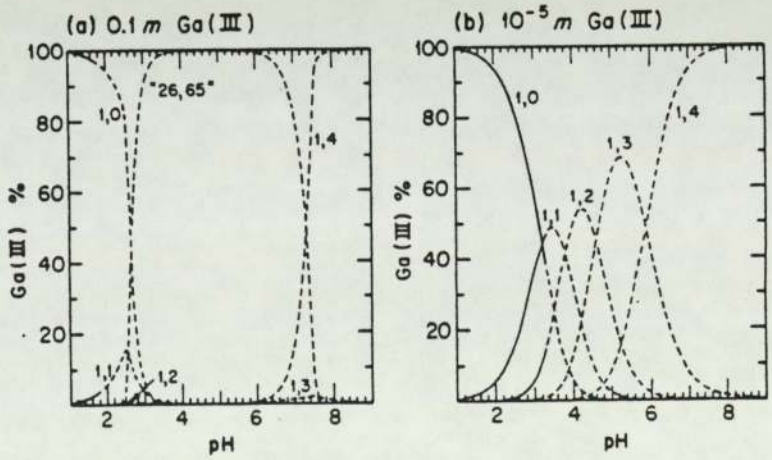


Figure 1.1C. Gallium



Notes.

The co-ordinates (e.g. 1,0; 1,2; 1,3; 1,4) in the above six diagrams indicates:
X = the number of aluminium, gallium or scandium ions in the complex;
Y = the number of hydroxides in the complex

For example, 1,0 = the unhydrolysed hexahydrated $[Al(H_2O)_6]^{3+}$ cation,

1,3 = the sparingly soluble $[Al(H_2O)_3(OH)_3]$ complex,

1,4 = the soluble anionic $[Al(H_2O)_2(OH)_4]^-$ complex.

See also equation 1.1. for full series of hydrolysis products.

characteristics although each have slightly different affinity constants for the ligand involved (Martell and Smith, 1982).

Most of the biological speciation chemistry of Group 3 elements is concerned with aluminium. The speciation of aluminium has been well documented in blood plasma. A binding to large molecular weight proteins, for example, albumin, globulins and tranferrins has been observed (Gardiner et al. 1984) using gel-filtration separation techniques. Cochran et al. (1984) suggested that the major large molecular weight protein was transferrin, since $Al^{3+}$and Fe^{3+} had similar ionic radii (Al^{3+} - 0.54Å, Fe^{3+} - 0.65Å) and that 70% of the available binding sites in transferrin were unoccupied in a normal situation. Although Al^{3+} cannot successfully displace Fe^{3+} from transferrin there was sufficient sites for the binding of both metal ions (Martin, 1986).

In addition to the protein-bound fraction in plasma there is evidence linking aluminium to a low-molecular weight complex which has not yet been identified (Gardiner et al. 1984). Possible suggestions as to the nature of this complex were low molecular weight oxygen donor ligands including phosphates, carbonates, oxalates, and citrates. Martin, (1986) considers citrate, because of its high concentration in plasma (0.1mmol/l), to be the major small molecule plasma binder. Aluminium has been observed to bindly strongly with fluoride, a common addition to many water supplies and tooth-paste preparations. There is a complex interaction of $Al(OH)_3$, F^-, PO_4^- and Ca^{2+} in the GI tract (Spencer et al. 1985) leading to bone fracturing in dialysis patients (as a result of high Al^{3+}, low Ca^{2+} and lowF^-).

The speciation chemistry of gallium is less well documented. In medicine it is primarily used as a cancer locating agent (Hoffer, 1980). Gallium has also been used as an analogue for aluminium in plasma binding studies, showing a similar transferrin binding characteristic (Cochran et al. 1983). Gallium has been observed to bind to sulfated acid mucopolysaccharides in stomach, small intestine, pancreas and muscle (Ando et al. 1985).

The biological chemistry of scandium has been little examined. As seen previously (section 1.7) its hydrolysis chemistry is similar to aluminium and it forms complexes with oxygen donor ligands (Venugopal and Luckey, 1978), as do aluminium and gallium.

1.9. AIMS.

The biological and clinical significance of aluminium has been previously mentioned (as have the difficulties in the quantification of aluminium) and it is obvious that further studies investigating aluminium absorption and metabolism are required. This thesis therefore proposes to investigate:

1. The suitability of gallium and scandium as physiological markers for aluminium.

2. The tissue distribution of gallium, scandium and aluminium after oral administration in fed and fasted rats.

3. The effects of citrate, maltol and fluoride (examples of food and water additives potentially affecting the bioavailability of aluminium) on the tissue distribution of gallium after oral administration in fed and fasted rats.

4. The kinetics of gallium, scandium and aluminium absorption in *in-vitro* everted sacs.

5. The effects of the above additives on the *in-vitro* intestinal absorption of gallium.

6. The *in-vivo* absorption of gallium using a jejunal perfusion system.

7. The effects the above additives on the *in-vivo* intestinal absorption of gallium.

8. The speciation of gallium within the lumen of the small intestine following oral administration and the nature of transportable species (i.e. blood-bound).

CHAPTER TWO

TISSUE DISTRIBUTION OF ALUMINIUM, GALLIUM AND SCANDIUM AFTER ORAL ADMINISTRATION

CHAPTER 2. TISSUE DISTRIBUTION OF ALUMINIUM, GALLIUM AND SCANDIUM AFTER ORAL ADMINISTRATION.

2.1. INTRODUCTION

The aim of this section of experiments is to establish whether the radioisotopes ^{67}Ga and ^{46}Sc could be utilized as suitable markers for aluminium. Tissue distribution experiments, both acute and chronic were performed to see if there were any similarities between levels of uptake and regions of organ distribution after the oral administration of aluminium, gallium or scandium.

No Group 3 metals have been found to be essential in animal or human nutrition. There are no reports either of the existence of any homeostatic mechanisms which regulate levels of these metals in mammals.

It was previously thought that aluminium, gallium and scandium were not absorbed from the gastrointestinal tract after oral administration (Campbell et al. 1957; Browning, 1969). Although the majority ($\approx$ 99%) of aluminium, gallium or scandium form highly insoluble hydroxide and phosphate complexes, and are thus rendered unavailable for absorption, a small proportion is transported into the body. In the case of aluminium, it is possible that this small percentage of absorbed aluminium is responsible for the neurotoxicity observed in dialysis dementia or senile dementia of the Alzheimer type.

Levels of absorption depend upon the form of the Group 3 salt administered (Yokel et al., 1988; Venugopal and Luckey, 1978), increased absorption of aluminium and gallium being observed when administered as the lactate or citrate form.

The toxicity of aluminium, gallium or scandium depends upon the mode of entry. Toxicity is least observed when all Group 3 elements are orally introduced emphasizing the extent of the gastrointestinal tract in acting as a barrier to prevent the effective absorption of the metal ions in question.

Tissue distribution measurements of aluminium have been made in both humans and animals. Skalsky and Carchman (1983) have noted the relative accumulative properties in certain organs. Bone and lung both showed tendencies to accumulate aluminium, whilst muscle and skin contained aluminium in proportion to the weight of that tissue. Fat tissue (adipose), blood, brain and liver retained aluminium in proportions which were lower than their relative organ weights, (table 2.1).

TABLE 2.1. ALUMINIUM DISTRIBUTION IN MAN.

Tissue	Total Al(mg) in tissue	Al/mg Kg	% Distribution in 70Kg man	
			Organ	Aluminium
Adipose	5.20	0.35	21	2
Bone	116.70	11.67	14	40
Muscle	118.20	4.22	40	41
Blood	2.14	0.39	8	1
Skin	10.06	3.87	3	3
Brain	0.47	0.336	2	1
Liver	3.64	2.02	2	1
Lungs	35.35	35.35	1	12

Data reproduced from Skalsky and Carchman (1983).

Group 3 elements are particularly known to be 'bone seekers' because of their phosphate binding capacities (Venugopal and Luckey, 1978). The surface of bone has a hydrated structure and substances present in the surrounding body fluids are able to freely move between the two media. In particular instances where there is phosphate depletion, it is possible that there is increased aluminium

deposition (Spencer and Lender, 1979), leading to imbalances in the mineralisation processes (Talwar et al. 1986).

The lung has the highest levels of aluminium content per unit weight of tissue of any organ measured (Skalsky and Carchman, 1983). This is probably due to the ubiquitous nature of aluminium in the environment. There is little evidence to suggest that inhaled aluminium is actually absorbed into the body although toxicity has been observed in one case (McLaughlin, 1962). Initially in this study aluminium, gallium and scandium levels were measured in the lung, however the results were wide ranging and inconsistent and the measurements discontinued.

2.1.2. CITRATE, MALTOL AND FLUORIDE.

The effects upon gallium bioavailability of three dietary ligands citrate, maltol and fluoride were also investigated. If dietary ligands are observed to complex with aluminium, there could be far reaching neurotoxicological implications if the bioavailability of aluminium is increased or if aluiminium is transported as a novel species within the body.

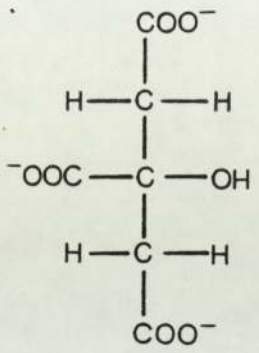
Citric acid is used as an acidity regulator in the manufacture of confectionery, fruit juices, ice-cream, marmalade and jelly. It is also used to retard the browning (oxidation) process in fruits and vegetables. Production of citric acid is based upon the fermentation of molasses by the microbe *Aspergillus niger* (Belitz and Grosch, 1987).

The highly charged Al^{3+} cation seeks oxygen donor ligands and the citrate ion provides these binding sites (figure 2.1). Citrate is present in blood plasma at a concentration of 0.1mmol per litre, a binding of aluminium to plasma citrate is possible (see chapter 5 for further details).

Maltol (3-hydroxy-2-methyl-4H-pyran-4-one) has a caramel like-odour. It is generally used as a flavour enhancer in carbohydrate rich food e.g. baked goods, marmalades and fruit jellies (Coultate, 1984; Belitz and Grosch, 1987). Maltol

FIGURE 2.1. STRUCTURE OF CITRATE AND MALTOL.

FIGURE 2.1A. CITRATE



2.1B ALUMINIUM-CITRATE

No one aluminium-citrate complex has been identified. The complexes formed are thought to be both concentration and pH dependent.
Suggested aluminium-citrate complexes in the pH range 3-8 and at physiological concentrations are $Al_3(OH)_4Cit_3$, AlCit and AlHCit (Slanina et. al., 1986)

FIGURE 2.1C. MALTOL

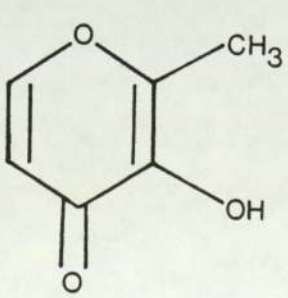
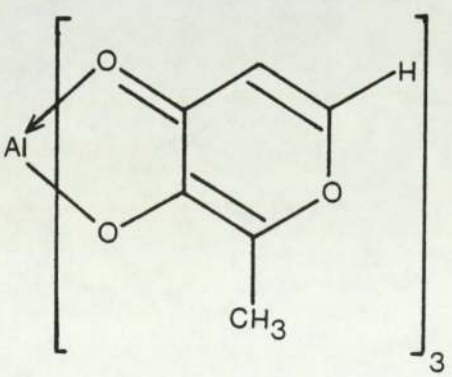


FIGURE 2.1D. ALUMINIUM-MALTOL COMPLEX



is formed by the cyclization of dicarbonyl compounds produced during caramelization reactions.

Both gallium and aluminium form complexes with maltol (figure 2.1). The complexes are formed in a 3:1 maltol to metal ratio and are stable over the pH range 4-9. They are thought to be resistant to hydrolysis and to be neutral and lipid soluble. The aluminium-maltol complex was considered to be unusually neurotoxic as it has been suggested to traverse the blood-brain barrier intact (Finnegan et al. 1986, 1987).

Aluminium complexes readily with fluoride producing a series of aluminium fluoride species (AlF^{2+}, AlF_2^{+}, AlF_3° and AlF_4^{-}) depending upon the concentration of uncomplexed fluoride. The insoluble AlF_3° species exists significantly between 0.02 to 5mM of free fluoride per litre (Martin, 1986). A similar series of species is expected to form with gallium.

Fluoride is an essential mineral, the adult human body containing approximately 2.6g (Belitz and Grosch, 1987). Fluoride is added to many household water supplies in the form of sodium fluoride at average concentrations of between 0.5-1.5ppm. The fluoridation of drinking water is however, a controversial issue **(Sticht, 1988).**

Advantages of fluoride addition include the prevention of tooth decay (caries) and the treatment of osteoporosis. Fluoride is thought to slow the process of tooth enamel solubilization and also inhibit bacteriological activity causing tooth decay (Belitz and Grosch, 1987). Osteoporosis is a gradual bone loss with age, primarily affecting older women (Spencer and Kramer, 1985). Bone demineralization occurs gradually over several decades. Fluoride is thought to decrease the process of bone resorption and to stabilize bone crystal.

Toxicity in the form of fluorosis (minor poisoning symptoms include abdominal pain, nausea, vomiting, diarrhea; severe poisoning symptoms include

hyperactivity, convulsions, respiratory and cardiac failure, Sticht, 1988) is thought to occur when water fluoride is over 2ppm.

2.2. MATERIALS AND METHODS

2.2.1. ANIMALS

Adult male Wistars (average weight 180-200g) were purchased from Bantin & Kingman (Hull, UK) and used throughout these investigations. Animals were housed in the University animal house at 20°C and maintained on Rat and Mouse Breeding Diet (Code 422), (Pilsbury's Limited, Birmingham, UK) with water *ad libitum*.

Where required, animals to be fasted were placed 18 hours before experimentation in cages with stainless steel wire grid bottoms. Access to tap water was allowed.

2.2.2. CHEMICALS

Analar grade reagents were supplied by BDH, Poole, UK; Sigma, Poole, UK; Aldrich, Gillingham, UK; Fisons, Loughborough, UK; Pharmacia, Milton Keynes, UK. Medical grade 95%O_2/5%CO_2 was supplied by the British Oxygen Company. Radioisotopes were purchased from Amersham International, Amersham, UK and New England Nuclear, Dupont, Boston, USA.

2.2.3. DOSING REGIMES

2.2.3.1. ACUTE DOSING

Animals either fed or fasted were lightly restrained using ether anaesthesia. O.3mls of the appropriate Al, Ga or Sc solution (see individual results tables for exact composition of dosing solution) was delivered to the stomach of the rat via a blunt curved needle attached to a hypodermic syringe. The animals were stunned and then killed by cervical dislocation 4 hours after oral dosing. The major internal

organs, the femur and a portion of skeletal muscle were removed. Ga and Sc concentrations were assessed by counting the γ-emissions of the radioisotopes ^{67}Ga and ^{46}Sc (see section 2.2.4, Radioisotopes). Tissue aluminium was measured using graphite furnace atomic absorption spectroscopy (see section 2.2.5). Uptake in the tissues was expressed as a percentage of the total dose administered (%TDA).The results included blood bound aluminium, gallium or scandium in all of the examined tissues.

2.2.3.2. CHRONIC DOSING REGIMES.

Experimental rats were acclimatised in Jencons Metabowls (Jencons Ltd. Leighton Buzzard, UK) until their daily faecal and urinary output was constant (daily urine release 11-15cm^3; daily faecal mass 9-15g; the Harvard Bioscience Whole Rat Catologue, 1983). Water and food was available *ad libitum*. Animals were divided into two groups. Both groups were dosed orally with 0.3mls 10^{-6}M $ScCl_3$ containing 10μCi ^{46}Sc for 12 days. Faeces and urine were collected daily and counted for ^{46}Sc content. 4 hours after the last oral dose the first group of animals were sacrificed and tissues counted for ^{46}Sc. The second group of rats received no more Sc after the 12th day, but their faecal and urinary output of ^{46}Sc was measured daily until the isotope content was negligible. 5 days after the last oral dose, on the 17th day of the experiment the rats were killed and the tissues counted for ^{46}Sc activity.

2.2.4. RADIOISOTOPES

^{67}Ga was supplied as carrier free $GaCl_3$ in 0.04M HCl, ^{46}Sc as $ScCl_3$ in 0.04M HCl (Amersham International, Amersham, UK).

The amount of ^{67}Ga or ^{46}Sc in tissues and fluids was assessed by measuring gamma emissions in an LKB Compugamma counter (LKB instruments, Milton

Keynes, UK). The isotopes were counted within the following energy ranges: ^{67}Ga, 50-420 KeV; ^{46}Sc, 350-1400 KeV. All samples were counted for a period of 60 seconds and counts were automatically corrected for radioactive decay.

2.2.5. MEASUREMENT OF ALUMINIUM BY GRAPHITE FURNACE ATOMIC ABSORPTION SPECTROSCOPY.

Animals were killed and the organs removed as described previously (2.2.3.1). The organs were freeze dried for 18 hours and then powdered to a homogenous mixture. 50mg of powdered tissue was added to 500µl of 1% EDTA in Analar water

(BDH, Poole, Dorset, UK) in a plastic microreaction vial and left overnight to chelate the aluminium. The vial was then centrifuged in a Beckman constant speed microfuge for 5 minutes and the supernatant removed. Aluminium standards (10-1000µg/ml) were prepared from a 1mg/l stock (BDH spectroscopy standards). All standards and sample solutions were matrix-matched with the addition of Triton X-100 surfactant (to a final volume of 0.5%) (Alfrey, 1983).

The concentration of aluminium in the supernatant was determined using a Perkin Elmer 560 atomic absorption spectrophotometer with a Perkin Elmer HGA 500 graphite furnace (Perkin Elmer Ltd. Beaconsfield, Bucks). All samples were analyzed in triplicate using non-pyrolysed graphite tubes and the program detailed in table 2.1.

TABLE 2.2. PROGRAM FOR THE DETERMINATION OF ALUMINIUM BY GFAAS.

Step Number	Temp. (^{O}C)	Ramp time (sec)	Hold time (sec)	Internal gas flow(ml Argon/min)
1	100	10	30	300
2	1550	10	55	300
3	2650	1	8	50
4	2700	1	5	50

-sample volume = 20µl

Uptake into the tissue was expressed as a % TDA. Unlike Ga or Sc the amount of aluminium in the tissue reflects both endogenous aluminium and the increase in aluminium concentration seen after oral dosing. The %TDA therefore will be an overestimation of aluminium uptake.

2.2.6. STATISTICAL ANALYSIS

For all statistical analysis (including two tailed unpaired t-tests, one-tailed t-test and one-way analysis of variance) see appendix 1. This includes statistics from this chapter and the other results chapter.The majority of results were expressed as the mean of 5 observations ± the standard error of the mean.

2.3. RESULTS AND DISCUSSION.

2.3.1. COMPARISONS BETWEEN THE SHORT-TERM TISSUE DISTRIBUTION OF ALUMINIUM, GALLIUM AND SCANDIUM AFTER ORAL ADMINISTRATION.

Table 2.3 compares the absorption of aluminium, gallium and scandium in fed and fasted animals. The values are not absolute levels of absorption being only the sum of liver, kidney, spleen, heart, brain, femur and muscle activities. Although total uptake from the gastrointestinal tract has not been measured, the values are useful as a comparative measure. The aluminium values will be an over-estimation of aluminium in the tissues after oral administration, because of the presence of endogenous aluminium. These 'background' levels of aluminium were not estimated because of difficulties in the analytical procedure (see 1.5). The presence of endogenous gallium or scandium does not arise; the use of radiotracers allows only the measurement of absorbed gallium or scandium in the tissues and furthermore only minute quantities (<0.01ppm) of the elements have been detected in organs (Bedford 1960; Lievens et al., 1976).

The results of table 2.3 indicate the importance of nutritional status in determining levels of uptake. In aluminium, gallium and scandium experiments, significantly greater amounts of the element are absorbed in the fasted state when compared to the fed. The reasons for the decreased absorption in the fed state (presence of food material to physically block uptake, chelation of aluminium, gallium or scandium by dietary components, reduction in concentration of the soluble 'free' hydrated ion, formation of non-absorbable metallo-protein complexes) are examined more fully in the discussion on speciation and bioavailability of aluminium and gallium (see chapters 5 & 6).

The low levels of permeation (<1% of the total dose administered) have been reported by other workers (Dudley et al. 1950; Luckey et al. 1977; Yokel et al. 1988).

TABLE 2.3. THE INTESTINAL ABSORPTION OF ALUMINIUM, GALLIUM AND SCANDIUM IN FED AND FASTED RATS (n=5, ±SEM).

DOSE (0.3mls)	FED RATS (%TDA)	FASTED RATS (%TDA)
$Al(OH)_3$ (10^{-2}M)	0.276 (±0.064)	0.7798 (±0.161)*
$Ga(NO_3)_3$ (10^{-3}M)	0.044 (±0.0035)	0.2445 (±0.0342)***
$ScCl_3$ (10^{-3}M)	0.0369 (±0.0026)	0.0447 (±0.0014)*

Notes.
1).The above values represent the sum of aluminium, gallium and scandium identified in the liver, kidney, spleen, heart, brain, femur and muscle (i.e. a percentage of the total dose administered, %TDA).
Although the above values do not represent an absolute measure of intestinal absorption, they are useful as a comparative measure.

2). *P<5%, **P<1%, ***P<0.1%, indicates a significant increase in the fasted values compared to the fed.

Scandium has been particularly noted to be poorly absorbed (<0.05% of an administered dose) because of the ready formation of colloidal forms of $Sc(0H_3)$ in the lumen of the gastrointestinal tract. The absorption of scandium is so minute that radiolabelled Sc_2O_3 has been used as a nutritional marker (Luckey et al. 1977).

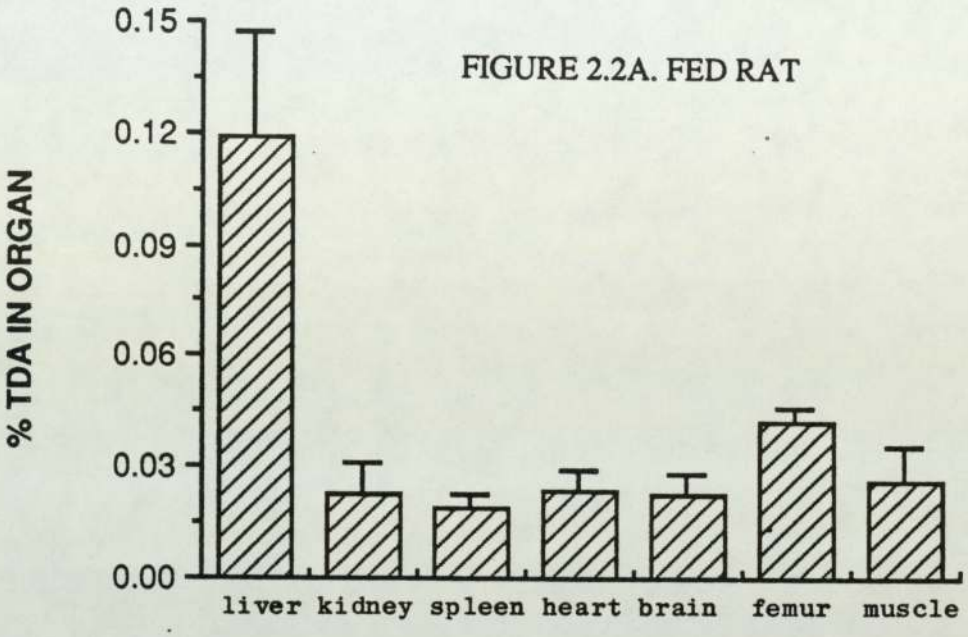
The absolute organ levels of aluminium, gallium and scandium are expressed in histograms (figures 2.2a - 2.4b). In all three elements measured, significantly more activity was observed in the livers and femurs of fasted rats when compared to fed. The greatest concentrations of aluminium and gallium were observed in liver (figures 2.2 and 2.3), whilst after scandium administration, the greatest concentrations were observed in muscle (figure 2.4). It is difficult to gauge a measure of tissue distribution and to compare and contrast between aluminium, gallium and scandium distributions because of the difference in weights of the organs measured. If the results are converted to a unit weight, then an idea of the relative accumulating ability (or alternatively depleting ability) of a tissue can be judged. These results, expressed for both chronic and acute experiments are discussed in section 2.3.3.

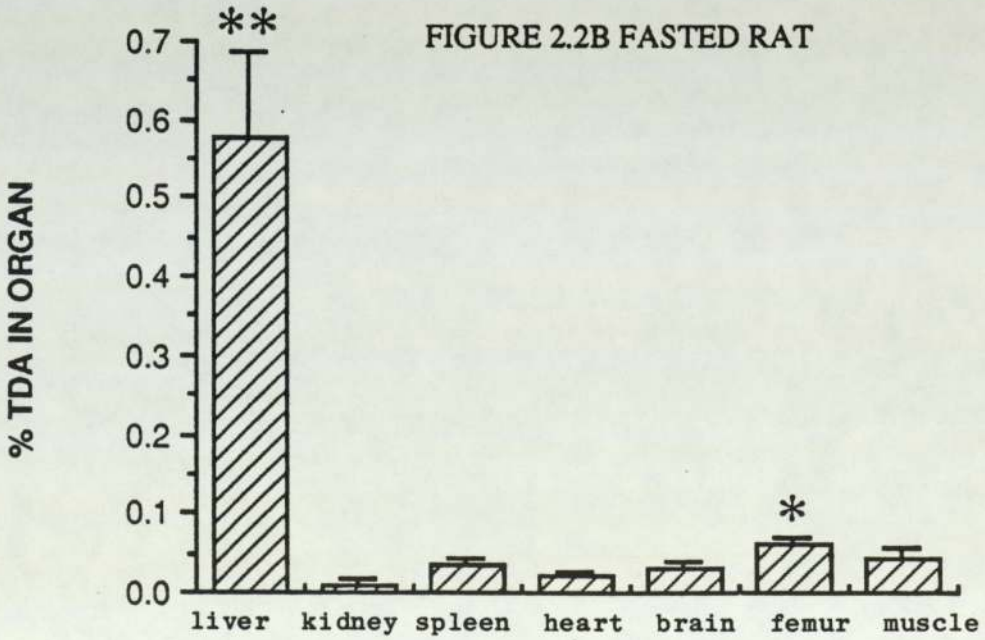
2.3.2. TISSUE DISTRIBUTION OF SCANDIUM AFTER CHRONIC (12 DAY) DOSING PERIOD.

A). Chronic dosing experiments.

Significantly more scandium was observed in all tissues examined, except spleen after a chronic dosing period of 12 days when compared to the four hour acute period (figure 2.5). In these results greater amounts of scandium were observed than if the acute results were summed for the 12 day dosing period. It appears therefore that an accumulative mechanism is occurring. In other chronic experiments with aluminium, Greger et al. (1986) observed an increase in aluminium content in bone, kidney and muscle. Other tissues however, e.g.liver,

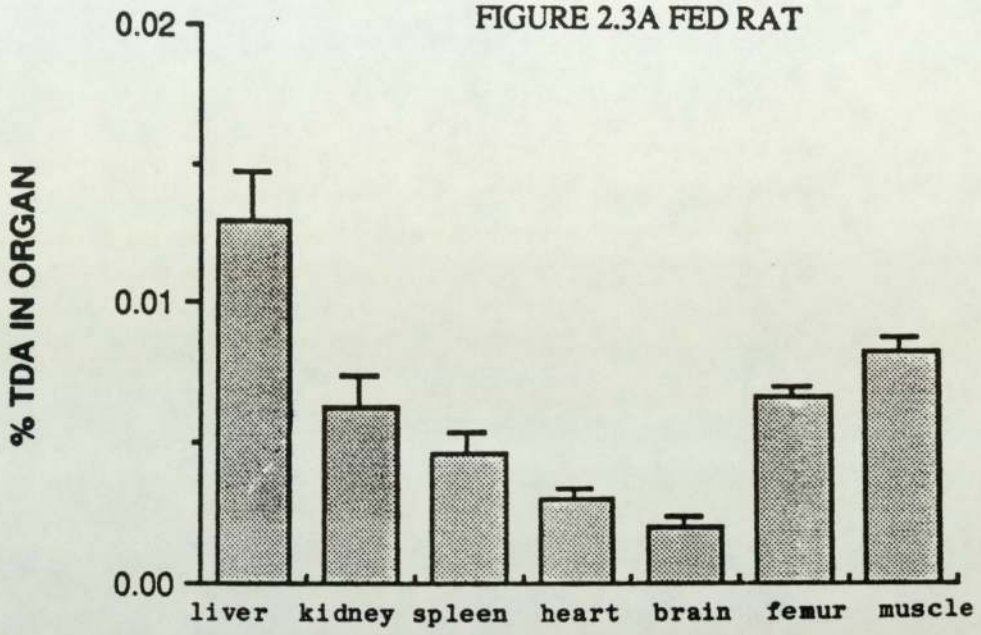
FIGURE 2.2. TISSUE DISTRIBUTION OF ALUMINIUM 4 HOURS AFTER THE ORAL ADMINISTRATION OF 0.3mls 10^{-2}M $Al(OH)_3$.

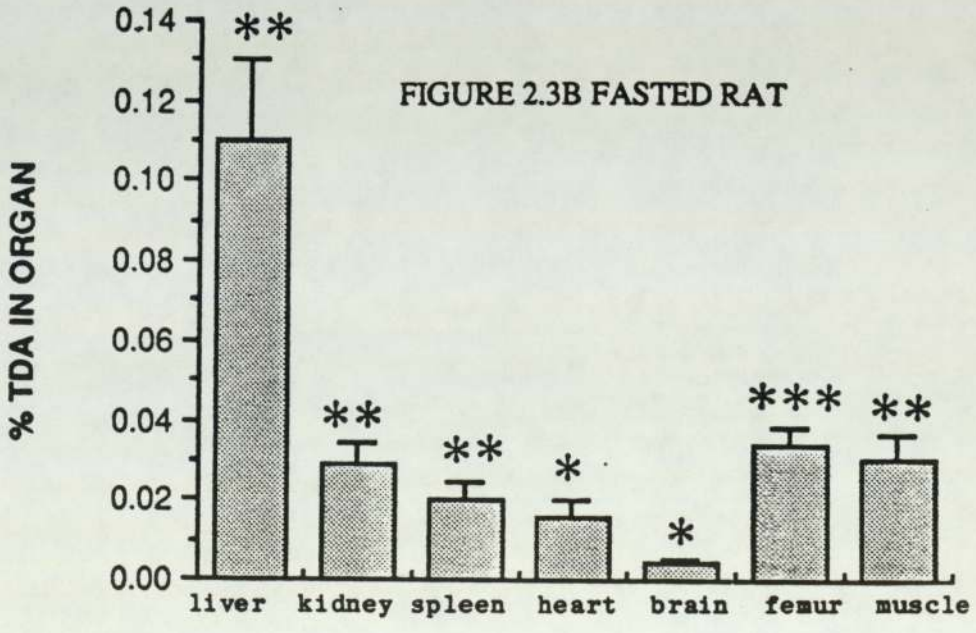




Notes.
1). *P<5%, **P<1% indicates a significant increase in aluminium identified in fasted tissues when compared to fed (n=5, ±SEM).
2) Results expressed as a percentage of the total dose administered (aluminium results include a proportion of endogenous aluminium) (% TDA)

FIGURE 2.3. TISSUE DISTRIBUTION OF GALLIUM 4 HOURS AFTER THE ORAL ADMINISTRATION OF 0.3mls 10^{-3}M $Ga(NO_3)_3$ + 10μCi ^{67}Ga





Notes.
1). *P<5%, **P<1%, ***P<0.1% indicates a significant increase in gallium identified in fasted tissues when compared to fed (n=5, ±SEM).
2). Results expressed as a percentage of the total dose administered (% TDA)

FIGURE 2.4. TISSUE DISTRIBUTION OF SCANDIUM 4 HOURS AFTER THE ORAL ADMINISTRATION OF 0.3 mls 10^{-3}M $ScCl_3$ +10μCi ^{46}Sc.

FIGURE 2.4A. FED RAT

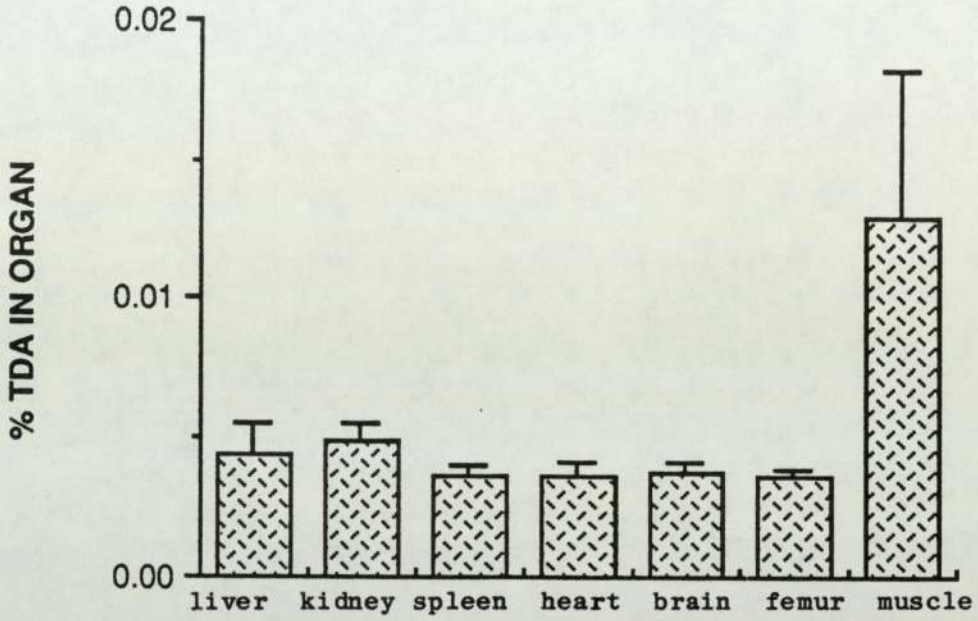
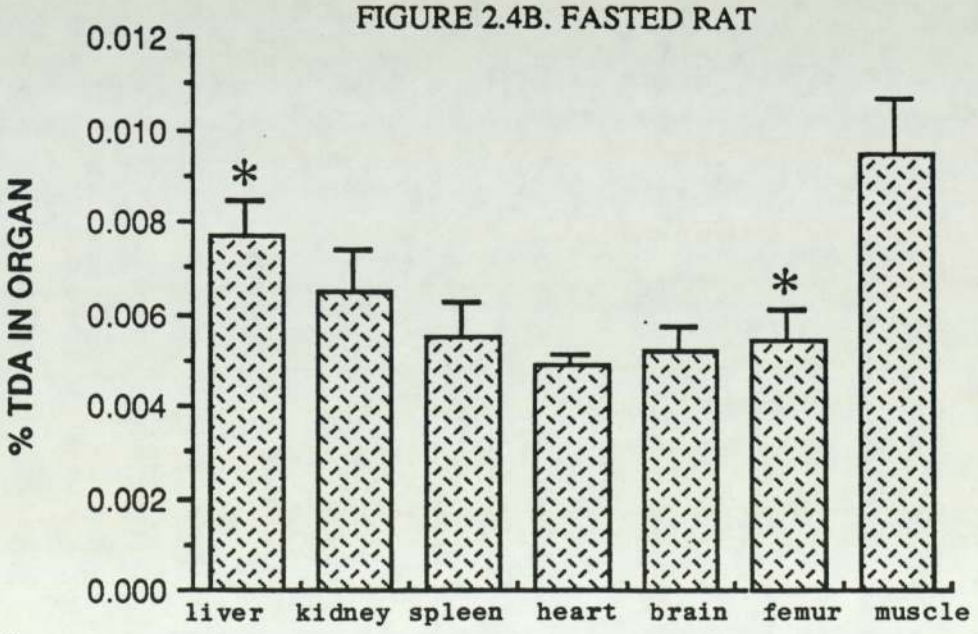
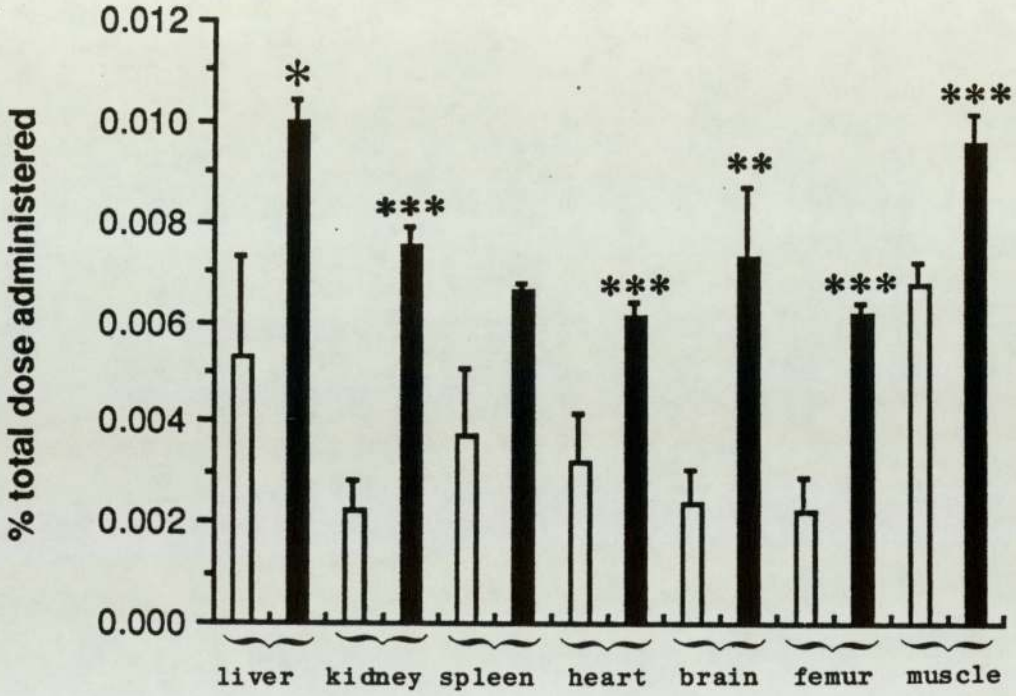


FIGURE 2.4B. FASTED RAT



Notes.
1). *P<5% indicates a significant increase in scandium identified in fasted tissues when compared to fed (spleen, heart and brain significant at P<10%) (n=5, ±SEM).
2). Results expressed as a percentage of the total dose administered (% TDA).

FIGURE 2.5. COMPARISON BETWEEN ACUTE (4 HOUR) AND CHRONIC (12 DAY) SCANDIUM TISSUE DISTRIBUTION AFTER ORAL ADMINISTRATION OF 0.3mls 10^{-6}M $ScCl_3$ + 10μCi ^{46}Sc.



Notes.
1). *P<5%, **P<1%, ***P<0.1%, indicates a significant increase in scandium activity in chronically dosed animals compared to acutely dosed animals (n=5, ±SEM).
2). Results expressed as percentage of total dose administered.
3). White bars = acute 4 hour dosing.
Black bars = chronic 12 day dosing.

spleen, heart and brain were not examined in this study. Mayor et al. (1977) have also noted an increase in aluminium content in bone after long-term aluminium administration.

Table 2.4 indicates that after 17 days (5 days after the last oral dose) scandium was found in liver, kidney, spleen, muscle and femur. It was not detected at all in heart and brain. The levels of scandium in liver, kidney and spleen were significantly lower than at the end of the 12 day dosing period, suggesting that scandium was excreted from the tissue reservoirs in these organs. If the experiment had continued beyond 17 days it would be likely that scandium would have been completely excreted as had been observed with heart and brain tissue. In femur and muscle however, there was no significant difference between the levels of scandium at 17 or 12 days suggesting a possible accumulation or retention mechanism.

These results may be explained by considering the work of Triplett et al. (1985), who assessed the tissue accumulation of gallium. Tissues were differentiated into 'vascular' or 'non-vascular'; the vascular tissues, such as liver, heart, spleen and kidney accumulating gallium rapidly because of their high proportion of blood vessels (up to 20% w/w blood in these tissues). In comparison muscle tissue contain only 1.5% w/w blood in the wet tissue.

At the end of the 17 day experiments scandium was not detected in heart or brain suggesting these tissues had good elimination mechanisms. Heart tissue, being a good example of Tripletts vascular type would have a continuous blood supply to remove any accumulated scandium after the discontinuation of further doses. Significantly lower levels were detected in liver, kidney and spleen and again the vascular nature of these tissues may account for the reduced levels of scandium identified.

The elimination of scandium from the brain is of particular importance. Although not a vascular type tissue no scandium was detected after the 5 day washout period. Preliminary encephalopathic symptoms have been reversed when the oral

TABLE 2.4. SCANDIUM ACTIVITY IN RATS DOSED DAILY FOR 12 DAYS AND IN RATS 5 DAYS AFTER THE 12TH DOSE (N=5, ±SEM).

	12 DAY RATS (%TDA)	12 + 5 DAY RATS (%TDA)
LIVER	0.010 ±0.00047	0.0042 ±0.00056***
KIDNEY	0.0075 ±0.00038	0.0047 ±0.0011*
SPLEEN	0.0066±0.00016	0.0037 ±0.0009*
HEART	0.0061 ±0.00032	not detected
BRAIN	0.0073 ±0.0014	not detected
FEMUR	0.0062 ±0.00024	0.0045 ±0.00092
MUSCLE	0.0096 ±0.00064	0.0070 ±0.0024

Notes.
1). *P<5%, ***P<0.1% indicates a significant decrease in tissue scandium activity after 5 day 'washout' period in liver, kidney and spleen.

2). All results expressed as a percentage of the total dose administered (%TDA).

3). Scandium was not detected in heart or brain after 5 day 'washout' period.

4). Muscle sample contains both Type I and II fibres.

administration of aluminium is discontinued suggesting a similar washout of aluminium from the brain as was observed with scandium (Masselot et al.1978).

The retention of scandium in bone confirms the observations of previous workers (Venugopal and Luckey, 1978). Although bone is a vascular type tissue, containing 12% w/w blood in tissue (and therefore would not be expected to retain scandium), Group 3 elements are characteristically thought to be 'bone seekers'. Aluminium in renal disease accumulates in bone (Alfrey, 1983). In over two thirds of dialysis dementia patients symptoms of osteomalacia are observed; aluminium thought to be replacing Ca^{2+} in the bone mineralisation process.

The retention of scandium in muscle tissue may be due to the less vascular nature of the tissue. If accumulation occurs over the 12 day period, subsequent elimination may be slow because of the low levels of blood (only 1.5% w/w blood in the tissue) circulating through the muscle.

B) Balance study experiments.

The above mentioned chronic experiments were performed in Metabowls and allowed the daily monitoring of scandium content in faeces (figures 2.6a &b). After several days of administering scandium a plateau was reached whereby the daily faecal output was approximately equal to the daily administered dose. 48hours after the last oral dose, the scandium content in faeces was considerably lower (figure 2.6a). A small plateau however, was observed in the 5 day 'washout' period (figure 2.6b). this continued faecal excretion of scandium appears to represent a small residual turnover. Venugopal and Luckey (1978) have suggested an enterohepatic circulation for aluminium, and it may be possible that the same occurs with scandium.

Urine content of scandium was also measured daily over the 17 day period. The pattern of scandium excretion was similar to that of faecal excretion, except for the extremely low levels detected (never greater than 0.3% of the daily dose). This

FIGURE 2.6. MEASUREMENT OF DAILY FAECAL OUTPUT OF SCANDIUM.

FIGURE 2.6A. SCANDIUM OUTPUT OVER 17 DAYS.

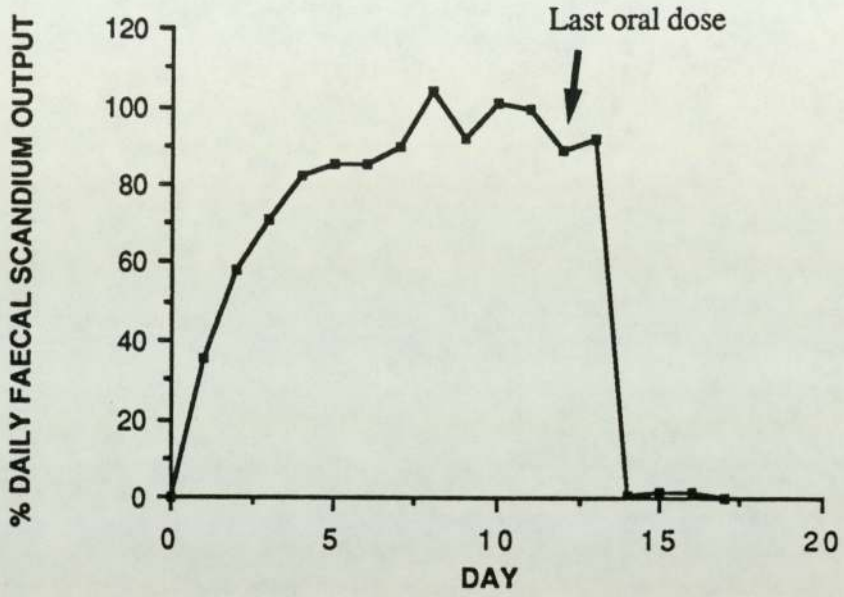
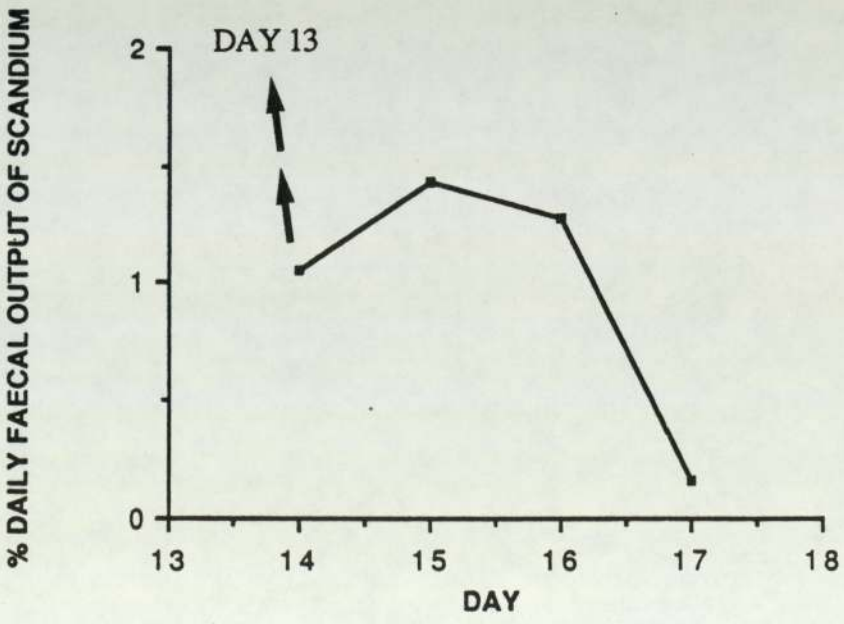


FIGURE 2.6B. ENLARGEMENT OF DAYS 13-17.



reflects the low percentage of the dose moving across the gut into the body similarly noted by Venugopal and Luckey (1978).

In order to explain the small residual plateau observed in the 5 day discontinuation period, rats were parenterally administered with 0.3mls 10^{-6}M $ScCl_3$ + 5μCi ^{46}Sc and kept in Metabowls for 4 days. Daily faecal and urine output were monitored for ^{46}Sc content. Scandium output was highest on the 3rd day, faecal output being 14% of the administered dose. Urinary output containing less than 0.5% of the dose. It is therefore possible that the presence of scandium in the faeces after intraperitoneal injection (and also as a small residual plateau after the discontinuation of scandium dosing) was due to an enterohepatic circulation (i.e. reentry/entry into the gut via the bile duct).

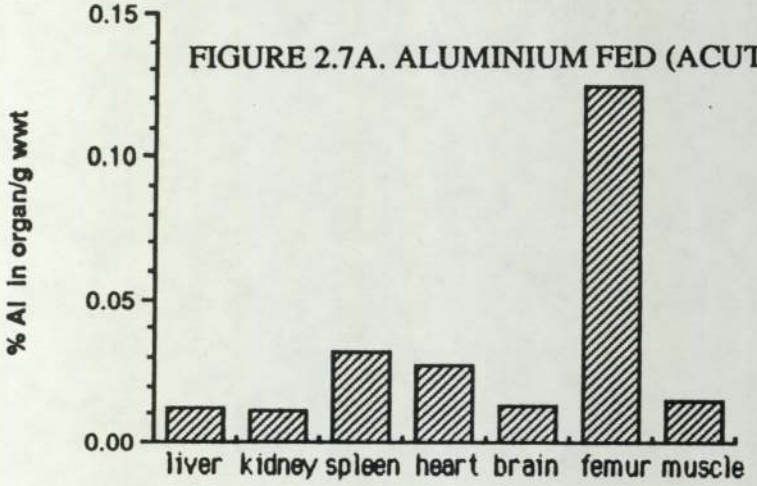
Secretory processes by intestinal epithelia are not thought to occur because of the lack of importance of aluminium in physiological processes. More important trace elements such as zinc, copper, and magnesium are known to have homeostatic mechanisms which regulate their turnover in the body. These include intestinal secretory processes (Underwood, 1977). Ligation of the bile duct (not done in this thesis) after both the oral and parenteral administration of scandium would confirm the existance of an enterohepatic circulatory system.

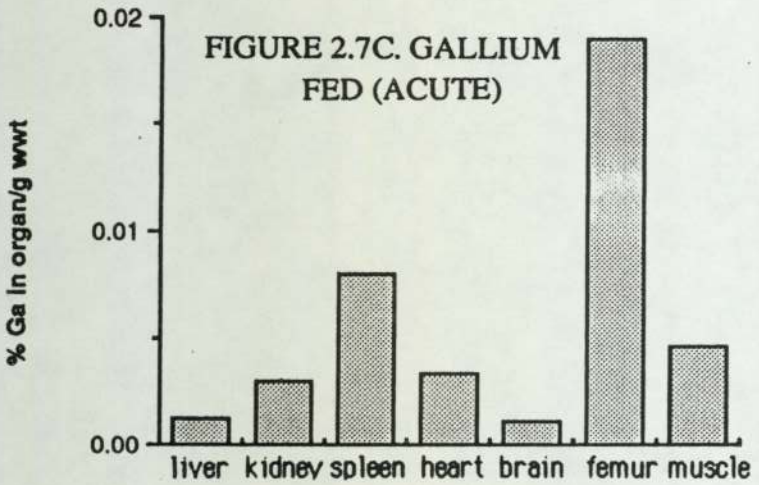
2.3.3. CONVERSION OF ACUTE AND CHRONIC ALUMINIUM, GALLIUM AND SCANDIUM TISSUE LEVELS TO UNIT WEIGHTS.

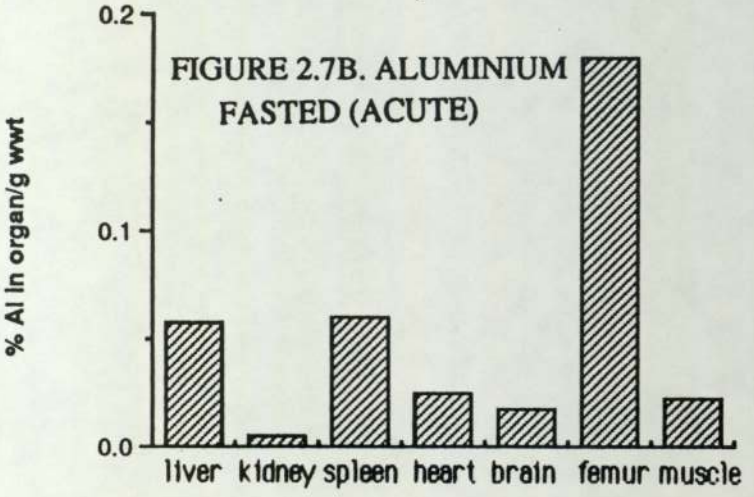
If the results from figures 2.2, 2.3, 2.4, and 2.5 are reexpressed from metal content in whole organ to metal content per gram wet weight of tissue, a direct comparison of tissue mass and aluminium, gallium, or scandium content can be made.

The most immediate point to note is the accumulation of aluminium, gallium and scandium in the femur of the rat after both chronic (scandium only) and acute dosing (figures 2.7 & 2.8). The retention of Group 3 metals in skeletal tissue is a characteristic which has been previously noted by Dudley and Levine, 1949;

FIGURE 2.7. CONVERSION OF ALUMINIUM AND GALLIUM ABSOLUTE ORGAN LEVELS TO ACTIVITY PER UNIT WEIGHT (n=5).







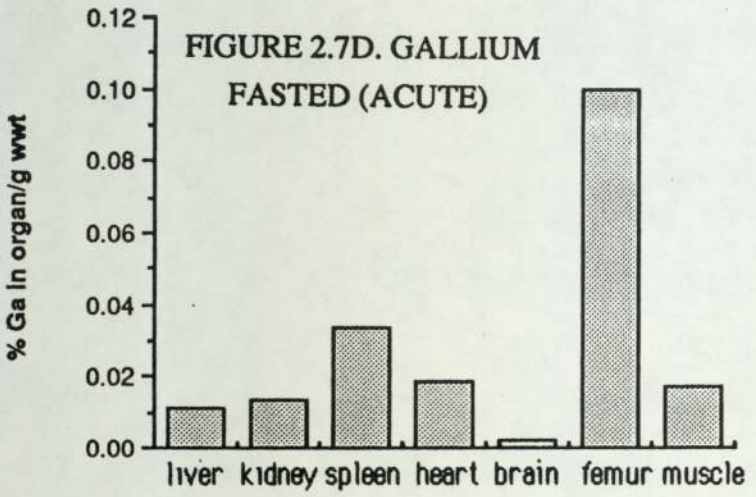
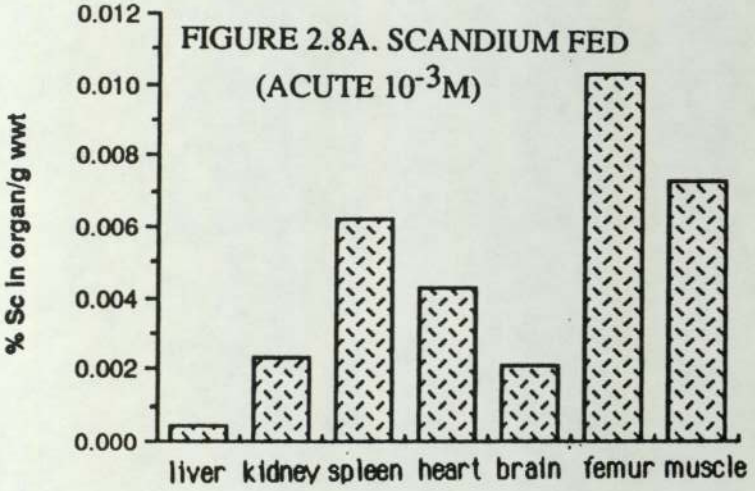
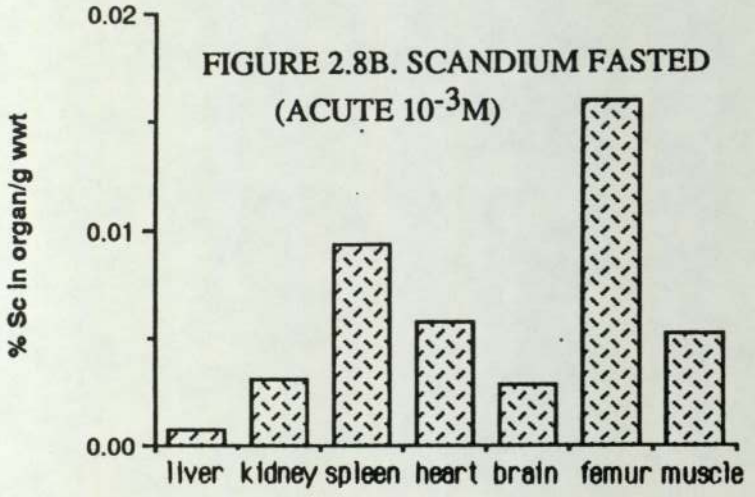
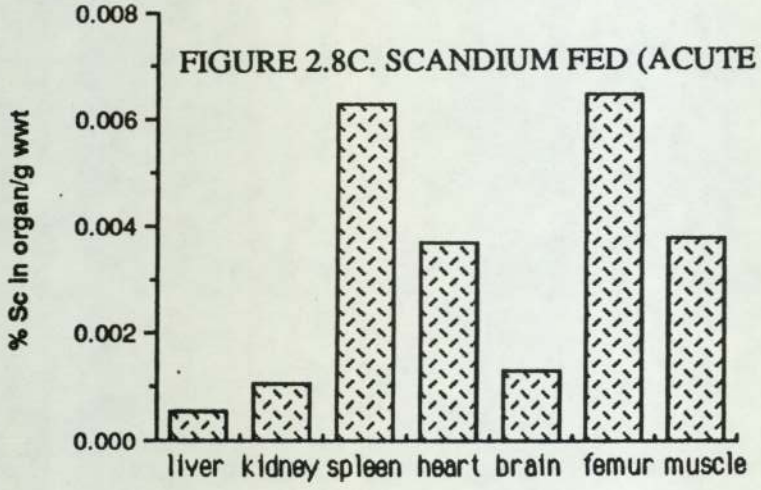
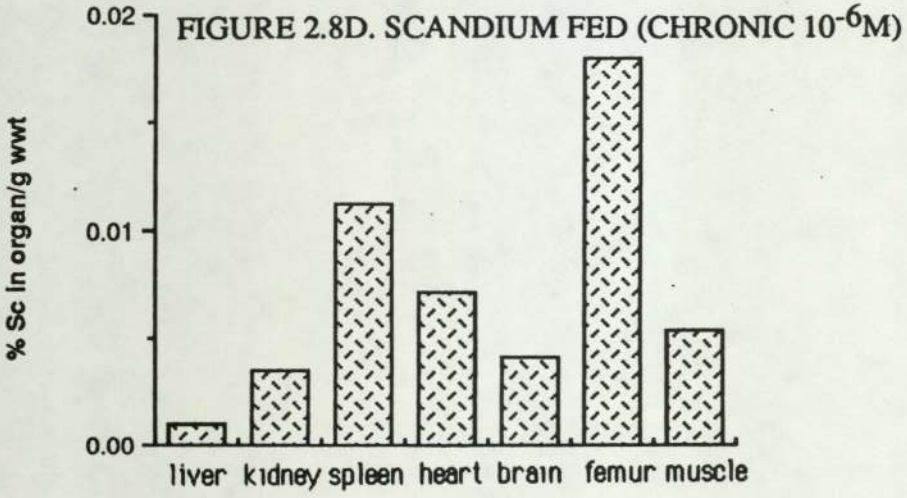


FIGURE 2.8. CONVERSION OF SCANDIUM ABSOLUTE ORGAN LEVELS TO ACTIVITY PER UNIT WEIGHT (n=5).









Browning, 1969; Mayor et al.1977; Venugopal and Luckey, 1978; Skalsky and Carchman, 1983; Alfrey, 1986.

Apart from the obvious accumulation in bone, aluminium, gallium and scandium were observed to be accumulating in spleen, heart and muscle (figures 2.7 and 2.8). The brain content of aluminium, gallium and scandium per unit weight of tissue was generally lower than that of other organs. The gallium and scandium content in liver was also present to a low extent. The high content of gallium and scandium in the uncorrected livers (see figures 2.2-2.4) was obviously due to the large size of the organ in comparison to the others measured. The large content of aluminium in the liver (figure 2.7b) may be due to high endogenous aluminium levels not related to accumulation after an oral dose being included in the measurement. It is also possible that an error occurred during the analytical procedure, the technique of graphite furnace atomic absorption spectroscopy being far less accurate than that of γ-counting (see 1.5).

In general nutritional status does not affect the relative tissue distributions of aluminium, only to increase the amount of absorbed material present in tissues in the fasted state.

2.3.4. EFFECT OF CITRATE, MALTOL AND FLUORIDE ON THE TISSUE DISTRIBUTION OF GALLIUM AFTER ORAL ADMINISTRATION.

A). Citrate.

The intestinal absorption of gallium in the presence of citrate was enhanced in fasted rats (table 2.5). Although enhanced absorption of gallium was observed in the presence of equimolar citrate the increase was not significant. A significant increase of gallium uptake was observed however, in fasted rats when the citrate concentration was increased 500 fold (this citrate dose was equivalent to the doses given to man by Slanina et al. 1986). In fed rats the concomitant administration of citrate at the higher dose did not significantly affect gallium absorption (table 2.5).

TABLE 2.5. THE EFFECT OF MALTOL, CITRATE AND FLUORIDE ON THE INTESTINAL ABSORPTION OF GALLIUM IN FED AND FASTED RATS (N=5, ±SEM).

DOSE (0.3mls)	FED RATS (%TDA)	FASTED RATS (%TDA)
$Ga(NO_3)_3$ (10^{-3}M)	0.0766 ±0.0066	0.342 ±0.0466
$Ga(NO_3)_3$/maltol (10^{-3}M/10^{-2}M)	0.0681 ±0.0113	0.6637 ±0.0749**
$Ga(NO_3)_3$ (10^{-6}M)	0.0311 ±0.0026	0.158 ±0.013
$Ga(NO_3)_3$ /citrate (10^{-6}M/10^{-6}M)	- -	0.265 ±0.076
$Ga(NO_3)_3$/citrate (10^{-6}M/5 x 10^{-4}M)	0.0281 ±0.0027	0.408 ±0.068**
$Ga(NO_3)_3$(10^{-6}M)	0.0331±0.0026	0.158 ±0.013
$Ga(NO_3)$/ NaF (10^{-6}M/25μM)	0.025 ±0.0028	0.0939 ±0.017*
$Ga(NO_3)_3$/ NaF (10^{-6}M/250μM)	0.0187 ±0.0021**	0.0395 ±0.0026***

Notes.
1) The above values represent the sum of gallium identified in liver, kidney, spleen, heart, brain, femur and muscle (%TDA). Although the above values do not represent an absolute measure of intestinal absorption, they are useful as a comparative measure.

2) *P<5%, **P<1%, ***P<0.1%, indicates a significant difference from the control value ($Ga(NO_3)_3$ only) of that group,(e.g. fed control versus fed test).

Subsequently tissue distribution of gallium in the 7 organs measured was not altered by the presence of citrate (figure 2.9).

In the fasted rats however, there was a step-wise increase in tissue gallium concentration. The control (gallium only dosed) rats had the lowest gallium content in tissues with increasing tissue gallium concentration observed with increasing doses of citrate (figure 2.10). Similarily to the absorption levels of table 2.5, tissue gallium content was not significantly enhanced in the presence of equimolar citrate (although an increase was observed in all tissues examined). Significantly higher levels of gallium were recorded in all tissues except muscle in the presence of the high dose of citrate (figure 2.10).

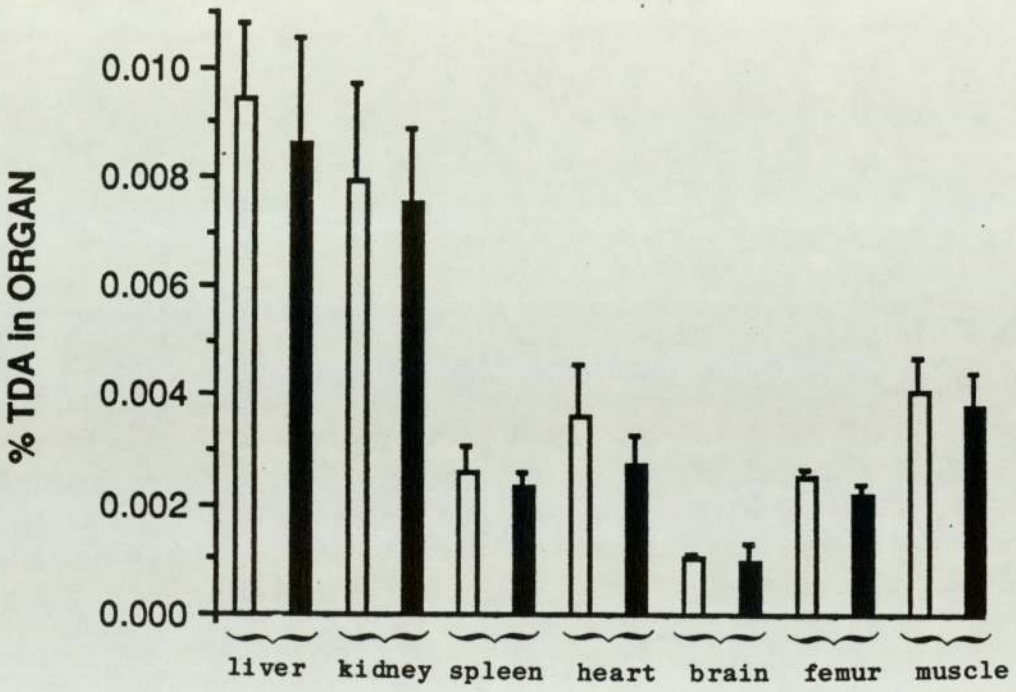
The presence of citrate did not alter the relative distribution of gallium between organs.

Dietary citrate has been previously observed to enhance the intestinal absorption of aluminium in humans, rabbits and rats (Slanina et al. 1984, 1986; Yokel & McNamara, 1988). In human subjects the simultaneous ingestion of aluminium hydroxide and citrate enhanced plasma aluminium from 5µg Al/l to 23µg Al/l ($P<0.001$)(Slanina et al. 1986). Yokel and McNamara (1988) observed the oral bioavailability of eight salts of aluminium, aluminium citrate having the highest uptake levels (2.2% of the total dose administered).

Citrate is believed to form strong complexes with both aluminium (Martin, 1986) and gallium. The species thought to be responsible for the enhanced absorption of both aluminium and gallium is the unionized lipid soluble aluminium/gallium-citrate ligand predominantly formed between pH 2-5. The stomach and proximal duodenum have been suggested as possible sites for absorption of this species (Slanina, et al. 1986).

An enhanced absorption of gallium was not observed in the fed state, again emphasizing the importance of nutritional status in controlling bioavailability levels. It is likely that the species responsible for increasing the transport of gallium into

FIGURE 2.9. EFFECT OF CITRATE ON THE TISSUE DISTRIBUTION OF GALLIUM IN THE FED RAT.



Notes

1). Rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ + 10μCi ^{67}Ga (white bars), or 0.3mls 10^{-6}M $Ga(NO_3)_3$ / 5 x 10^{-4}M citrate (black bars).

2). All rats sacrificed after 4 hours.

3). No significant differences were observed between fed tissues (white bars) dosed with gallium only, or fed tissues (black bars) dosed with gallium and citrate (n=5, ±SEM).

4). All results expressed as a percentage of the total dosed administered (%TDA).

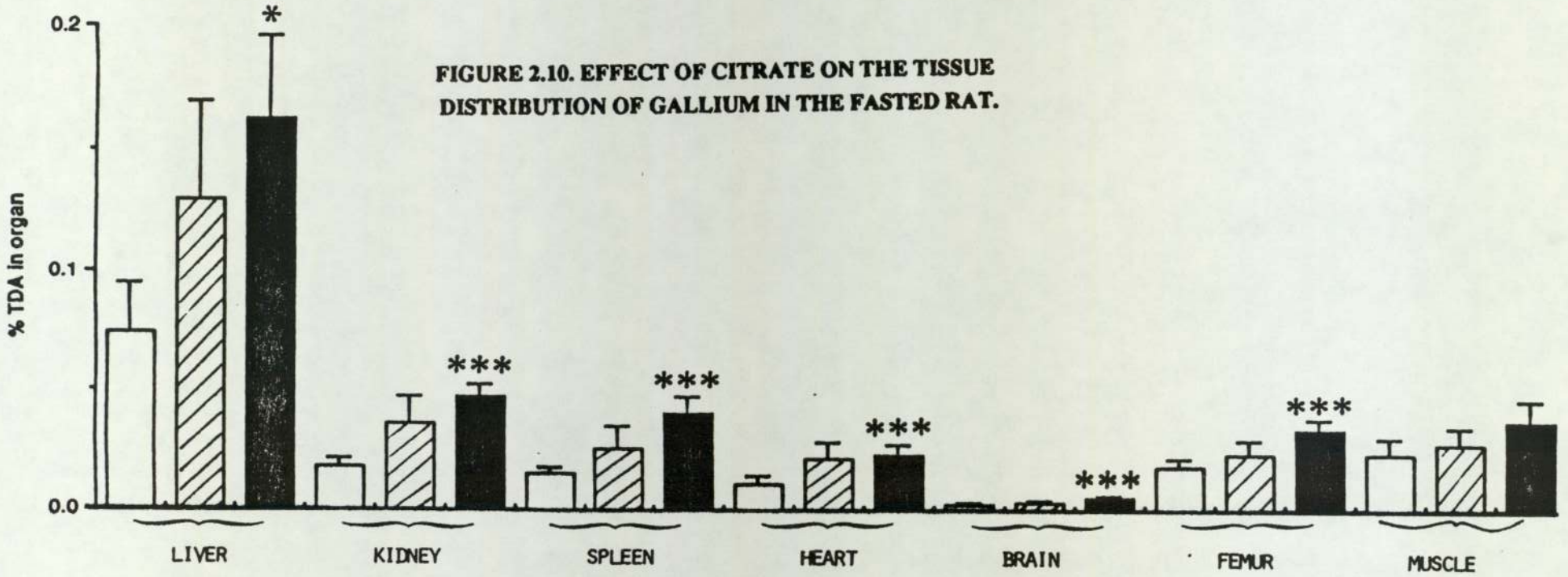


FIGURE 2.10. EFFECT OF CITRATE ON THE TISSUE DISTRIBUTION OF GALLIUM IN THE FASTED RAT.

Notes

1). White bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ + 10µCi ^{67}Ga (control).
Striped bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ / 10^{-6}M citrate + 10µCi ^{67}Ga.
Black bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ / 5 x 10^{-4}M citrate + 10µCi ^{67}Ga.

2). All rats sacrificed 4 hours after dosing.

3). *P<5%, ***P<0.1%, indicates a significant difference from the control value (gallium only dosed) of that tissue (n=5, ±SEM).

4). All results expressed as a percentage of the total dose administered (%TDA).

the body was decomposed by other dietary components leaving gallium to form insoluble unabsorbable hydroxide or phosphate compounds. The previous enhancement of aluminium absorption mentioned by the other workers was always performed in a gut lumen that had been fasted and therefore the lipid soluble aluminium-citrate complex would persist.

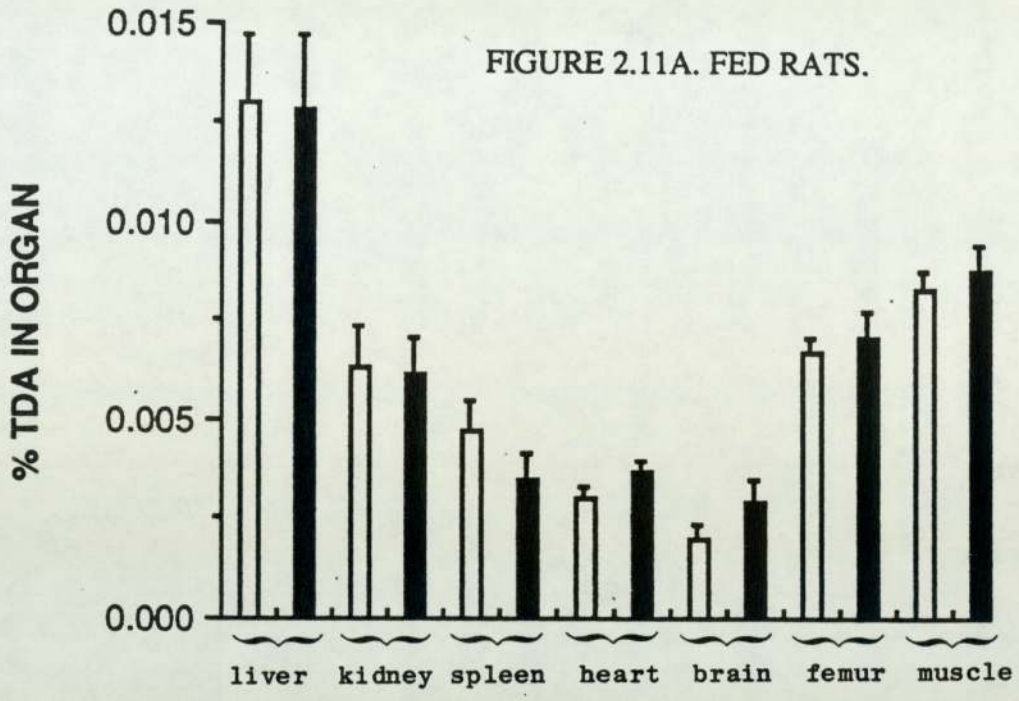
B). Maltol.

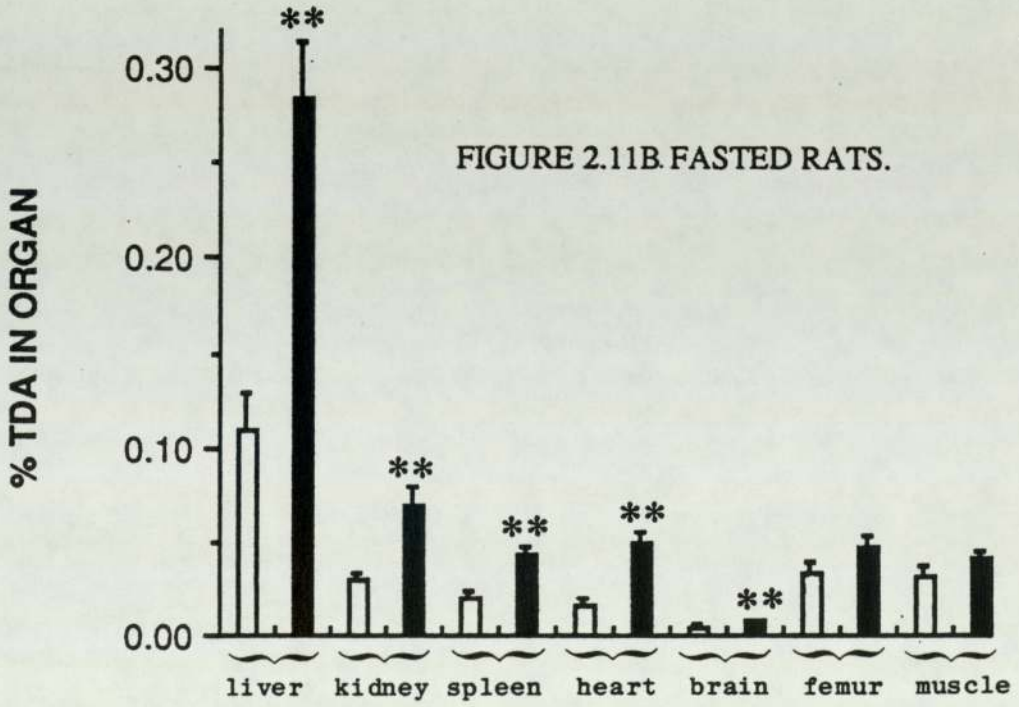
In animals that had been fasted overnight, the presence of maltol was shown to enhance gallium uptake into the liver, kidney, spleen, heart and brain (figure 2.11b). The values for all tissues examined were summed to give an estimate of the intestinal absorption of gallium; this indicated that over twice as much gallium had been absorbed in the fasted state when maltol and gallium were administered together than when gallium was given alone (table 2.5). The presence of maltol does not however, appear to alter the relative tissue distribution of gallium in the fasted animals.

This enhanced uptake of gallium probably indicates that gallium-maltol, like aluminium-maltol (Finnegan et al. 1986 & 1987) is soluble and carries a neutral charge thereby facilitating its movement across the brush border membrane of the intestinal epithelial cell. The aluminium or gallium cation is not likely to move across the membrane, but to traverse the epithelium via the tight-junctions and paracellular spaces (see chapters 3 & 4).

In fed animals there was no significant difference in gallium concentration between animals given gallium or gallium and maltol for any tissue examined (figure 2.11a). Furthermore the total value for gallium was similar in both groups (table 2.5). The failure of maltol to enhance the uptake of gallium in the fed state may be due to the decomposition of the gallium-maltol complex by dietary components thereby reducing the gallium-maltol concentration in the gut. This was supported by the finding that in fed animals ion exchange chromatography could only demonstrate

FIGURE 2.11. EFFECT OF MALTOL ON THE TISSUE DISTRIBUTION OF GALLIUM IN FED AND FASTED RATS.





Notes

1). Rats dosed with 0.3mls 10^{-3}M $Ga(NO_3)_3$ + 10μCi ^{67}Ga (white bars), or 0.3mls 10^{-3}M $Ga(NO_3)_3$ / 10^{-2}M maltol + 10μCi ^{67}Ga (black bars).

2). All rats sacrificed after 4 hours.

3). **P<1%, indicates a significant difference from the control (gallium only dosed) value for that tissue (n=5, ±SEM).

4). All results expressed as a percentage of the total dosed administered (%TDA).

the gallium ion in the soluble phase of gut washings after administration of gallium and maltol. In fasted animals however, a combination of gallium and gallium-maltol species was shown by ion-exchange chromatography to exist. This indicated that the complex proposed by Finnegan et al. was available for absorption by the small intestinal epithelial cells (see chapter 5 for results and discussion concerning gallium and maltol intestinal speciation).

As maltol is a food additive, any aluminium-maltol complex which is formed as a result of the simultaneous ingestion of these compunds should not be absorbed over and above the normal levels for aluminium transport (average intake is 5mg/day; Bertholf et al. 1988). Although aluminium is acknowledged to be a possible neurotoxin, this study suggests that the aluminium-maltol complex in fed animals may not pose an increased neurotoxic threat over aluminium as has been previously been suggested by Finnegan et al.(1986 & 1987).

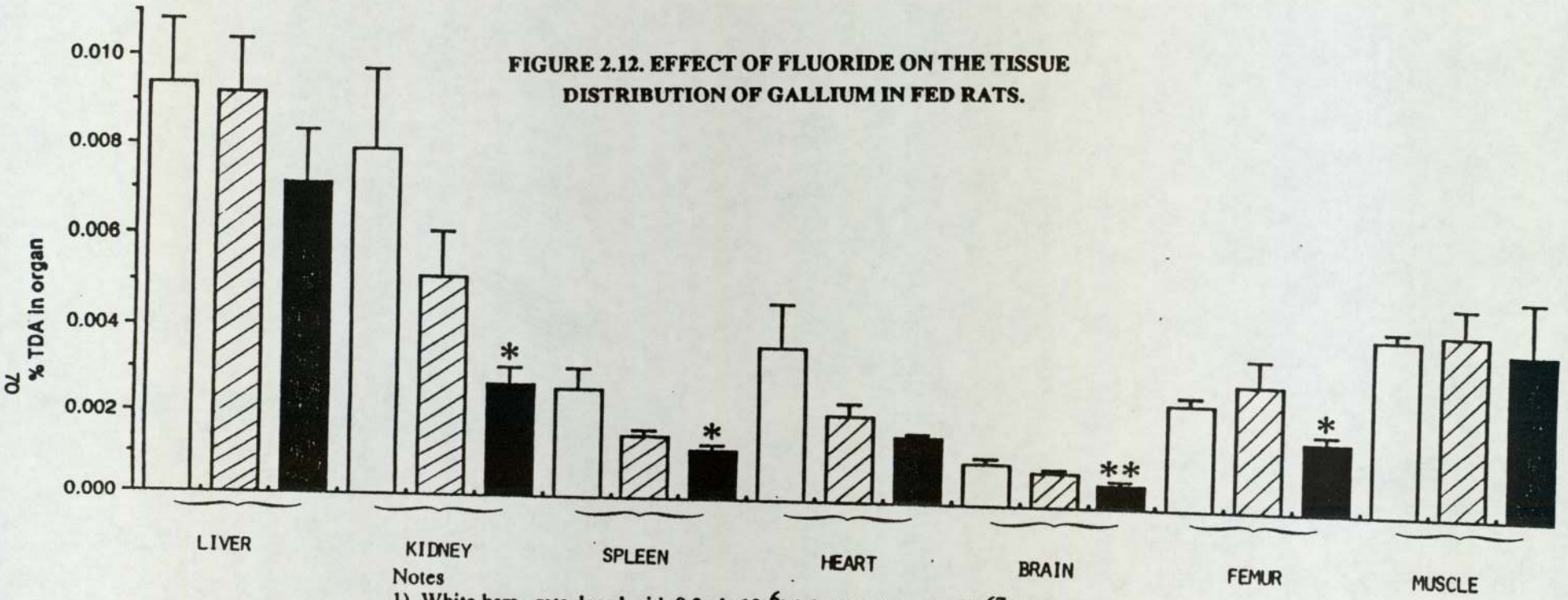
C). Fluoride.

Addition of 25μM fluoride significantly reduced gallium absorption in the fasted rat but not in the fed (table 2.5). When 250μM fluoride was simultaneously dosed with gallium, there was a signficant reduction in uptake in both fed and fasted rats (table 2.5).

These whole absorption values of gallium are reflected in the tissue distribution gallium levels after oral administration. In both fed and fasted rats there was a step-down trend observed, with the control values (gallium only dosed tissues) the highest. With increasing amounts of fluoride added to the dose, there was decreasing concentrations of gallium identified in the tissues (figures 2.12 & 2.13).

In both fed and fasted rats there was no significant decrease in gallium tissue content in any of the tissues examined when fluoride was added at the lower dose (25μM). At the higher dose (250μM) however, there was reduced tissue gallium

FIGURE 2.12. EFFECT OF FLUORIDE ON THE TISSUE DISTRIBUTION OF GALLIUM IN FED RATS.



Notes

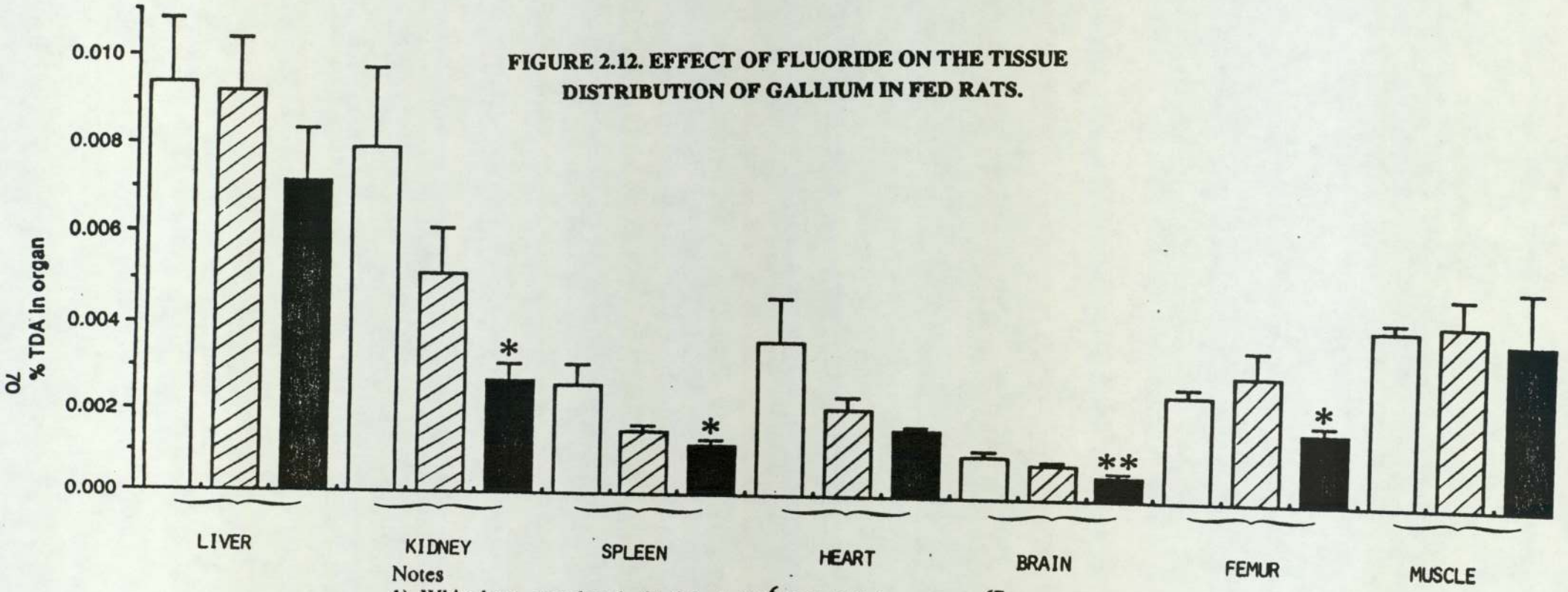
1). White bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ + 10μCi ^{67}Ga (control).
Striped bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ / 25μM fluoride + 10μCi ^{67}Ga.
Black bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ /250μM fluoride + 10μCi ^{67}Ga.

2). All rats sacrificed 4 hours after dosing.

3). *P<5%,**P<1%, ***P<0.1%, indicates a significant difference from the control value (gallium only dosed) of that tissue (n=5, ±SEM).

4). All results expressed as a percentage of the total dose administered (%TDA).

FIGURE 2.12. EFFECT OF FLUORIDE ON THE TISSUE DISTRIBUTION OF GALLIUM IN FED RATS.



Notes

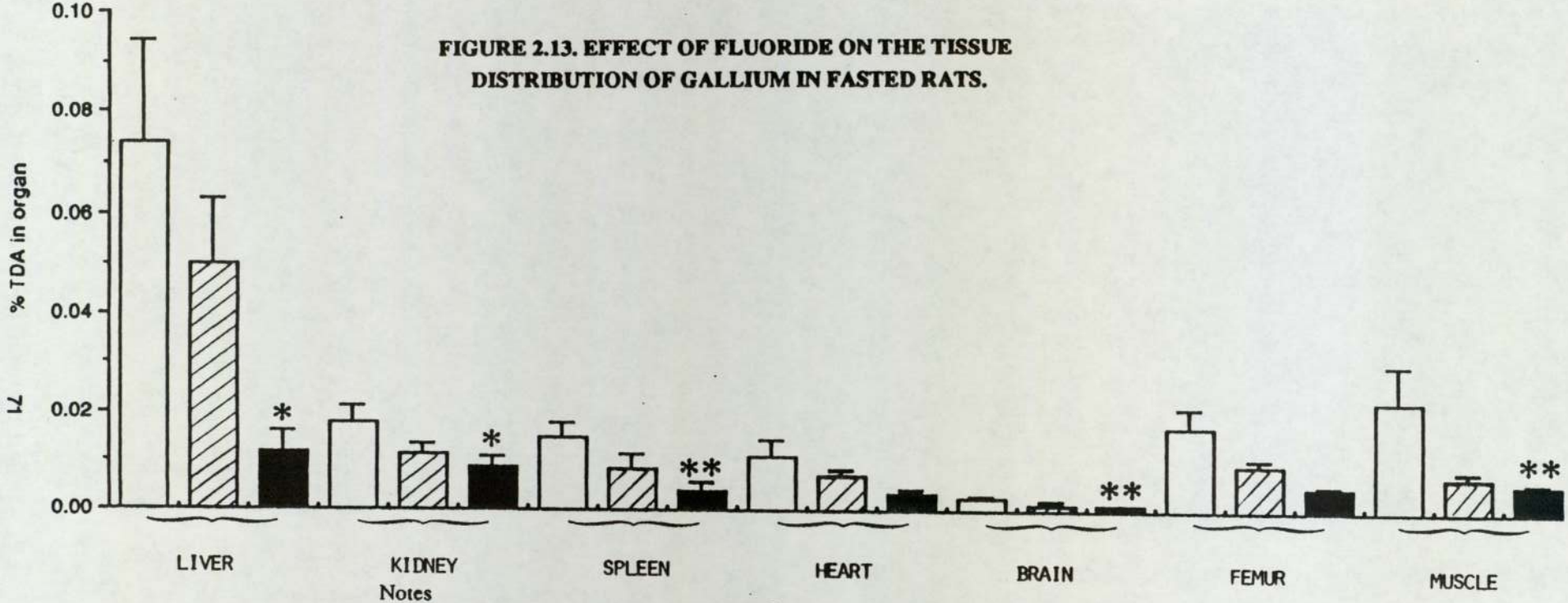
1). White bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ + 10μCi ^{67}Ga (control).
Striped bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ / 25μM fluoride + 10μCi ^{67}Ga.
Black bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ /250μM fluoride + 10μCi ^{67}Ga.

2). All rats sacrificed 4 hours after dosing.

3). *P<5%,**P<1%, ***P<0.1%, indicates a significant difference from the control value (gallium only dosed) of that tissue (n=5, ±SEM).

4). All results expressed as a percentage of the total dose administered (%TDA).

FIGURE 2.13. EFFECT OF FLUORIDE ON THE TISSUE DISTRIBUTION OF GALLIUM IN FASTED RATS.



Notes

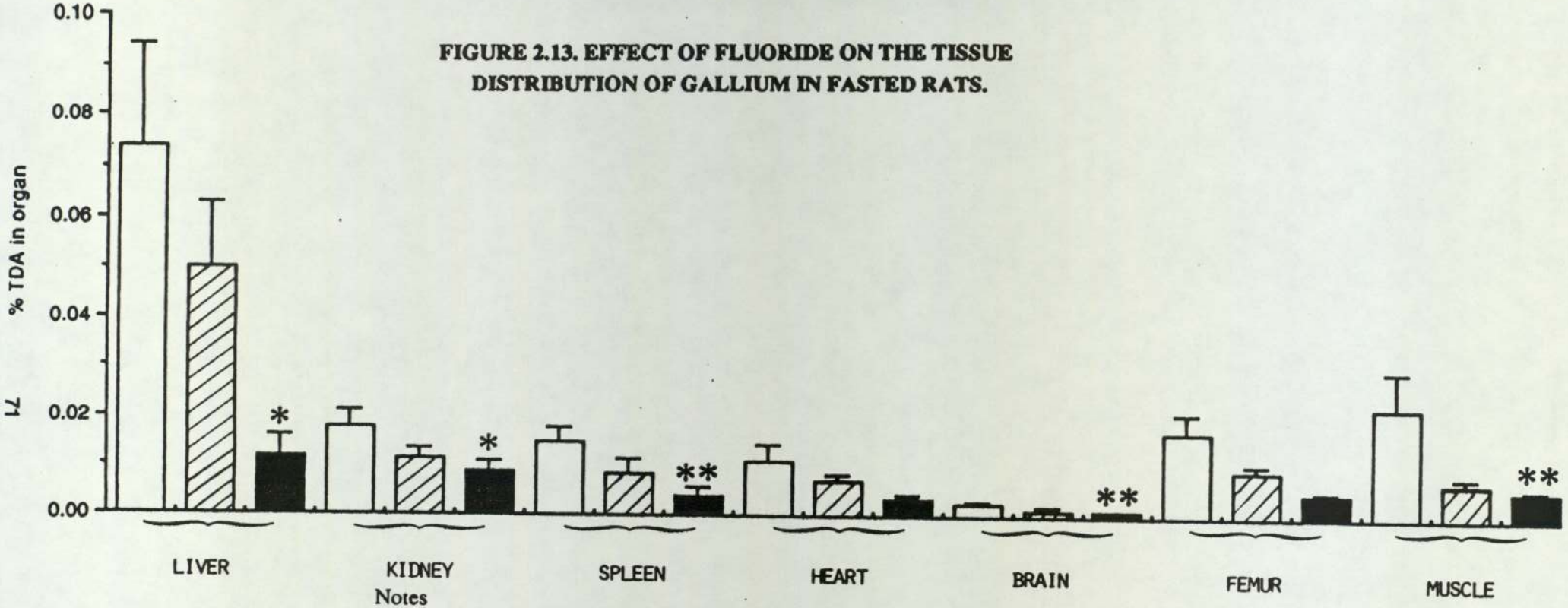
1). White bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ + 10µCi ^{67}Ga (control).
Striped bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ / 25µM fluoride + 10µCi ^{67}Ga.
Black bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ /250µM fluoride + 10µCi ^{67}Ga.

2). All rats sacrificed 4 hours after dosing.

3). *P<5%,**P<1%, ***P<0.1%, indicates a significant difference from the control value (gallium only dosed) of that tissue (n=5, ±SEM).

4). All results expressed as a percentage of the total dose administered (%TDA).

FIGURE 2.13. EFFECT OF FLUORIDE ON THE TISSUE DISTRIBUTION OF GALLIUM IN FASTED RATS.



Notes

1). White bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ + 10μCi ^{67}Ga (control).

Striped bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ / 25μM fluoride + 10μCi ^{67}Ga.

Black bars - rats dosed with 0.3mls 10^{-6}M $Ga(NO_3)_3$ /250μM fluoride + 10μCi ^{67}Ga.

2). All rats sacrificed 4 hours after dosing.

3). *P<5%,**P<1%, ***P<0.1%, indicates a significant difference from the control value (gallium only dosed) of that tissue (n=5, ±SEM).

4). All results expressed as a percentage of the total dose administered (%TDA).

levels in kidney, spleen, brain and femur in both fed and fasted rats (figures 2.12 & 2.13). The liver content of gallium was also significantly lower in the fasted rat in the 250μM fluoride dosed animals (figure 2.13).

Unlike citrate and maltol, the addition of fluoride affected the bioavailability of gallium in both fed and fasted rats. The citrate and maltol complexes formed with gallium are thought to persist only in the fasted animal, having been decomposed by other dietary components in the fed state. Fluoride however, appears to be able to resist this decomposition, since its effects were observed both in both nutritional status.

There has been previous interest in interactions between aluminium and fluoride since both are used in the treatment of drinking water. Aluminium forms strong complexes with fluoride (Martin, 1986), the resulting species formed are even stronger than those of Fe^{3+}. Out of 14 cations tested aluminium has been found to be the strongest fluoride binder (Brudevold et al. 1972).

Previous intestinal interactions between aluminium and fluoride have been noted. The addition of aluminium inhibits fluoride absorption (Spencer et al. 1981) and is used as a treatment for fluorosis in sheep and cattle (Underwood, 1977). Still and Kelley (1980) observed that in areas with high drinking water fluoride concentrations the incidence of Alzheimers disease was less than in areas with normal water fluoride levels.

Since the presence of aluminium reduces fluoride uptake and increases both aluminium and fluoride excretion, Shore et al. (1985) have suggested that fluoride could be used as an aluminium binder to lower levels of aluminium absorption.

2.4. CONCLUSIONS.

The short term tissue distribution of aluminium, gallium and scandium has been observed after oral administration. Levels of absorption were less than 1%,

scandium being least absorbed from the gastrointestinal tract. Nutritional status was an important factor in the bioavailability of aluminium, gallium and scandium. Significantly more aluminium, gallium and scandium was absorbed and identified in the tissues in fasted rats when compared to the fed.

The 7 tissues measured, liver, kidney, spleen, heart, brain, femur and muscle varied in weight (from between 0.34g to 9.96g). It was not entirely possible therefore, to make comparisons between the elements and to measure accumulative ability of the tissues. When the aluminium, gallium and scandium tissue results were reexpressed as activity per unit weight the most obvious accumulation in all three elements was noted in the femur. Skeletal accumulation of Group 3 elements is a characteristic which has been noted previously (Venugopal and Luckey, 1978). Accumulation of aluminium, gallium and scandium was also observed in spleen, heart and muscle. Brain and liver levels of aluminium, gallium and scandium were consistently lower than the concentrations observed in other tissues.

After a chronic 12 day period of oral administration there was significant accumulation of scandium in all tissues examined except spleen when compared to the acute 4 hour experiments. When the absolute organ levels of scandium were reexpressed as concentrations per unit weight of tissue, accumulation of scandium was again observed in skeletal tissue. Moderate levels of scandium accumulation were recorded in heart, spleen and muscle.

When the rats were left for a further 5 days scandium activity was not recorded in heart or brain suggesting that any accumulated scandium had been excreted. Significantly lower levels were recorded in liver, kidney and spleen suggesting that scandium was being released from the tissue reservoirs and was in the process of being excreted. The levels of scandium in bone and muscle tissues at 17 days did not significantly differ from those observed at 12 days. It is possible that ingested scandium was slowly being incorporated into skeletal tissue, the 5 day 'washout' period not sufficient for turnover to be completed. Retention of scandium in muscle

may be a result of the lower vascular nature of the tissue having a limited ability to remove any accumulated scandium.

Assessment of daily faecal and urine scandium content over the 12 day period confirmed that a low proportion of the administered dose was being transported into the body. The daily faecal scandium content was approximately 100% of the administered dose, whilst urine scandium content was never greater than 0.3% of the daily dose. In the 5 day period after the last administered dose, scandium in the gastrointestinal tract was completely removed except for the presence of a small plateau ($\approx$ 1%) identified in the last four days of the experiment. This small residual turnover of scandium may be the result of an enterohepatic circulation where transported scandium re-entered the gastrointestinal tract via the bile duct.

Addition of citrate, maltol and fluoride, common additives to food and water supplies affected the bioavailability of gallium after oral administration. Citrate and maltol enhanced the absorption of gallium in fasted rats only. The addition of fluoride however inhibited the absorption of gallium in both fed and fasted rats. It is possible that the gallium-citrate or gallium-maltol ligands were decomposed by other dietary components in the fed state (or that other metal cations e.g. Fe^{3+} competed successfully for the anionic binding sites). The gallium-fluoride complex however, resisted competition from other ligands and metal cations and persisted in both nutritional states.

Addition of citrate, maltol or fluoride did not affect the relative tissue distribution of gallium in either fed or fasted states.

The results outline the importance of nutritional status and food and water additives in influencing the bioavailability and possible neurotoxicity of aluminium. Any further understanding into the mechanisms of intestinal absorption need to carefully consider the complex interactions between dietary ligands and aluminium.

CHAPTER THREE

IN-VITRO INTESTINAL TRANSPORT STUDIES

CHAPTER 3. *IN-VITRO* INTESTINAL TRANSPORT STUDIES

3.1. INTRODUCTION

3.1.1. THE EVERTED SAC TECHNIQUE AND VIABILITY STUDIES.

The everted intestinal sac technique of Wilson and Wiseman (1954) has been extensively used over the past four decades. Although *in-vivo* techniques (see Chapter 4), such as intestinal perfusion have the advantages of intact hormonal and neuronal control, the experimenter is faced with a lack of control over conditions on both sides of the intestinal wall. The main advantages of the *in-vitro* everted sac preparation are the ready sampling of the serosal and mucosal compartments and the precise control which is allowed over conditions on both sides of the intestinal membrane.

Eversion of the intestinal sac allows radiation of villi, where the brush border lining has access to a large volume of oxygenated buffer. If mucosal uptake and transport occur, the large volume of fluid bathing the mucosal surface ensures that insignificant changes occur in the concentration of the test substance in the incubating buffer.

Measurement of the viability of everted sacs must take place to ensure the preparation is suitable for measuring absorption. In this investigation viability was assessed in four ways.

A). Maintenance of a constant (approximately 5-9mV) transmural potential difference (PD). The PD observed across the small intestine is generated by the continual movement of ions across the epithelial cell layer (Barry et al., 1964; Schultz, 1981).

B). Movement of glucose. The epithelial cell membrane is thought to contain specific binding sites for the active uptake of glucose and its subsequent release into the serosal compartment. These carrier mechanisms are thought to be substrate specific and sodium dependent (Crane et al. 1965), thus movement of

glucose is against a concentration gradient but is facilitated by the movement of sodium across its concentration gradient.

C). Water transport. Water uptake studies consist of i) fluid uptake, ii) tissue uptake and iii) subsequent transfer to the serosal compartment. The 'standing gradient' hypothesis of Diamond (1977) is still the favoured model to explain water transport by the small intestine (Freidman, 1986). The presence of solutes (mainly sodium and chloride ions) entering the interlateral spaces initiates an osmotic gradient. In addition, the active extrusion mechanisms of Na^+/K^+ ATPase in the basolateral membrane creates a hypertonic environment in the lateral intracellular space, generating an osmotic flow to the serosal side.

D). Permeation of polyethylene glycol (PEG900). Polyethylene glycols are used in both animals and humans to demonstrate intestinal pèrmeability (Hamilton, 1986). Movement of PEG900 across rodent mucosa is thought to be as a result of paracellular transport (Fischer and Lauterbach 1984). Permeation of PEG900 has been restricted to 0.5% of total buffer PEG900 (over a 45 minute incubation period) in *in-vitro* intestinal preparations (Karim, 1985). This is thought to be due to the large size of the molecule and to the presence of bulky side chains (Hamilton, 1986).

The anatomical integrity of everted intestinal tissue has also been studied using histological methods. Levine et al. (1970) found everted sacs to progressively lose structural integrity with time during the course of incubation. Maiti and Banerjee (1978) found after 30 minutes of incubation at 37°C, the epithelium of everted mouse gut sacs were completely intact. At 60 minutes however, slight oedema of the villi and partial rupturing of the epithelial cells was noticed. The majority of studies in this investigation (excepting viability and time based experiments) were for a set period of 20 minutes only.

3.1.2. THE *IN-VITRO* INTESTINAL ABSORPTION OF ALUMINIUM, SCANDIUM AND GALLIUM.

Since Berlyne et al. (1970) observed elevated aluminium serum levels in patients orally administered with aluminium resins, there has been increasing evidence to suggest that oral aluminium is an important factor in the aetiology of neuropathological disease (Ihle and Becker, 1985; Mayor et al. 1985). The presence of aluminium antacid formulations has also been suggested as a possible cause of toxicity.

There have been limited studies of the mechanisms of aluminium absorption across epithelial membranes, and the characteristics of uptake are little understood. Intestinal work has obviously been hampered by the lack of a suitable aluminium radionucleotide, other available techniques rendering a wide range of results (see 1.5). Chapter 2 has outlined the similarities between aluminium, gallium and scandium tissue distribution in the rat after oral administration, suggesting the elements are similarly metabolised. The availability of the radioisotopes ^{46}Sc and ^{67}Ga adds to the ease of experimentation, but more importantly should contribute to greater accuracy in attempting to define the characteristics of aluminium uptake and transport.

3.2. MATERIALS AND METHODS

3.2.1. ANIMALS. See section 2.2.1.

3.2.2. CHEMICALS. See section 2.2.2.

3.2.3. PHYSIOLOGICAL BUFFERS

The following physiological buffers were used:

A. Krebs Henseleit bicarbonate buffer (KHBB)

NaCl (112mM), KCl (4.5mM), $CaCl_2$ (2.4mM), $MgSO_4$ (1.12mM), KH_2PO_4 (1.12mM), $NaHCO_3$ (24mM), and glucose (28mM).

$NaHCO_3$ was bubbled with CO_2 before addition. Glucose was added last and freshly made up for each experiment. Solutions were buffered to pH7.4 and kept on ice until use. Throughout use the KHBB was gassed was gassed with 95% O_2/5% CO_2.

B. KHBB minus phosphate.

Preparation as before except for the exclusion of KH_2PO_4.

C. Simple physiological buffer.

NaCl (120mM), KCl (5mM), glucose (20mM), mannitol (20mM).

D. HEPES physiological buffer.

NaCl (125mM), KCl (3.5mM), HEPES (16mM), glucose (20mM).

E. Krebs-Tris buffer.

NaCl (112mM), KCl (4.5mM), $CaCl_2$ (2.4mM), $MgSO_4$ (1.12)
Tris (25mM), glucose (20mM).

All buffers at pH 7.4.

Physiological buffers contained $Ga(NO_3)_3$, $ScCl_3$, or $Al(NO_3)_3$ at concentrations ranging from 5ng/ml- 4000ng/ml (see results tables for exact concentrations). ^{67}Ga and ^{46}Sc was counted by γ-counting (see 2.2.4). Aluminium was measured by graphite furnace atomic absorption spectroscopy (see 2.2.5). In certain experiments the metabolic inhibitors 2,4-dinitrophenol and phloridzin were added (see individual results tables for details).

3.2.4. PREPARATION OF EVERTED INTESTINAL SACS.

A single 100mg/kg intraperitoneal injection of Inactin (5-secbutyl-5-ethyl-2-thiobarbituric acid) was used to anaesthetise fasted rats. The abdomen was opened and the small intestine excised into cold oxygenated KHBB.

Debris was removed from the gut by flushing through with isotonic saline. 6-7cm lengths of intestine were everted over thin glass rods. During all everted sac studies, work was performed on 0.9% saline saturated filter paper (Whatmans No. 1). Between each of the preparative stages sacs were blotted carefully to remove excess moisture.

The sacs were sealed, filled with 0.5ml KHBB and incubated at 37°C, 80 oscillations min^{-1} for varying time periods (see results tables) in 10mls physiological buffer (see 3.2.3). After incubation the serosal fluids were removed from the sac by a hypodermic needle and syringe to prevent contamination from the mucosal tissue. Serosal and mucosal fluids were counted for ^{67}Ga or ^{46}Sc activity (see 2.2.4). Aluminium content was measured by GFAAS (see 2.2.5).

3.2.5. MEASUREMENT OF VIABILITY.

3.2.5.1. TRANSMURAL POTENTIAL DIFFERENCE.

An everted sac was taken from the jejunal region and ligated at one end. The other end was ligated onto a fluted glass cannula (diam. 4mm). 0.5ml KHBB was introduced into the sac via the cannula and the sac placed into 30mls gassed KHBB at 37°C. Electrodes (3.8M KCl-Agar salt bridges) were placed opposite, one in the mucosal buffer, the other down the glass cannula into the serosal compartment. Potential difference measurements from the serosal to mucosal side were recorded continuously for 1 hour using a Philips PD meter (now Pye-Unicam, Cambridge. UK).

3.2.5.2. WATER TRANSPORT STUDIES.

Everted sacs were prepared (see 3.2.4) and incubated in 10mls KHBB for 10-60 minutes. The sacs were weighed four times during the preparation as follows:

W1=Weight of empty sac before incubation,
W2=Weight of filled sac before incubation,

W3=Weight of filled sac after incubation,
W4=Weight of empty sac after incubation.

Water movement can be assessed in the following ways:

W3-W2=Total fluid uptake,
W4-W1=Tissue fluid uptake,
(W3-W2)-(W4-W1)=serosal fluid uptake.

The weight of the ligature threads were considered negligible and not considered in the calculations.

3.2.5.3. GLUCOSE TRANSPORT.

The mucosal and serosal fluids from the 60 minute incubations (see 3.2.5.2) were analysed for glucose by the method of Morton (1982).

50µl sample was added to 2.5mls reagent (reagent consisting of 500mM/l Tris-HCl, pH 7; 70mg/l glucose oxidase; 15mg/l peroxidase, 1.14g/l ABTS [2,2-Azinodi-(3 ethyl benzthiazoline sulphonic acid)]. The mixture was left at room temperature for 1 hour and the extinction read at 436nM against a reagent blank (Shimadzu UV spectrophotometer, Japan). The calibration curve was between 5-500nM/ml of glucose and samples were diluted accordingly to lie within this range.

3.2.5.4. PERMEABILITY OF POLYETHYLENE GLYCOL 900 (PEG900).

0.2µCi of polyethylene glycol, [1,2-^{3}H]-(M.W.800-1000) was added to the incubating medium of each everted sac. After incubation the serosal fluid was carefully removed from the sac using a long needle so as to avoid contamination with the mucosal surface. The serosal fluids were made up to aliquots of 1ml and had 10ml of 'Optiphase' scintillant added. A Beckman LS7500 ß-scintillation counter preprogrammed with a tritium quench curve, was used to assess the presence of ^{3H}PEG900 in the serosal fluid.

3.2.5.5. MEASUREMENT OF EXTRACELLULAR FLUID (ECF).

Measurement of ECF by ^{3H}PEG900 was performed in conjunction with the previous experiment (see 3.2.5.4). In these experiments it was assumed that a, PEG900 in the ECF was in equilibrium with that of the incubating buffer and b, 1µl water is equivalent to 1mg in tissue weight (inc. ECF). 0.2µCi ^{3H}PEG900 was added to the buffer as before and the everted sac incubated for time periods of between 2-30 minutes. After the removal of the serosal fluid not more than 0.1mg tissue was added to 1ml of tissue solubiliser 'Optisolve'. and placed in a shaking water bath at 50°C until digested. Not more than 4 drops H_2O_2 was then added to each vial to bleach the solution. 10mls of scintillant 'Optiphase' (now containing 7mls/l glacial acetic acid) was finally added to each of the vials. These were placed in the dark for 24 hours to eliminate chemiluminescence. Counting was again performed by the Beckman LS 7500. When measuring tissue samples, the preprogrammed tritium quench curve was corrected for increased quenching due to the addition of tissue solubiliser.

3.3. RESULTS AND DISCUSSION.

3.3.1. VIABILITY STUDIES

The use of viability studies in assessing the suitability of the everted sac preparation for metal ion transport studies have been well documented in the past (Blair et al. 1974; Gardner, 1978; Coleman 1979; Coogan, 1983; Plumb et al. 1987). The results are briefly summarized over the next few pages.

A). Maintenance of a constant transmural potential difference (PD).

PD reading were made every 10 minutes for a 60 minutes incubation period. The values observed showed a insignificant drop from 6.2 to 5.2mV ($\bar{x}$=5.58mV ±0.18, n=5) and indicate the electrical integrity of the preparation.

B. Movement of glucose.

Glucose measurements from mid-jejunal sacs at the end of 60 minutes incubation gave a serosal to mucosal ratio of 1.32($\pm$0.10, n=5). This value is statistically greater than unity and is indicative of active transfer and a metabolically viable preparation.

C). Water transport.

Total fluid uptake by the jejunum was linear with time with an overall rate of 1086 $\pm$106 µl water per gram wet weight of tissue per hour (r=0.97,P< 1%). Similar results (1162µl/g wwt/hr) have been obtained by Parsons et al. (1984) again in jejunal everted sacs.

There was an initial rapid uptake of fluid in the tissue in the first 10 minutes of incubation (0 -425 µl H_2O/g wwt). This is probably due to water entering the extracellular spaces surrounding the brush border of the epithelia and also to the reformation of the unstirred water layer (Winne, 1977). After 10 minutes incubation tissue uptake plateaus with only a slight increase in tissue uptake observed at the end of 60 minutes.

Transfer of water to the serosal compartment is linear with time with an overall rate of 530$\pm$68 µl H_2O/g wwt/hr (r=0.92, P<5%)

The addition of gallium (500ng/ml) to the incubation medium did not affect fluid uptake, tissue uptake or fluid transfer.

D). Permeation of polyethylene glycol (PEG900).

In 60 observations from all regions of the small intestine, no more than 0.4% of the total PEG900 in the bathing medium was found in the serosal fluid. Over the period 2 to 30 minutes the permeation of PEG900 was linear in all regions (duodenum, r=0.97, P<1%; jejunum, r=0.98, P<0.1%; ileum r=0.95, r=5%).

3.3.2. THE TRANSPORT OF ALUMINIUM, SCANDIUM AND GALLIUM.

3.3.2.1. TIME BASED STUDIES.

Tissue uptake of aluminium, scandium and gallium by mid-jejunal everted sacs was measured (figure 3.1a, 3.2a and 3.3a). Results indicate that aluminium and scandium tissue uptake saturated over the 30 minute incubation period (figure 3.1a/3.2a). Gallium tissue uptake followed a similar pattern but the saturation was less pronounced (figure 3.3a). The gallium results were also corrected for the presence of gallium in the extracellular fluid (see also 3.3.2.2), this correction however, did not change the pattern of uptake (figure 3.3a). The results suggest that tissue uptake of all three elements was rapid in the initial phase of incubation, subsequent absorption however, being slower.

The transfer of aluminium, gallium and scandium to the serosal compartment was linear with time (figures 3.1b, 3.2b and 3.3b). The serosal transfer of aluminium, gallium and scandium was qualitatively less than the overall tissue uptake, the latter possibly being due to non-specific tissue binding where the majority of adsorbed aluminium, gallium or scandium was not available for subsequent transfer. The results suggest a passive diffusive route of transport into the serosal compartment.

If the results for aluminium, gallium or scandium uptake and transfer (at 20 minutes incubation) are reexpressed as a serosal to mucosal ratio (x 100) an idea is gained of the percentage of mucosally bound element transported into the serosal compartment (table 3.1)

FIGURE 3.1A. TIME BASED UPTAKE OF ALUMINIUM (750ng/ml $Al(NO_3)_3$) BY MID JEJUNAL EVERTED SACS (n=5, ±SEM).

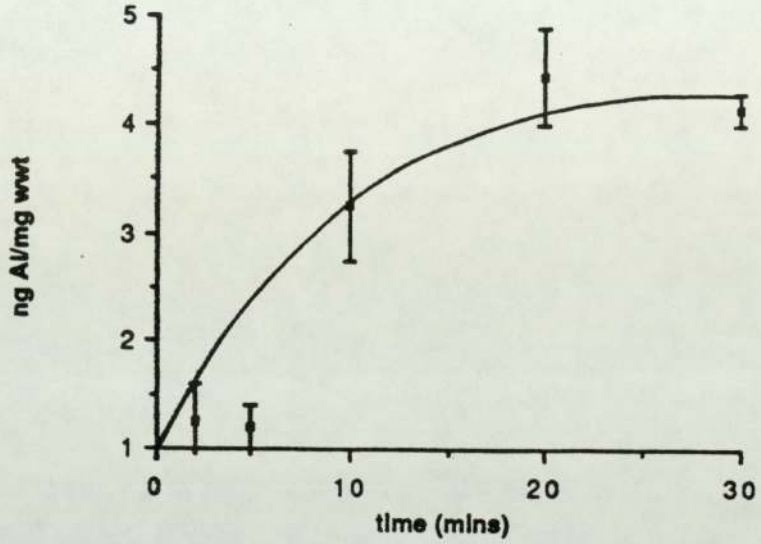
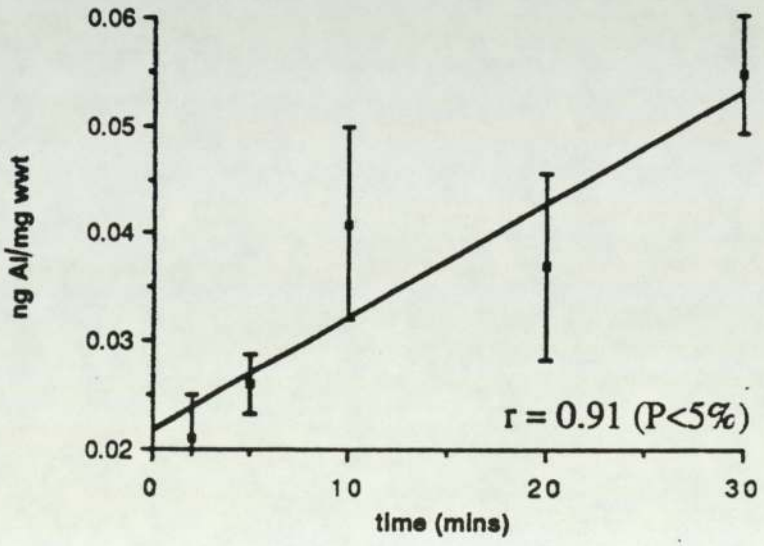


FIGURE 3.1B. TIME BASED SEROSAL TRANSFER OF ALUMINIUM (750ng/ml $Al(NO_3)_3$) BY MID JEJUNAL EVERTED SACS (n=5, ±SEM).



Notes
1). All results expressed as ng Al / mg wet weight tissue (n=5, ±SEM for each observation).

FIGURE 3.2A. TIME BASED TISSUE UPTAKE OF SCANDIUM (500ng/ml $ScCl_3$) BY MID JEJUNAL EVERTED SACS (n=5, ±SEM).

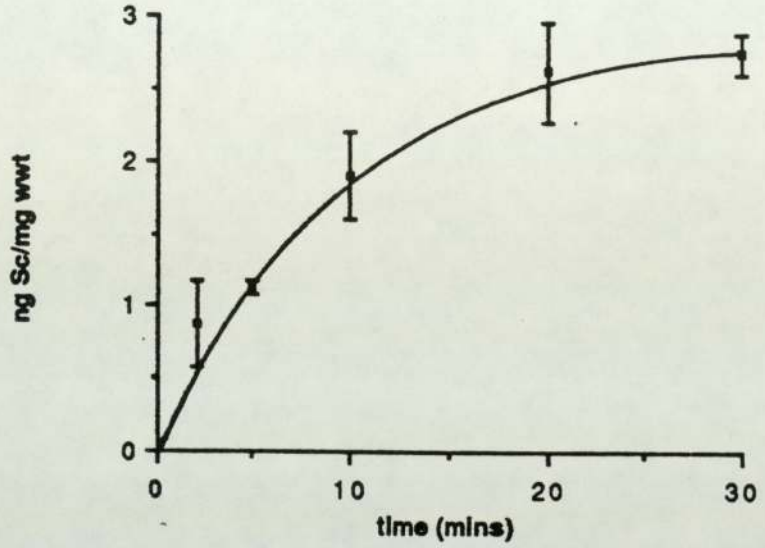
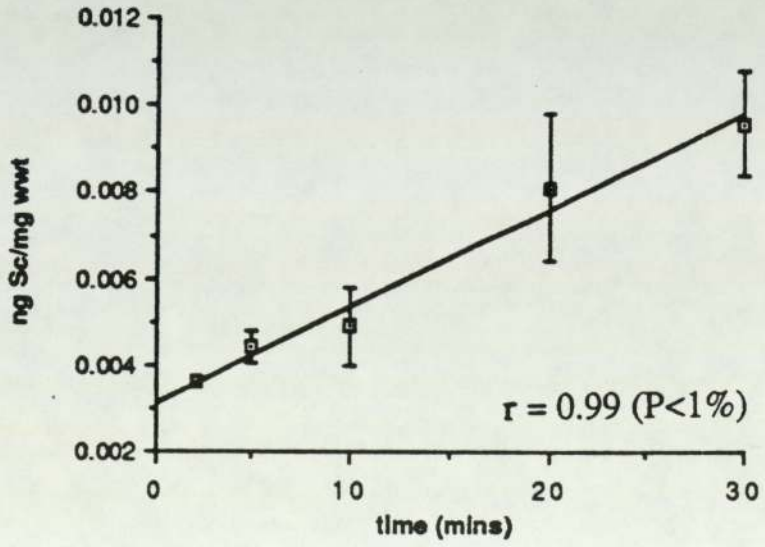


FIGURE 3.2B. TIME BASED SEROSAL TRANSFER OF SCANDIUM (500ng/ml $ScCl_3$) BY MID-JEJUNAL EVERTED SACS (n=5, ±SEM).



Notes
1). All results expressed as ng Sc / mg wet weight tissue (n=5, ±SEM for each observation).

FIGURE 3.3A. TIME BASED TISSUE UPTAKE OF GALLIUM (500ng/ml $Ga(NO_3)_3$) BY MID JEJUNAL EVERTED SACS (n=5, ±SEM).

(small round circles indicate a correction for gallium in ECF, see section 3.3.2.2. for further explanation)

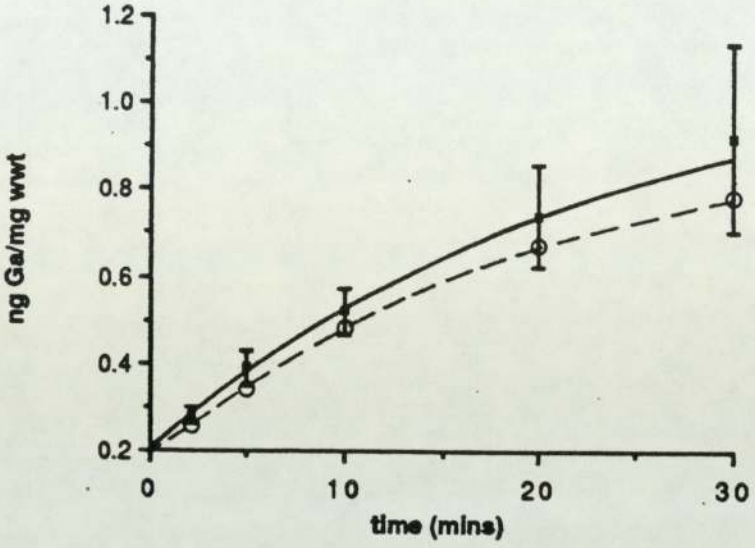
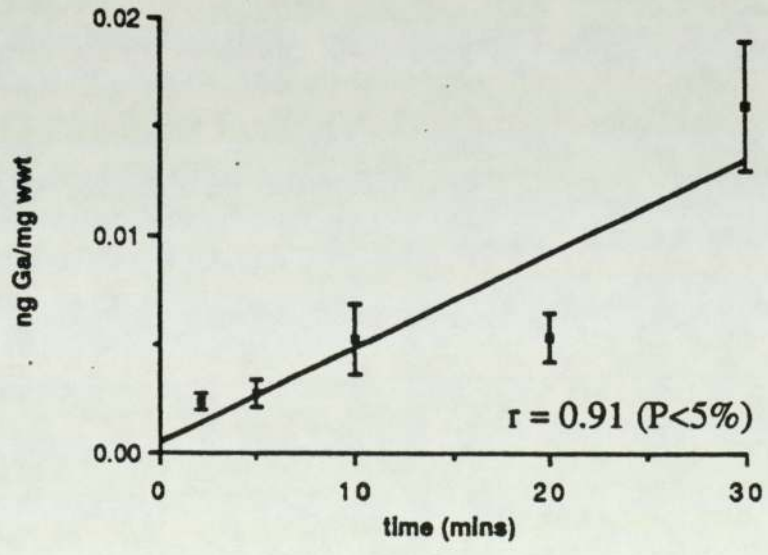


FIGURE 3.3B. TIME BASED SEROSAL TRANSFER OF GALLIUM (500ng/ml $Ga(NO_3)_3$) BY MID JEJUNAL EVERTED SACS (n=5, ±SEM).



Notes
1). All results expressed as ng Ga / mg wet weight tissue (n=5, ±SEM for each observation).

TABLE 3.1. REPRESENTATION OF JEJUNAL EVERTED SAC RESULTS AS A SEROSAL TO MUCOSAL RATIO (X100).

	S/M RATIO (X100)
ALUMINIUM	0.83 ±0.22
GALLIUM	0.78 ±0.18
SCANDIUM	0.31 ±0.032*

Notes.
1) The aluminium and gallium results do not significantly differ from one another, whilst the scandium ratio is significantly less (P<5%)

From the previous chapter it was observed that in the fasted state, (where a greater proportion of the element is expected to be in the soluble hydrated form, see chapter 5 for further details) scandium absorption was significantly less than that of aluminium and gallium, the *in-vitro* everted sac results confirming these observations.

3.3.2.2. MEASUREMENT OF EXTRACELLULAR FLUID (ECF) AND CORRECTION FOR GALLIUM PRESENT IN THE ECF.

A large molecular weight marker polyethylene glycol 900 (PEG900) was used to assess both extracellular fluid and the proportion of gallium in the mucosal tissue attributed to ECF. The experiments were performed in conjunction with the permeability studies used to assess viability (see 3.3.1).

The equilibrium of PEG900 in the ECF of the jejunal everted sac took less than 2 minutes to achieve (figure 3.4a). The average extracellular fluid volume over the 30 minute period of incubation was 165 ±2.58 µl ECF/g wwt (≈16.5% of tissue). Similar results have been achieved in human duodenal tissue by Cox and Peters (1979) using [^{57}Co]cyanocobalamin as the extracellular marker. Equilibrium of the marker, however was slower than PEG900, taking 5 minutes to plateau. This

FIGURE 3.4A. MEASUREMENT OF EXTRACELLULAR FLUID (ECF) IN MID-JEJUNAL EVERTED SACS (n=5, ±SEM).

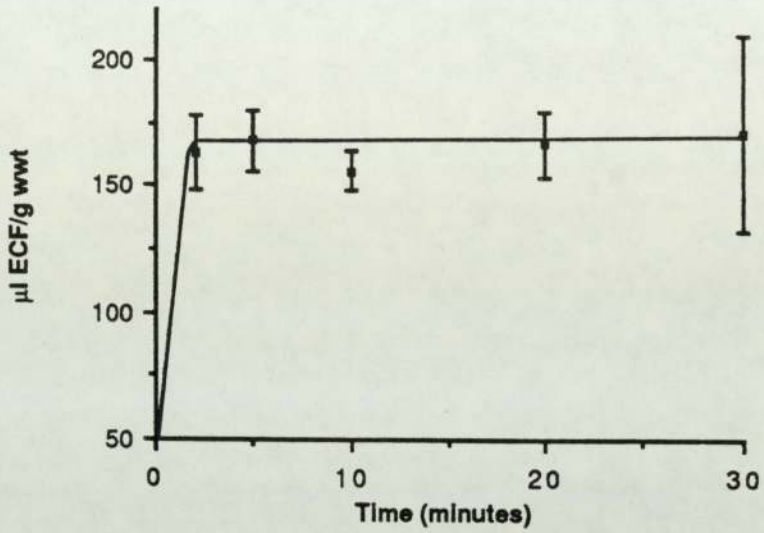
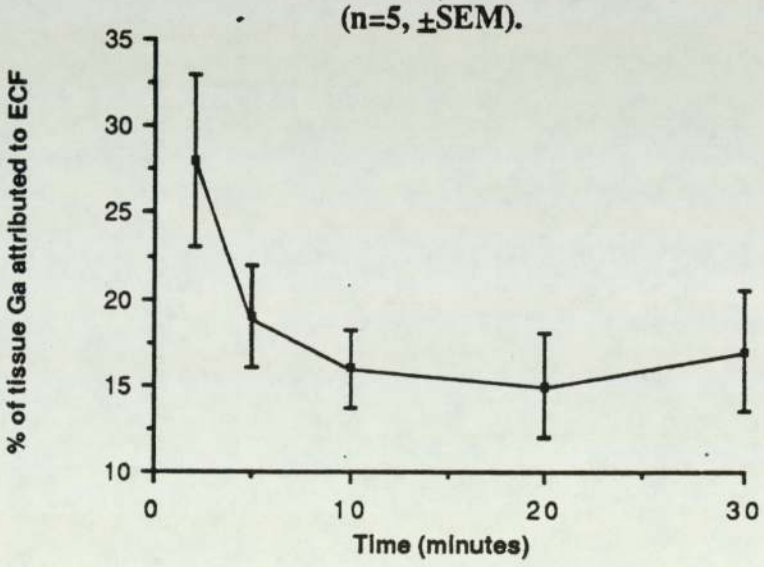


FIGURE 3.4B. PERCENTAGE OF TISSUE GALLIUM ATTRIBUTED TO ECF IN MID-JEJUNAL EVERTED SACS (n=5, ±SEM).



Notes
1).(n=5, ±SEM for each observation).

may be due to inter-species variation (man rather than rat) or that cyanocobalalamin has a larger molecular weight (1355) than PEG900, taking longer to diffuse into the extracellular spaces.

A wide variety of results have been recorded in the measurement of extracellular fluid, the values mainly due to the varying molecular weight markers used. Esposito and Csasky (1974) observed ECF's between 7.5% and 32% when using the molecular weight markers [^{14}C]inulin, [^{14}C]PEG, [^{3}H]inulin and [^{3}H]raffinose. Tritiated inulin and raffinose were considered to be unacceptable as ECF markers because of the wide variety of results measured compared to the constant results obtained with carbon labelled inulin and polyethlene glycol. The values achieved in this investigation however, were constant over the time range ($\approx$ 16.5%), similar levels being recorded in both duodenal and ileal mucosal tissue.

Measurement of gallium in the ECF allowed a correction to be made excluding gallium not associated with the tissue (see figure 3.3a). Although aluminium and scandium tissue absorption results were not corrected for ECF activity, the pattern of gallium tissue uptake after this correction factor had been applied did not change the overall pattern of absorption.

Generally over the 30 minute incubation period gallium in the extracellular fluid remains at a stable level (figure 3.4b). At the two minute incubation period however, the percentage of gallium in the extracellular fluid was significantly higher. This may represent a disequilibrium in the process of gallium movement into the extracellular spaces. Ando et al. (1985) have suggested that gallium binds rapidly to acid mucopolysaccharides and glycoproteins. As these molecules form the basis of the glycocalyx radiating from the brush border membrane and extending into the extracellular spaces it is possible that rapid binding to these structures occurs during the first few minutes of incubation. A rapid binding of aluminium, gallium and scandium was observed in the time-based studies

(3.3.2.1) before the slower phase of tissue uptake was observed. A similar rapid tissue uptake of aluminium has been observed by Van der Voet and de Wolff (1984) using an *in-vitro* perfusion technique.

3.3.3. CONCENTRATION BASED STUDIES.

Tissue uptake and serosal transfer of gallium by jejunal everted sacs over the concentration range 5ng/ml to 4000ng/ml $Ga(NO_3)_3$ was linear with time (figure 3.5a and 3.5b) This is further evidence of transport via a passive route probably via the tight junctions and paracellular spaces. The results are in contrast to those of Feinroth et al. (1982) who observed a plateau in both aluminium uptake and transfer at 1500ng/ml $Al(NO_3)_3$. As a result of this saturation and other inhibitory studies Feinroth et al. suggest that aluminium transport is via an 'energy-dependent, carrier-mediated mechanism'. The observed transport however, in their study was always down a concentration gradient and therefore active transport was not actually observed. It is possible that the plateau found was a result of aluminium forming insoluble complexes, this rendering it unavailable for absorption.

Both the time and concentration based studies in this thesis suggest linear transport and the lack of any inhibition of gallium tissue uptake or transport by the inhibitors 2,4-dinitrophenol and phloridzin (see next section) support these findings.

The results of this chapter agree with those of Provan and Yokel (1988) who likewise observed aluminium to be transported via a passive route. The large differences between mucosal uptake and serosal transfer of gallium have also been shown in aluminium studies by Van der Voet and de Wolff (1984).

FIGURE 3.5A. CONCENTRATION BASED TISSUE UPTAKE OF GALLIUM NITRATE BY MID JEJUNAL EVERTED SACS (n=5, ±SEM).

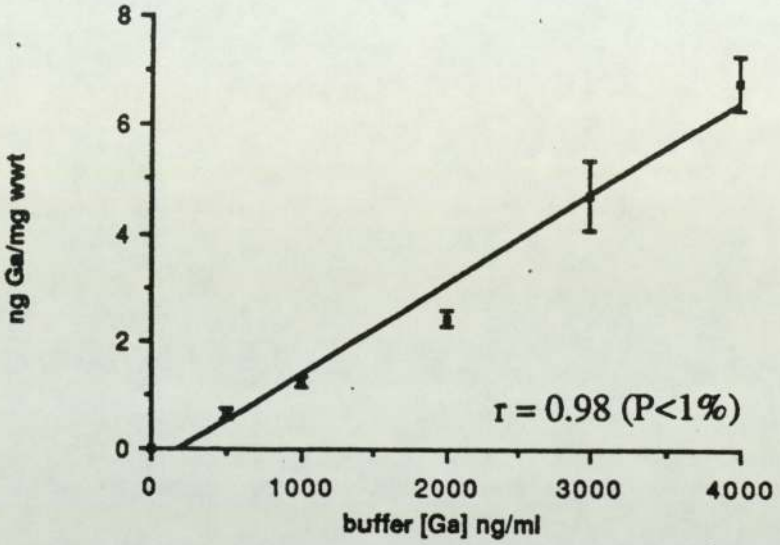
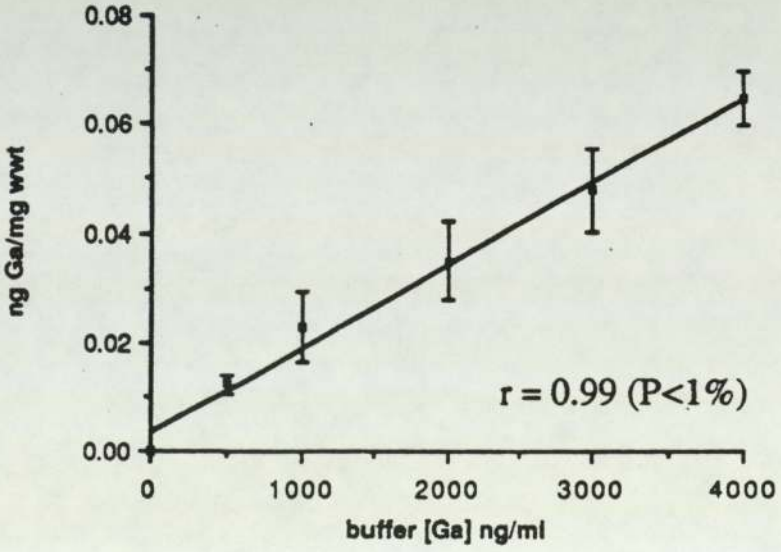


FIGURE 3.5B. CONCENTRATION BASED SEROSAL TRANSFER OF GALLIUM NITRATE BY MID JEJUNAL EVERTED SACS (n=5, ±SEM).



Notes.
1). All sacs incubated for 20 minutes in Krebs buffer, pH 7.4.
2). All results expressed as ng Gallium / mg wet weight tissue (n=5, ±SEM for each observation).

3.3.4. INHIBITOR STUDIES.

The mucosal uptake and serosal transfer of aluminium, scandium or gallium were not significantly altered by the addition of the metabolic inhibitor 2,4-dinitrophenol (10^{-4}M) to the mucosal incubating buffer (figures 3.6a and 3.6b). These results are further evidence to support the hypothesis that the Group 3 hydrated ion is passively transported via the paracellular route. The results are in agreement with those of de Wolff et al. (1987). If the results had suggested an active mechanism as proposed by Feinroth et al. (1982), this would have been confirmed by the inhibitor studies where dinitrophenol as a respiratory uncoupler would abolish any metabolically driven ion fluxes.

In experiments where two inhibitors were used (2,4-dinitrophenol and phloridzin, both at 10^{-4}M) there was again, no inhibition of gallium mucosal uptake or serosal transfer (figures 3.7a and 3.7b). The inhibitor sodium fluoride was not used because of the formation of insoluble gallium fluoride complexes (see 3.3.7).

3.3.5. EFFECT OF REDUCING pH ON THE UPTAKE AND TRANSFER OF GALLIUM.

Reducing the pH of the Krebs buffer from a physiological pH7.4 to 3.4 significantly increased both the mucosal uptake and serosal transfer of gallium (table 3.2).

FIGURE 3.6A. EFFECT OF THE INHIBITOR 2,4-DINITROPHENOL ON THE TISSUE UPTAKE OF ALUMINIUM, SCANDIUM AND GALLIUM (n=5, ±SEM).

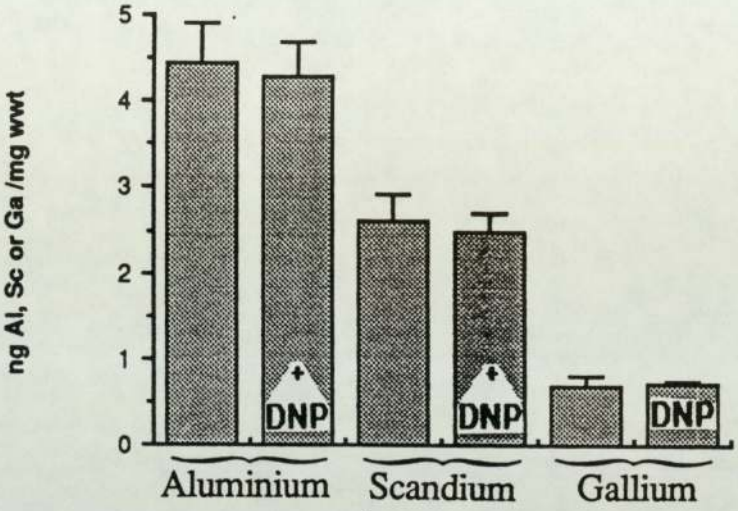
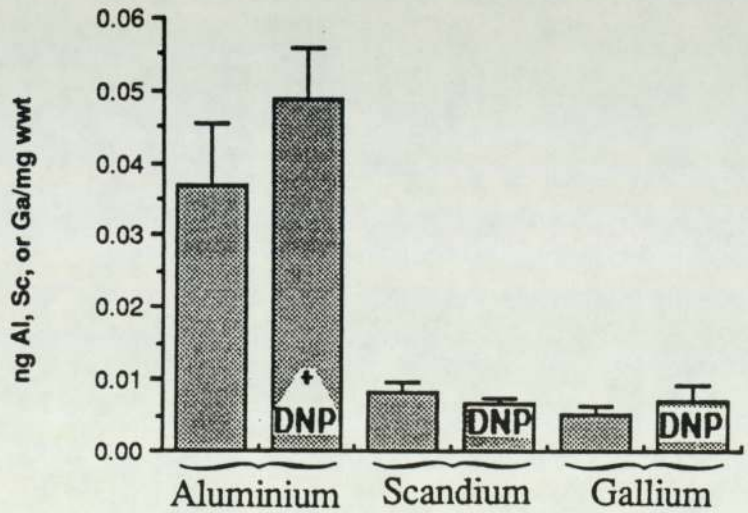


FIGURE 3.6B. EFFECT OF THE INHIBITOR 2,4-DINITROPHENOL ON THE SEROSAL TRANSFER OF ALUMINIUM, SCANDIUM AND GALLIUM (n=5,±SEM).



Notes.
1). $[Al(NO_3)_3]$ = 750 ng/ml, $[Ga(NO_3)_3]$ = 500ng/ml, $[ScCl_3]$ = 500ng/ml
2). [DNP] = 10^{-4}M.
3). All sacs incubated for 20 minutes.
4). No significant differences observed between test and control in any element.
5). All results expressed as ng Al, Ga or Sc / mg wet weight tissue.

FIGURE 3.7A. EFFECT OF THE INHIBITORS 2,4-DINITROPHENOL AND PHLORIDZIN ON THE TISSUE UPTAKE OF GALLIUM (n=5, ±SEM).

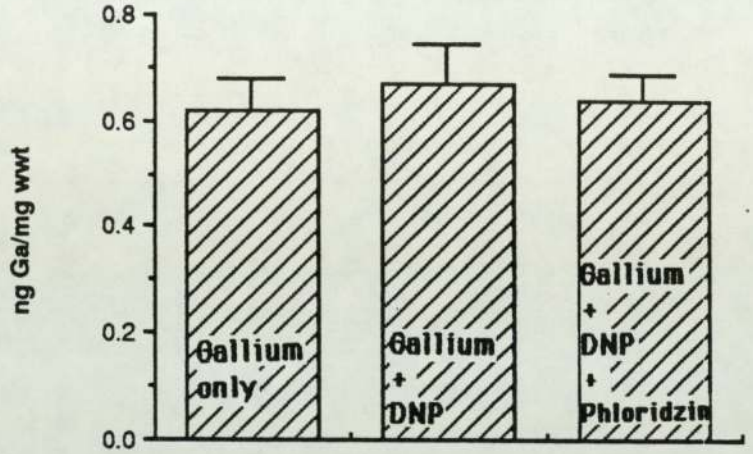
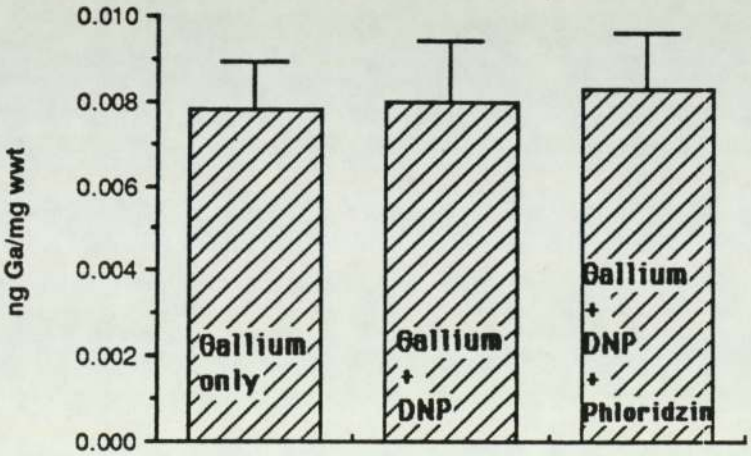


FIGURE 3.7B EFFECT OF THE INHIBITORS 2,4-DINITROPHENOL AND PHLORIDZIN ON THE SEROSAL TRANSFER OF GALLIUM (n=5, ±SEM).



Notes.
1). $[Ga(NO_3)_3]$ = 500ng/ml, [DNP] = 10^{-4}M, [phloridzin] = 10^{-4}M in Krebs buffer.
2). All sacs incubated for 20 minutes.
3). No significant differences observed.
4). All results expressed as ng Gallium / mg wet weight tissue.

TABLE 3.2. EFFECT OF REDUCING THE pH ON THE TISSUE UPTAKE AND SEROSAL TRANSFER OF GALLIUM.

	Tissue Uptake ng Ga/mg wwt	Serosal transfer ng Ga/mg wwt
pH 7.4		
Gallium only	0.56 ±0.034	0.0070 ±0.0013
Gallium + DNP	0.607 ±0.024	0.0069 ±0.0016
pH 3.4		
Gallium only	$1.13 \pm 0.10^{***}$	$0.0175 \pm 0.00147^{***}$
Gallium +DNP	$0.98 \pm 0.08^{**}$	$0.016 \pm 0.0011^{**}$

Notes.
1). Results expressed as ng Gallium taken up or transported per mg of initial wet weight tissue (±SEM, n=5).
2). Jejunal everted sacs incubated for 20 minutes.
3). $[Ga(NO_3)_3]$ = 500ng/ml, [DNP] = 10^{-4}M in standard Krebs buffer.
4). $^{*}P<5\%$, $^{**}P<1\%$, $^{***}P<0.1\%$, indicates a significant increase in gallium uptake and transfer when the pH is lowered to 3.4.

An increase in gallium uptake and transfer in everted sacs is probably due to the amphoteric nature of Group 3 elements. Gallium is hydrolysed at pH 7.4, the most probable species being the anionic $Ga(H_2O)_2(OH)_4^-$, with possibly a small proportion of the insoluble $Ga(H_2O)_3(OH)_3$ present (figure 1.1). With increasing acidity gallium becomes increasingly hydrated finally forming the hexahydrated $Ga(H_2O)_6^{3+}$ cation (see equation 1.1). At pH 3.4 therefore greater concentrations of gallium are available for absorption than at pH 7.4 and consequently increased uptake and transport is observed. Similar hydrolysis chemistry is seen for aluminium, and to a certain extent scandium (Baes and Mesmer, 1976). Van der Voet and de Wolff (1984, 1986) demonstrated similarly that a decrease in pH significantly increases the absorption of aluminium. Intraluminal pH, therefore, appears to be an important factor when considering the bioavailability of aluminium. In areas with lower than

normal pH (e.g. stomach, proximal duodenum) aluminium may be preferentially transported (Kaehny et al., 1977).

With a reduction in pH, there is a subsequent reduction in fluid transport (541±97 µl H_2O/g wwt at pH 7.4 to 285±29 µl H_2O/g wwt at pH 3.4, P<5%) due to the lowering of optimal absorptive processes at sub-physiological pH. If the passively transported gallium and aluminium ions moved concomitantly to the movement of water as do other cations in simple diffusion mechanisms (Turnberg, 1973) then gallium and aluminium uptake would be expected to decrease accordingly. The results therefore, indicate the increase in the concentration of 'free' gallium cations available at pH 3.4 when compared to pH 7.4 (see also figure 1.1).

3.3.6. EFFECT OF BUFFER TYPE ON THE UPTAKE AND TRANSFER OF GALLIUM.

Gallium uptake and transfer was assessed using five different buffer types (see 3.2.3). The results are outlined in figures 3.8a and 3.8b. Mucosal uptake of gallium is significantly increased in 3 buffers: the simple physiological solution, HEPES and Krebs-Tris buffer. The increase observed in the simple physiological solution may be due to an increased solubility (and therefore availability) of gallium, due to the lack of inorganic oxygen donor ligands in the buffer. The increased mucosal uptake of gallium in both HEPES and Krebs-Tris buffer is probably due to a soluble Ga-HEPES, or Ga-Tris species forming in preference to insoluble phosphate or hydroxide complexes.

Serosal transfer was not significantly increased in any of the buffers considered when compared to the normal Krebs buffer. There was however, a significant decrease in the transport of gallium in the simple physiological buffer (which was surprising since in this buffer the actual tissue uptake of gallium was increased).

FIGURE 3.8A. EFFECT OF BUFFER TYPE ON THE TISSUE UPTAKE OF GALLIUM (n=5, ±SEM).

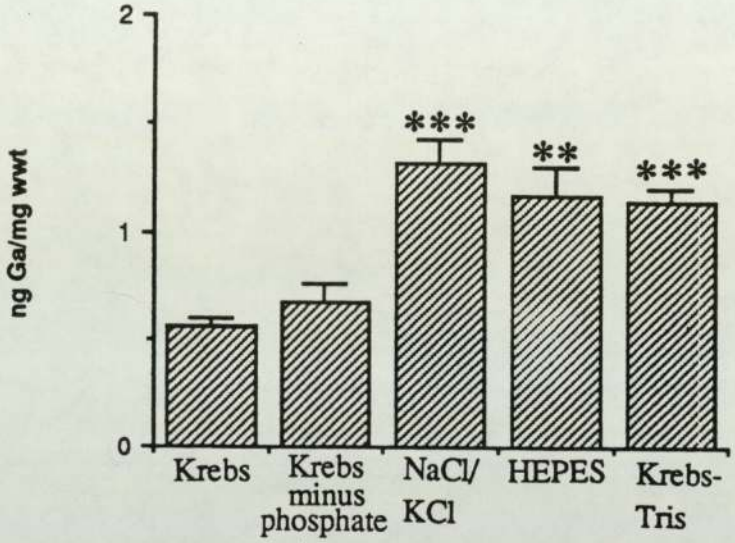
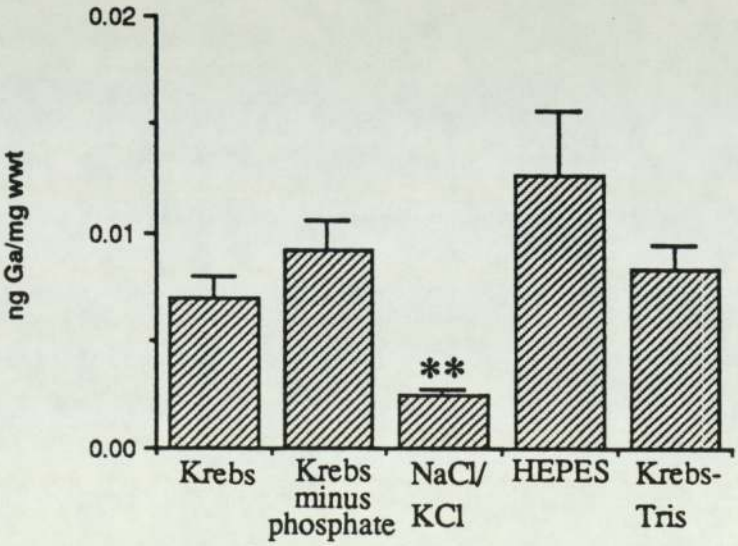


FIGURE 3.8B. EFFECT OF BUFFER TYPE ON THE SEROSAL TRANSFER OF GALLIUM (n=5, ±SEM).



Notes.
1). [$Ga(NO_3)_3$] = 500ng/ml
2). All sacs incubated for 20 minutes.
3). *P<5%, **P<1%, ***P<0.1% indicates a significant difference from the control (Krebs buffer) value.
4). All results expressed as ng Gallium / mg wet weight tissue.

An explanation for these observations may lie in the measurement of buffer osmolalities. The simple physiological buffer was on average 15% hypotonic (250 mOs/Kg) than the other buffers (288-294 mOs/Kg). A hypotonic buffer would cause increased water movement into the epithelial cell compressing the tight junctions (i.e. the 'leaky' tight junctions of the jejunum would become less leaky). If gallium transport occurred via the tight junctions (the evidence of this thesis so far points to a passive paracellular route) then reduced transport might occur due to the limited spaces at the tight junction complex.

3.3.7. EFFECT OF CITRATE, MALTOL AND FLUORIDE ON THE UPTAKE AND TRANSFER OF GALLIUM.

A). Citrate.

Mucosal uptake and serosal transfer of gallium in the presence of citrate was assessed at two pH's, 3.4 and 7.4 (figures 3.9a-d).

As seen previously (3.3.5) there is a significant increase in mucosal retention of gallium (with and without the presence of 2,4-dinitrophenol) when the pH is lowered to 3.4. The effect of citrate however, is to significantly reduce tissue uptake of gallium. This phenomena occurred at both pHs and in the presence of dinitrophenol (figures 3.9a and 3.9b). These differences are possibly due to the formation of different species of gallium in the test and control experiments. In the control experiments gallium was present in the Krebs buffer as the hydrated ion, with greater proportions of the cationic $Ga(H_2O)_6^{3+}$ species present at pH 3.4. Nicolson (1976) has found the glycocalyx to have a negative charge (to prevent adjacent cells from adhering), the brush border membrane therefore, having the capacity to attract highly charged cationic species. The rapid non-specific binding of aluminium, gallium and scandium to intestinal tissue has already been observed (3.3.2). In the presence of citrate however, it is possible that a neutral or negatively charged gallium-citrate species forms (Martin, 1986)

FIGURE 3.9. EFFECT OF CITRATE (1mM) ON THE UPTAKE AND TRANSFER OF GALLIUM (500ng/ml $Ga(NO_3)_3$) AT pH 3.4 AND 7.4 (n=5, ±SEM).

FIGURE 3.9A. pH 3.4. TISSUE UPTAKE

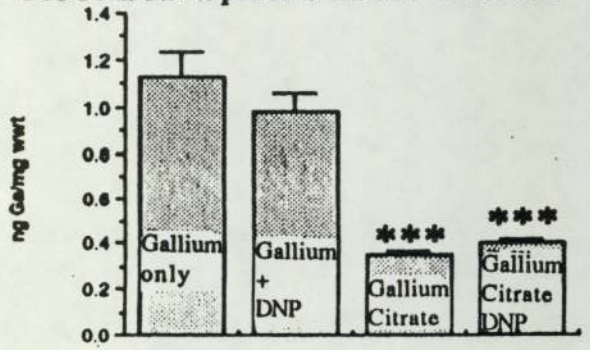


FIGURE 3.9B. pH 7.4. TISSUE UPTAKE

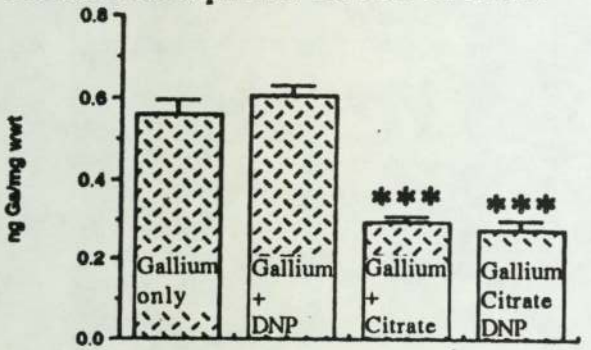


FIGURE 3.9C. pH 3.4. SEROSAL TRANSFER

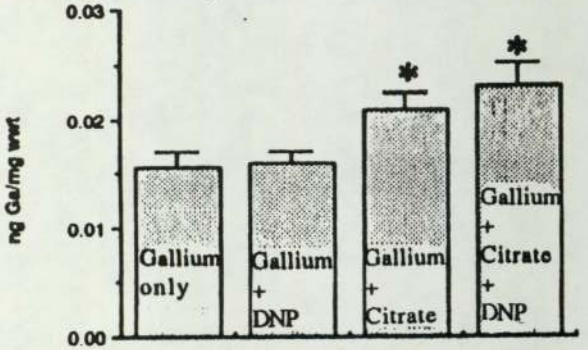
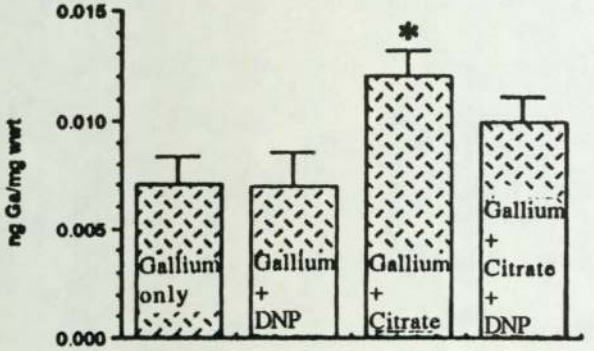


FIGURE 3.9D. pH 7.4. SEROSAL TRANSFER



Notes

1). All results expressed as ng Ga / mg wet weight tissue (n=5, ±SEM).
2). All sacs incubated for 20 minutes in Krebs buffer.
3). *P<5%, ***P<0.1%, indicates a significant difference from the control (either gallium only or gallium and DNP) value.

and that this complex did not avidly bind to the brush border membrane in the same manner as the gallium ion.

Although mucosal uptake of gallium in the presence of citrate was diminished, the actual transfer of gallium to the serosal side was significantly increased (figures 3.9c and 3.9d). It appears therefore, that although reduced levels of gallium are being taken up by the intestinal tissue, more transport is occurring. This is possibly due to the formation of neutrally charged gallium-citrate complexes predominant in the pH range 2-5. At pH 3.4 significantly more gallium in the presence of citrate is transported than at pH 7.4 (pH 3.4, 0.021$\pm$0.0015 ng Ga transported/mg wwt; pH 7.4, 0.012$\pm$0.0011 ng Ga transported /mg wwt; $P<1\%$). The neutrally charged gallium-citrate formed preferentially in the pH range 2-5 is thought to be membrane soluble, the intracellular route allowing greater levels of transport to be achieved compared to the rate-limiting paracellular route (thought to be the route for the hydrated uncomplexed gallium ion).

Studies with gel-filtration experiments (see chapter 5 for techniques) indicated that the gallium-citrate complex was present in the serosal incubating fluids and was therefore the transported species.

The addition of the metabolic inhibitor 2,4-dinitrophenol did not affect the serosal transfer of gallium either at pH 3.4 or 7.4 or with or without the presence of citrate (figure 3.9c & d).

The effect of citrate on the mucosal retention and transport of aluminium have been studied by de Wolff et al. (1987). Although they observed increased transport in the presence of citrate, they also found citric acid to stimulate the mucosal retention of aluminium. In the presence of dinitrophenol they observed both mucosal uptake and transfer of aluminium to be reduced. From these results they concluded that aluminium alone was transported passively whilst in the prsence of citrate , aluminium was transported via an energy-dependent process. The findings of this thesis do not support these observations.

B). Maltol.

The addition of the pyrone maltol to Krebs buffer did not significantly alter the mucosal uptake of gallium (figure 3.10a). Addition of the metabolic inhibitor DNP did not affect tissue uptake of gallium in the presence of maltol (figure 3.10a).

The serosal transfer of gallium however, was significantly enhanced in the presence of maltol (figure 3.10b). The inhibitor DNP did not significantly reduce this enhancement.

The *in-vitro* uptake of Fe^{3+} was enhanced in the presence of maltol (Barrand et al. 1986). However, as they used *in-vitro* intestinal tissue slices it was impossible to differentiate between non-specific tissue uptake and actual transfer of material.

Maltol has been shown to bind to aluminium, gallium and iron in a 3:1 ratio (Finnegan et al. 1987; Barrand et al. 1987) forming a neutrally charged complex which is stable over the pH range 2-8. Partition coefficient characteristics indicate that the species (like-that of gallium-citrate) would favour a transmembranal diffusion route (Bakaj, 1984). Ion-exchange chromatography (see chapter 5) indicated that the gallium-maltol species was transported across the basolateral membranes and was present in the serosal compartment.

The results of this *in-vitro* everted sac study with gallium and maltol support the findings of the tissue distribution experiments discussed in the previous chapter.

C).Fluoride.

The addition of fluoride to the incubating fluids did not alter the tissue uptake of gallium (figure 3.11a). Again the presence of dinitrophenol did not have any further effects upon tissue uptake either when gallium or gallium and fluoride were present in the Krebs buffer (3.11a).

The serosal transfer of gallium however, was significantly inhibited in the presence of fluoride (figure 3.11b). Addition of dinitrophenol did not cause any

FIGURE 3.10. EFFECT OF MALTOL (20μM) ON THE UPTAKE AND TRANSFER OF GALLIUM (500ng/ml $Ga(NO_3)_3$) (n=5, ±SEM).

FIGURE 3.10A. TISSUE UPTAKE

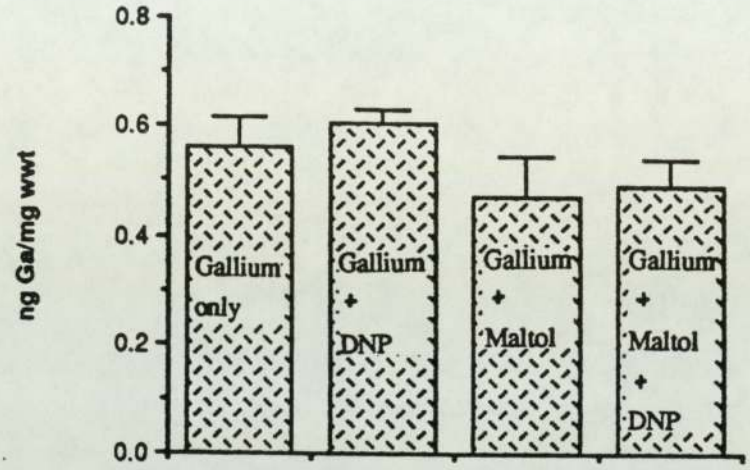
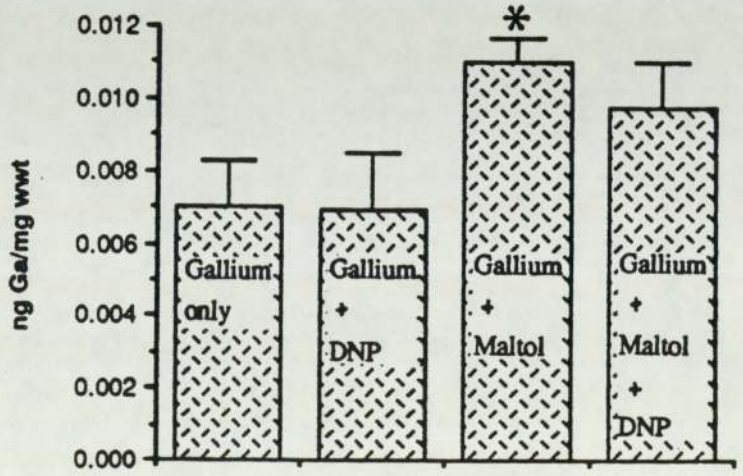


FIGURE 3.10B. SEROSAL TRANSFER.



Notes.
1). All sacs incubated for 20 minutes in Krebs buffer, pH 7.4.
2). All results expressed as ng Gallium / mg wet weight tissue
3). *P<5%, indicates a significant difference from the control (gallium only) value.

FIGURE 3.11. EFFECT OF FLUORIDE (0.5mM) ON THE UPTAKE AND TRANSFER OF GALLIUM (500ng/ml $Ga(NO_3)_3$) (n=5, ±SEM).

FIGURE 3.11A. TISSUE UPTAKE

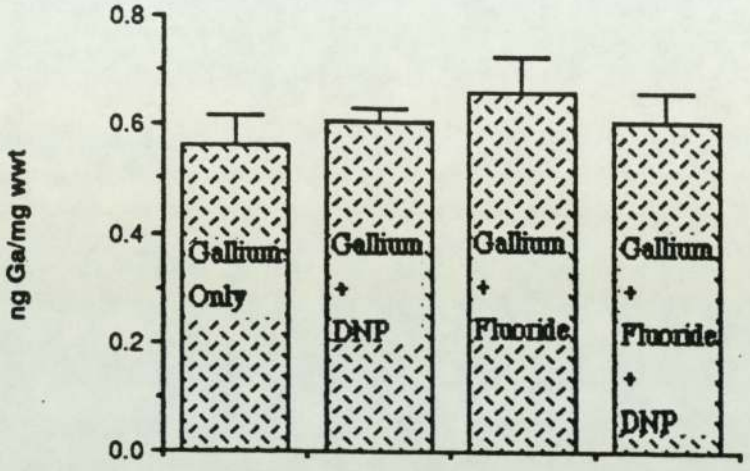
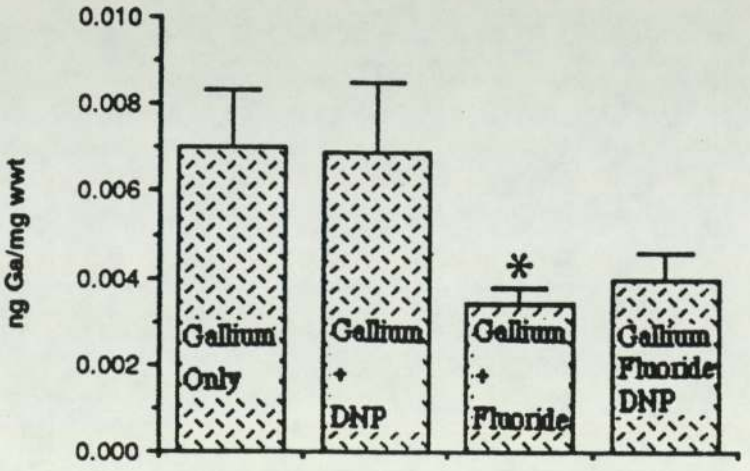


FIGURE 3.11B SEROSAL TRANSFER.



Notes.
1). All everted sacs incubated for 20 minutes in Krebs buffer.
2). Results expressed as ng Gallium / mg wet weight tissue.
3). *P<5%, indicates a significant difference from the control (gallium only) value.

further reduction of gallium transfer in the presence of fluoride (figure 3.11b). Aluminium and gallium forms strong complexes with fluoride and unlike the species formed with citrate and maltol these species are not membrane diffusible. The complexes formed with fluoride (AlF_3 and GaF_3) are also likely to be excluded from the tight-junctions causing a reduction in transport. An intraluminal interaction of aluminium and fluoride has been previously observed by Spencer et al. (1981) causing a simultaneous decreased absorption of both aluminium and fluoride (see previous chapter).

It could be argued that as NaF (sodium fluoride) is a metabolic inhibitor of glycolysis, the observed reduction of gallium transport in the presence of fluoride was due to an inhibitory effect rather than a gallium-fluoride complex reducing the amount of 'free' gallium available for transfer. Addition of other metabolic inhibitors, dinitrophenol and phloridzin did not reduce transport of gallium, therefore it was unlikely that NaF would act in this way.

3.4 CONCLUSIONS.

The mucosal uptake and serosal transfer of aluminium, gallium and scandium by everted intestinal sacs has been assessed in various physiological situations.

After preliminary viability studies to ensure the suitability of the everted sac preparation for metal ion studies, aluminium, gallium and scandium uptake was measured and found to be passive and energy independent. Both time-based and concentration based kinetic studies were undertaken. In the time based experiments aluminium, scandium and gallium saturated the non-specific binding sites available at the brush-border surface. Transport to the serosal compartment was however, linear with time. The concentration based uptake and transfer of gallium (at a constant 20 minutes incubation) was linear. Metabolic inhibitors did not affect the results of the above experiments.

Reducing the pH of the incubating buffer was found to significantly increase both mucosal uptake and serosal transport of gallium. This is probably due to the amphoteric nature of the metal, a greater proportion of the readily transported $Ga(H_2O)_6^{3+}$ cation being present at pH 3.4 than at 7.4.

Buffer type also influenced the mucosal uptake and serosal transfer of gallium, the main differences observed in the presence of soluble metallo-organic complexes (i.e. HEPES and Krebs-Tris).

Citrate, maltol and fluoride affected the serosal transfer of gallium, citrate only affecting the mucosal uptake of gallium. The formation of neutral membrane soluble species in the presence of citrate and maltol enhanced the serosal transfer of gallium, whilst the formation of insoluble gallium fluoride reduced the transfer of gallium. The metabolic inhibitor 2,4-dinitrophenol did not affect uptake or transfer of gallium in the presence of citrate, maltol or fluoride.

The above results are consistent with mucosal tissue binding and serosal transfer of a passive nature. This appears to be the case when gallium is present as a hydrated ion (transport via the paracellular tight-junctions) or when gallium is in formation with a membrane soluble ligand (transport via a transmembranal route).

In general the results of this *in-vitro* intestinal chapter support the findings of the previous chapter. The magnitude of serosal transfer, when compared to tissue uptake is considerably less (by approximately 100 times less in the case of aluminium and gallium, 300 times less in the case of scandium). This emphasises the avidity of aluminium, gallium and scandium for the mucosal surface, but the minute proportions transported. Similarly in the tissue distribution experiments less then one hundreth of the original dose was observed in the tissues.

A similar enhanced absorption of gallium in the presence of citrate and maltol was observed in fasted rats, whilst fluoride inhibited the absorption of gallium in both fed and fasted rats. The behaviour of complexing agents therefore appears to affect the bioavailability of gallium in both *in-vivo* and *in-vitro* systems.

CHAPTER FOUR

IN-VIVO INTESTINAL TRANSPORT STUDIES

CHAPTER 4 *IN-VIVO* INTESTINAL TRANSPORT STUDIES.

4.1. INTRODUCTION.

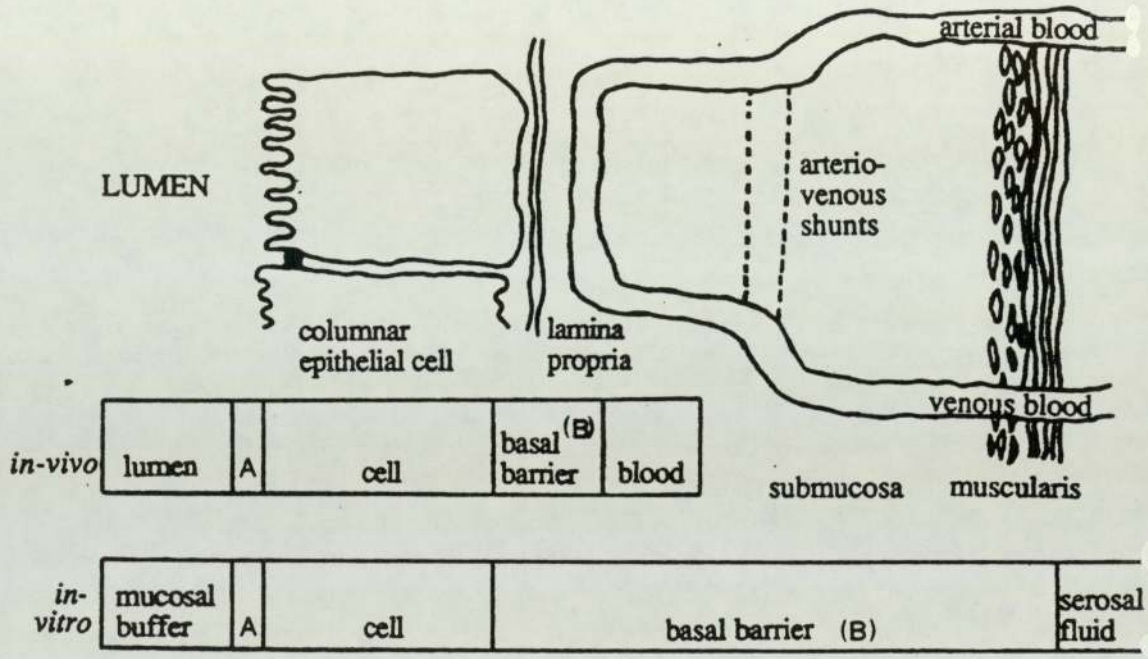
4.1.1. THE *IN-VIVO* PERFUSION SYSTEM AND VIABILITY STUDIES.

The main advantage of an *in-vivo* preparation is the maintenance of the mesenteric blood supply. Temporary anoxia is probably the main cause of loss of viability in an *in-vitro* preparation (Plumb et al. 1987). In the *in-vivo* preparation the mesenteric blood supply is immediately adjacent to the absorptive epithelial cell ensuring a continual supply of oxygen. In addition to supplying oxygen and other nutrients the proximity of the blood supply has the advantage of being able to transport any substances away from the epithelia and thus avoid any build up of material creating a rate-limiting barrier. In the *in-vitro* everted sac substances must pass through both the epithelial barrier and the basal barrier (consisting of lamina propria, submucosa and muscularis) before appearing in the serosal compartment. The differences between the *in-vitro* and *in-vivo* barriers are shown in figure 4.1. As previously mentioned in Chapter 3 there is a need for stringent viability testing in *in-vitro* preparations. The same applies for *in-vivo* systems although some of the previously mentioned tests cannot be applied (it is difficult to achieve transmural potential difference readings or to measure water uptake into epithelial cells).

It is possible to quantify absorption of glucose (measured by luminal loss) over four, 15 minute incubation periods. Uptake of glucose in an *in-vivo* situation should be continual as the system should not show any capacity to accumulate.

The permeation of polyethylene glycol (PEG900) was also used to assess the viability of the preparation. Polyethylene glycols are synthesized to varying molecular weights, the greater the molecular weight (e.g. PEG4000), the more useful the polymer as an impermeability marker (Hamilton, 1986). In addition to being used as a permeability marker (thus giving an idea of the structural integrity

FIGURE 4.1. SCHEMATIC DIAGRAM OF LONGITUDINAL SECTION OF INTESTINE COMPARING *IN-VIVO* AND *IN-VITRO* TISSUE PREPARATIONS (REDRAWN FROM BARR & RIEGELMAN, 1970)



Legend
A= apical barrier (same for both *in-vivo* and *in-vitro* systems).
B= basal barrier (note the increased width of basal barrier in the *in-vitro* system creating a rate-limiting step for the appearance of material in the serosal fluid).

of the intestine) polyethlylene glycol can be used to quantify extracelluar fluid (ECF). The measurement of ECF is used as a further test of viability in this *in-vivo* perfusion system. Other molecules utilised as ECF and impermeability/permeability markers (particularly in the assessment of coeliac disease, Crohns disease and other intestinal abnormalities) are [^{57}Co]cyanocobalamin (vitamin B_{12}), [^{51}Cr]EDTA and mannitol (Schutz and Rezenstein, 1963; Laker et al. 1982; Bjarnason and Peters, 1984).

4.1.2. THE *IN-VIVO* INTESTINAL ABSORPTION OF GALLIUM.

The previous *in-vitro* everted sac results (Chapter 3) highlighted the large proportion of tissue binding of aluminium, gallium and scandium in comparison to the amounts actually transported. A similar phenomonen was observed by Van der Voet and de Wolff (1984) when measuring the *in-vivo* intestinal absorption of aluminium. It is for this reason that disappearance of the metal ion from the perfusate cannot wholly represent the actual transport of a substance into the mesenteric blood supply. In addition to luminal loss measurements (probably reflecting non-specific tissue binding not wholly related to transport) further measurements involving collection of hepatic portal blood should be made to assess the transported species. Van der Voet and de Wolff (1984) cannulated the hepatic portal vein, diverting the blood supply into a loop which could be sampled at frequent intervals for a kinetic assessment of transport mechanisms. Technically this operation proved difficult to achieve due to the low pressure of the mesenteric bed and hepatic portal vein blood flow. It was decided therefore, to remove the visceral organs (liver, kidney and spleen) and a small volume of blood at the end of the experiment, and to use the ^{67}Ga content of these tissues as a measure of transported gallium.

There are however, complicating factors of metabolism and tissue distribution within the body after gallium has been transported (for example gallium, scandium

and aluminium showed a particular accumulation in bone, see 2.3.3). The measure of ^{67}Ga in liver, kidney, spleen and blood is therefore only intended to be a 'comparative' measure of transport (e.g. against different buffer gallium concentrations, or to assess the effects of complexing agents in the incubating buffer). It is not intended to measure total gallium transport from the *in-vivo* preparation or to measure rates of transfer.

4.2. MATERIALS AND METHODS.

4.2.1. ANIMALS. See section 2.2.1.

4.2.2. CHEMICALS. See section 2.2.2.

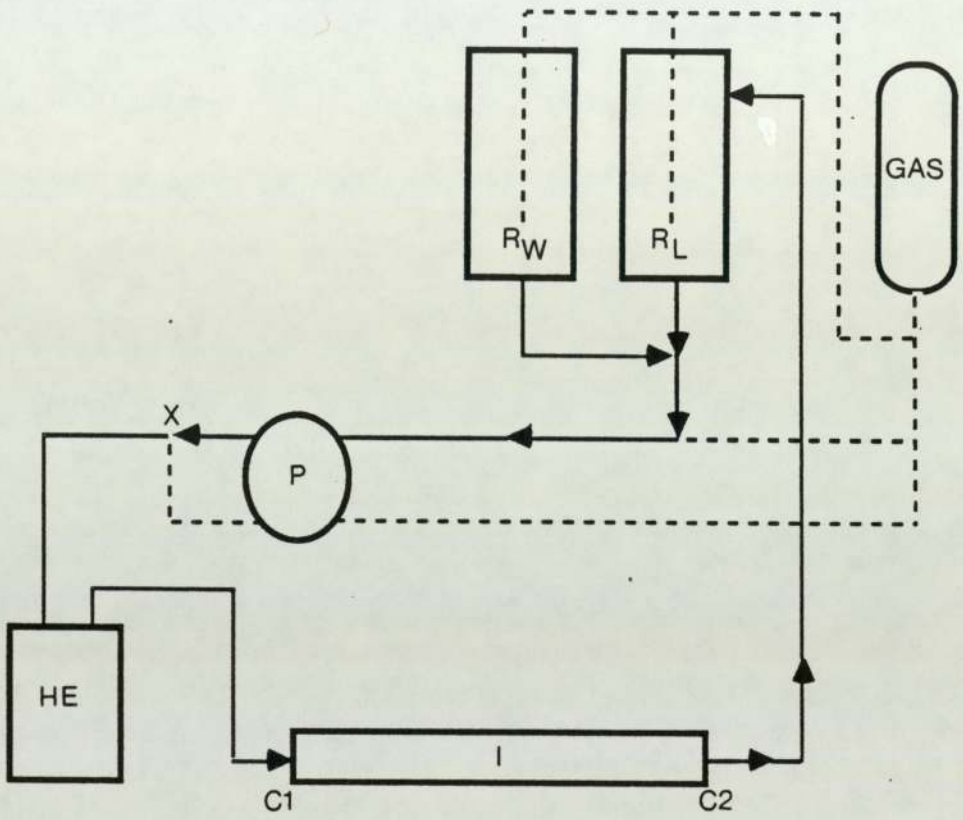
4.2.3. PHYSIOLOGICAL BUFFERS.

All studies used a Krebs buffer (see 3.2.3). $Ga(NO_3)_3$ concentrations in the buffer ranged from 5-4000ng/ml. 10μCi ^{67}Ga was added to each individual 25ml perfusion solution. In the viability experiments 0.2μCi $^{3H}PEG900$ was added to each solution. Additions to the Krebs buffer were 2,4-dinitrophenol ($10^{-4}M$), citrate (1mM), maltol (20μM) and fluoride (0.5mM).

4.2.4. PERFUSION APPARATUS

The perfusion apparatus centred around two reservoirs, the perfusion reservoir and the washout reservoir (see figure 4.2). The temperature of both containers was maintained at 37°C by the circulation of water from a heated bath, through jackets surrounding each of the reservoirs. A 3-way tap allowed either the perfusate or the washout medium to perfuse the lumen of the small intestine. This was essential for the cleaning of the gut in between perfusion periods. A heat exchanger was placed prior to the inlet cannula (figure 4.2) to maintain the perfusate at 37°C and a 60W lamp shone approximately 45cm above the rat to ensure bodily warmth.

FIGURE 4.2. SCHEMATIC DIAGRAM OF PERFUSION APPARATUS.



Legend

R_L - luminal reservoir

R_W - washout reservoir

P - peristaltic pump

HE - heat exchanger

I - intestine

X - segmented flow created

C1 - inlet cannula

C2 - outlet cannula

Dotted lines - flow of gas

Solid lines - fluid movement

4.2.5. PERFUSION PROCEDURE

Neuroleptanalgesia was obtained by an intramuscular injection of Hypnorm (fentanyl citrate), 0.3ml/kg body weight. A further intraperitoneal injection of diazepam (2.5mg/kg body weight) was needed to provide enough muscle relaxation for intra-abdominal surgery (Green, 1982). The abdomen was opened with a 3cm midline incision and the intestines wetted with 5ml warm 0.9% saline. A glass cannula (length 2.5cm, external diameter 4mm, internal diameter 2.5mm) was inserted 3cm distal to the ligature of Trietz and the duodenum tied off to prevent leakage. Approximately 20cm of jejunum was located and a small incision made into the intestine. Prior to the insertion of the distal cannula the jejunum was washed with 20ml warm Krebs buffer and the lower jejunum tied off, again to prevent leakage. The cannulated intestine was placed back into the body cavity and the incision covered with cotton wool gauze soaked in 0.9% saline. The perfusion medium (25mls Krebs + gallium) was pumped (Gilson Minipuls peristaltic pump, Villiers-le-Bel, France) through the system at a flow rate of 1.8mls min^{-1} using segmented flow. Each of the reservoirs was aspirated with 95% O_2/5% CO_2.

A 15 minute perfusion period was timed from when the segmented flow of KHBB reached the inlet cannula. Each perfusion experiment consisted of four, 15 minute perfusion periods. At the end of each 15 minute period the intestine was perfused with 'washout' medium (Krebs minus gallium). Both perfusate and washout media were collected at the end of this 23 minute period and the solution made up to 100mls. Aliquots of the solution were removed and counted for ^{67}Ga activity. Fresh solutions were introduced into the perfusate reservoir at the beginning of each 15 minute period.

Mucosal uptake of gallium was measured by luminal loss of gallium from the perfusate. At the end of each experiment (i.e. after four, 15 minute perfusion periods, and four, 8 minute washout periods) the liver, kidneys and spleen and

1ml blood were removed for counting of ^{67}Ga. To a limited extent the gallium content of these tissues allows a measure of transport to be assessed.

4.2.6. METHODS OF ASSESSING VIABILITY

4.2.6.1. GLUCOSE UPTAKE

The depletion of glucose in the perfusate over the four, 15 minute perfusion periods was measured by the method of Morton (1982), (3.2.5.3).

4.2.6.2. PERMEABILITY OF POLYETHYLENE GLYCOL (PEG900)

0.2μCi ^{3H}PEG900 was added to each 25ml perfusate fluid. At the end of the experiment blood was drawn from the animal whilst under anaesthetic. 50μl blood was added to 1ml of the tissue solubilizer 'Optisolve' and heated to 50°C until digested. ^{3H}PEG900 was counted following the procedures outlined in 3.2.5.5.

4.2.6.3. MEASUREMENT OF EXTRACELLULAR FLUID (ECF).

Measurement of ECF by ^{3H}PEG900 was performed in conjunction with the above experiment (4.2.6.2). The procedure was similar to that of 3.2.5.5.

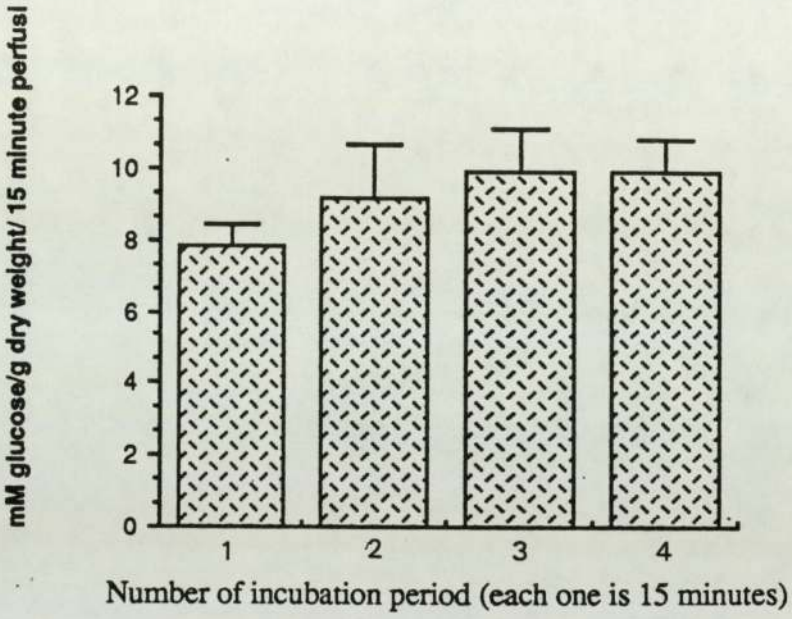
4.3. RESULTS AND DISCUSSION.

4.3.1. VIABILITY EXPERIMENTS

A). Luminal loss of glucose.

The depletion of glucose from the perfusate (i.e. uptake of glucose from the intestine) was measured over the four, 15 minute incubation periods. A one-way analysis of variance recorded there to be no difference between any of the four incubation periods (figure 4.3). Addition of $Ga(NO_3)_3$ to the incubating buffer did not significantly affect the depletion of glucose from the perfusate.

FIGURE 4.3. LUMINAL LOSS OF GLUCOSE MEASURED OVER 4, 15 MINUTE INCUBATION PERIODS.



Notes.
1). Uptake of glucose measured by luminal loss.
2). A one-way analysis of variance indicated there to be no significant difference in uptake between the 4 periods.
3). n=4, ±SEM for each period.

B). Permeation of polyethylene glycol (PEG900).

Experiments measuring the permeation of PEG900 were performed in conjunction with the above glucose viability tests. Results were expressed as a percentage of the total PEG900 exposed to the intestine to what was present per ml of blood. Approximately 0.13% ($\pm$0.012, n=4) was found per ml of blood at the end of the four 15 minute incubation periods. If the PEG molecule is assumed to be inert and not metabolised in any manner, the majority of permeated PEG900 would expected to be found in the blood system before being passed into the kidneys for subsequent excretion. If a 180g rat has 12.2mls of blood in its circulatory system (6.78 mls blood/100g body weight, unpublished observations) then 1.58% of the total PEG900 in the circulatory fluids would have permeated the epithelial barrier (assuming at this stage that no PEG900 had passed into the bladder). Bjarnason et al. (1983) measured the urinary excretion of the permeability marker [^{51}Cr]EDTA in control and coeliac diseased patients. After 3 hours the urinary excretion of [^{51}Cr]EDTA in control patients is less than 1% (obviously some [^{51}Cr]EDTA would be still present in blood), the remaining [^{51}Cr]EDTA being excreted over a 24 hour period bringing the percentage of absorbed marker to just below 2.5%. The results of this viability test indicate that the intesine of the *in-vivo* perfused rat is structurally intact and suitable for further metal ion absorptive studies.

C). Measurement of extracellular fluid (ECF).

Measurement of ECF was again performed in conjunction with the above permeability experiments. The average extracellular fluid volume over the four, 15 minute incubation period was 102.5µl ECF/g wwt (10.25% $\pm$0.47, n=4).

4.3.2. CONCENTRATION BASED UPTAKE OF GALLIUM.

Tissue uptake of gallium (measured as a result of luminal loss of the perfusate) was linear with increasing concentration over the range 5ng/ml to 4000ng/ml

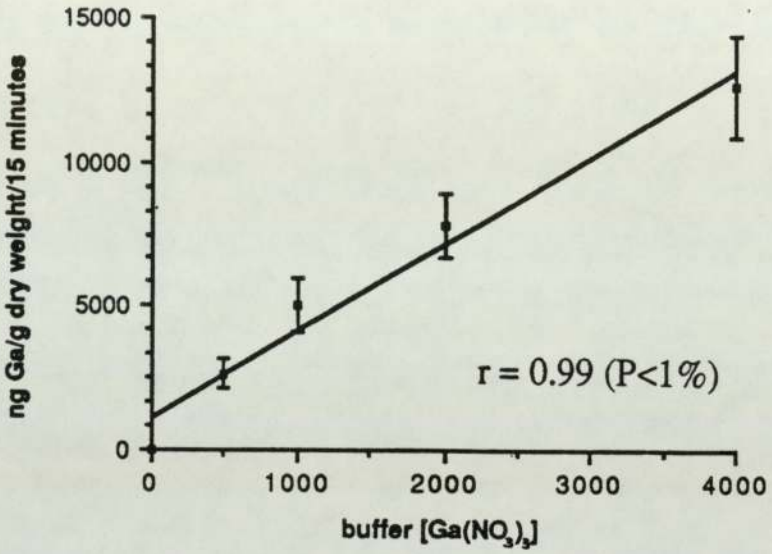
$Ga(NO_3)_3$ (figure 4.4). The results support the findings of the *in-vitro* everted sac concentration based experiments suggesting passive mechanisms of absorption. At all five concentrations measured a one-way analysis of variance indicated that significantly greater amounts of gallium were absorbed by the mucosal tissue in the first 15 minute period than in the subsequent three 15 minute incubations periods (see figure 4.5 for examples). A similar phenomenon was observed with the rapid binding of aluminium, gallium and scandium in the first few minutes of incubation with jejunal everted sacs. Van der Voet and de Wolff (1984) noted a similar rapid tissue binding of aluminium with their perfusion/cannulation technique.

The transport of gallium as measured by the sum of ^{67}Ga activity in liver, kidney and spleen show a similar linear uptake with increasing buffer gallium concentration, as does transport measured by blood ^{67}Ga activity (figures 4.6a/b). The actual amounts of gallium quantified in the tissues and blood represent a minute proportion of the gallium which is actually tissue bound in the lumen of the intestine. Similar observations have been made in both the tissue distribution experiments (chapter 2) and *in-vitro* everted sac experiments (chapter 3).

4.3.3. INHIBITOR STUDIES

Addition of the metabolic inhibitor 2,4-dinitrophenol ($10^{-4}M$) to the incubating buffer at both 5ng/ml and 4000ng/ml $Ga(NO_3)_3$ did not affect either the tissue uptake or the transfer of gallium as measured in liver, kidney, spleen and blood (figures 4.7/4.8). This is further evidence to confirm that the *in-vivo* intestinal absorption of gallium is via a passive energy-independent route. Proven and Yokel (1988) found aluminium uptake to be similarly unaffected by the inhibitor 2,4-dinitrophenol in an *in-situ* rat gut preparation similar to the technique used in this thesis.

FIGURE 4.4. CONCENTRATION BASED UPTAKE OF GALLIUM.



Notes
1). Intestinal uptake of gallium measured by luminal loss (and therefore includes transported gallium).
2). Uptake measured as ng Ga / gram of dry weight tissue over the 15 minute incubation period.
3). Each point is the mean of 4 values corresponding to uptake over each four 15 minute perfusion period (n=4, ±SEM).

FIGURE 4.5. TISSUE UPTAKE OF GALLIUM INDICATING THE LARGE PROPORTION OF UPTAKE IN THE FIRST INCUBATION PERIOD.

FIGURE 4.5A. At 500ng/ml $Ga(NO_3)_3$.

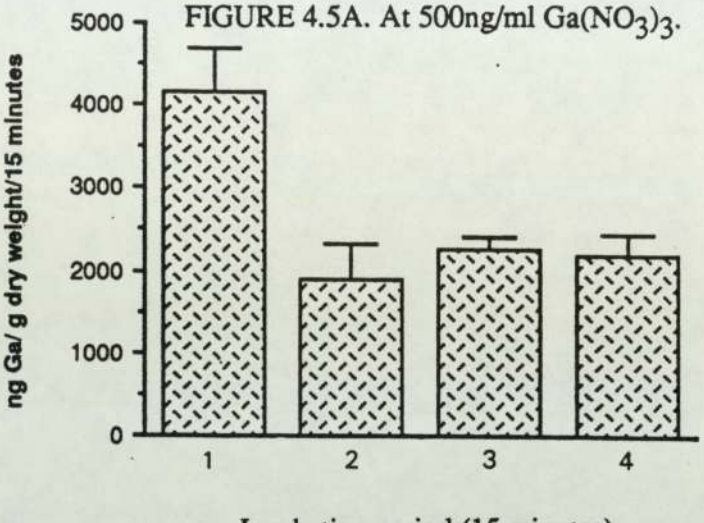
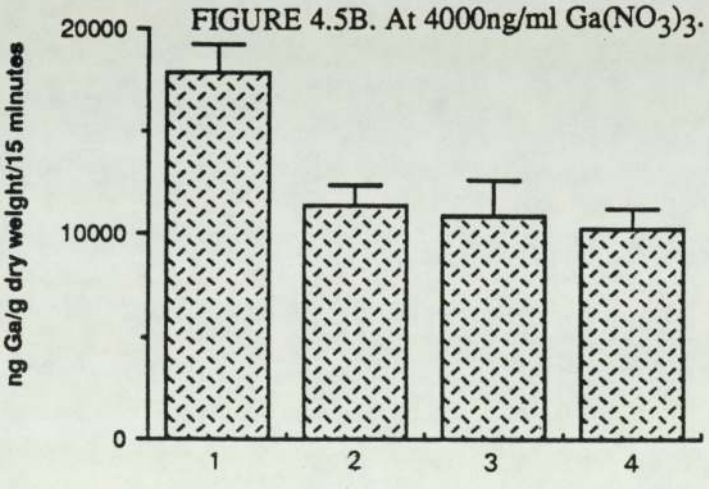


FIGURE 4.5B. At 4000ng/ml $Ga(NO_3)_3$.



Notes

1). Intestinal uptake of gallium measured by luminal loss (and therefore includes transported gallium)

2). Uptake measured as ng Ga / gram of dry weight tissue over each 15 minute incubation period.

3). A one way analysis of variance indicates that significantly more gallium was absorbed in the first incubation period compared to the remaining three.

4). Similar results recorded for the other concentrations measured (see figure 4.4).

FIGURE 4.6. TRANSPORT OF GALLIUM INTO LIVER, KIDNEY AND SPLEEN (FIG 4.6A) AND BLOOD (FIG. 4.6B).

FIGURE 4.6A. GALLIUM IN LIVER, KIDNEY AND SPLEEN.

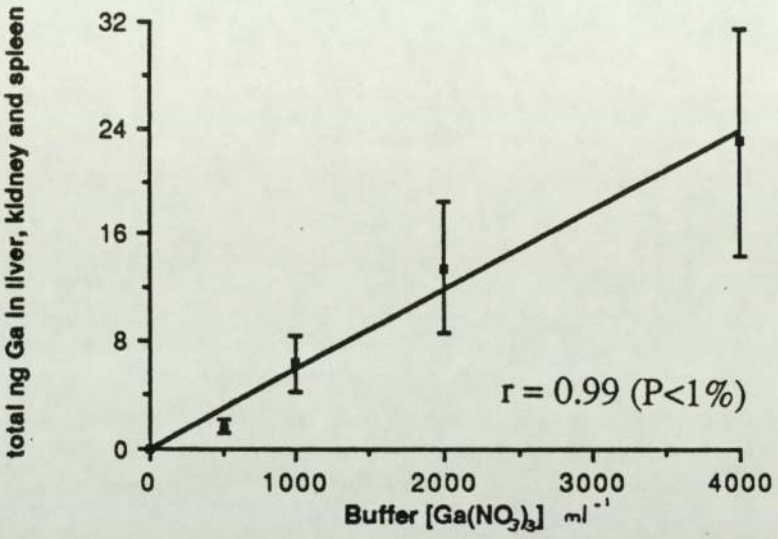
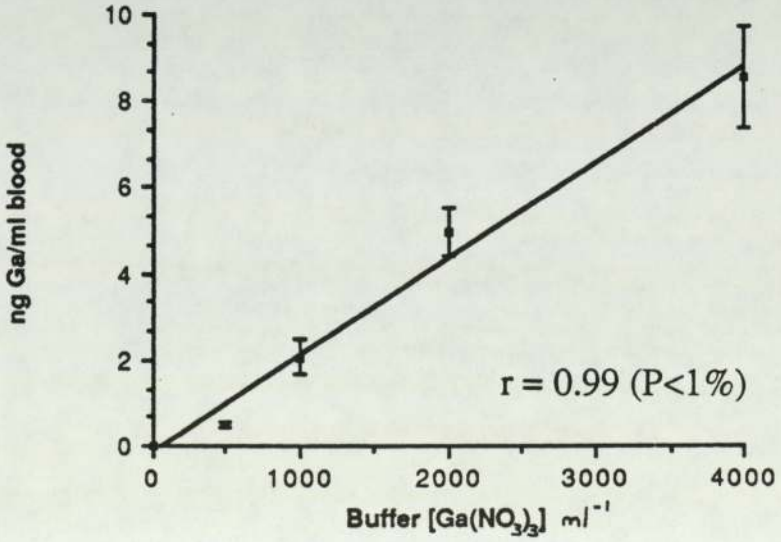


FIGURE 4.6B. GALLIUM PER ml OF BLOOD.



Notes
1). Tissues and blood taken from the rat at the end of four, 15 minute incubation periods (n=4, ±SEM).

FIGURE 4.7. EFFECT OF 2,4-DINITROPHENOL ON THE TISSUE UPTAKE OF GALLIUM AT 5ng/ml AND 4000ng/ml.

FIGURE 4.7A. TISSUE UPTAKE AT 5ng/ml $Ga(NO_3)_3$

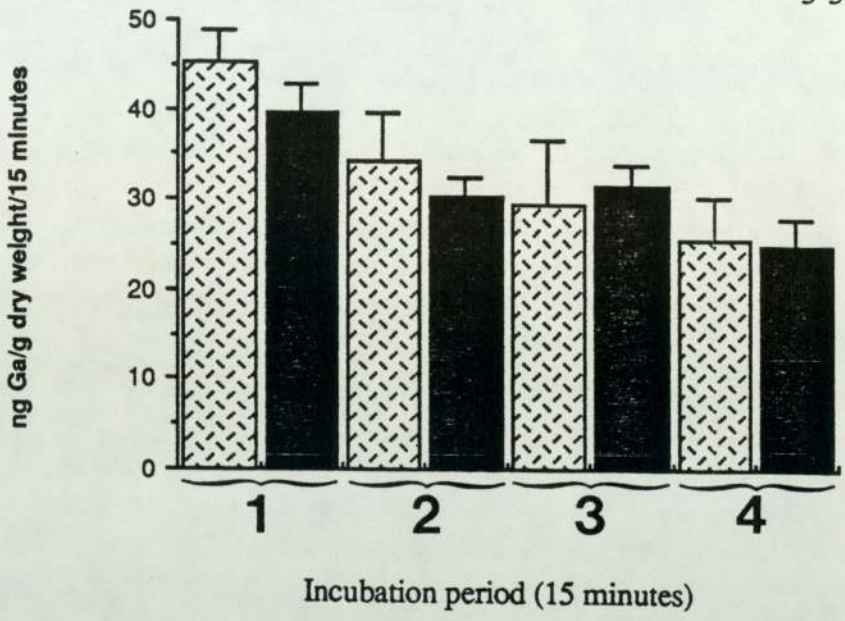
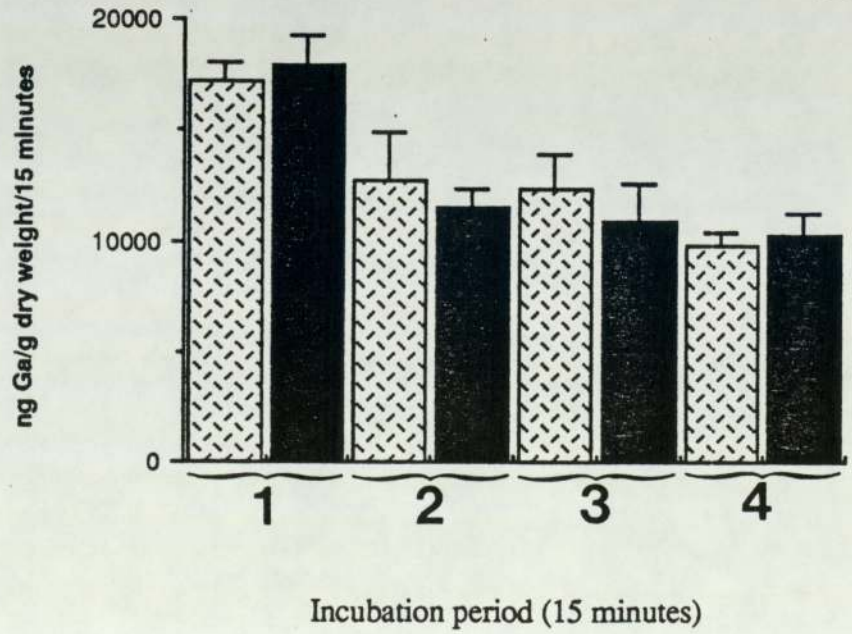


FIGURE 4.7B. TISSUE UPTAKE AT 4000ng/ml $Ga(NO_3)_3$



Notes

1). Intestinal uptake of gallium measured by luminal loss (and therefore includes transported gallium)

2). Striped bars = Addition of gallium only to Krebs buffer (control)
Black bars = Addition of 2,4-dinitrophenol (10^{-4}M) + gallium to Krebs buffer.

3). No significant differences observed between test and control levels of gallium absorption in any of the observed incubation periods (n=4, ±SEM).

FIGURE 4.8. EFFECT OF 2,4-DINITROPHENOL ON THE TRANSFER OF GALLIUM TO LIVER, KIDNEY, SPLEEN AND BLOOD AT 5ng/ml AND 4000ng/ml $Ga(NO_3)_3$

FIGURE 4.8A. TRANSFER AT 5ng/ml $Ga(NO_3)_3$

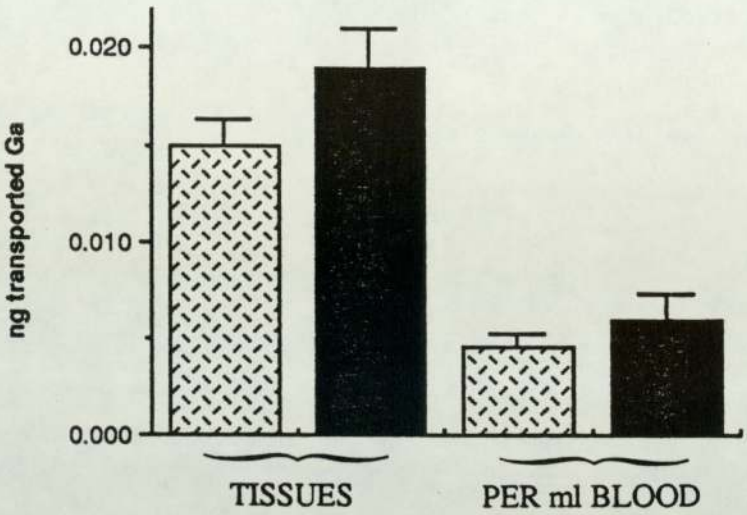
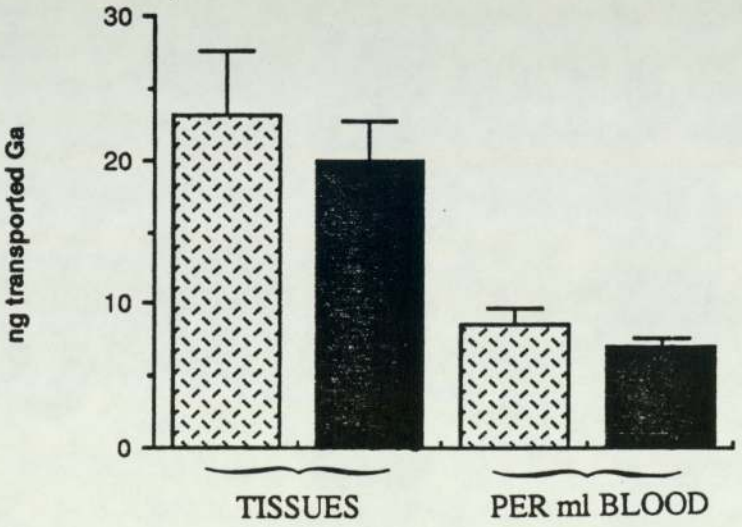


FIGURE 4.8B. TRANSFER AT 4000ng/ml $Ga(NO_3)_3$



Notes
1). Striped bars = Addition of gallium only to Krebs buffer (control)
Black bars = Addition of 2,4-dinitrophenol (10^{-4}M) + gallium to Krebs buffer.
2). No significant differences observed between test and control levels of transport in any of the examined tissues (n=4, ±SEM).

4.3.4. COMPARISON OF *IN-VIVO* AND *IN-VITRO* RESULTS.

In both the everted sac and perfusion experiments tissue uptake of gallium over the concentration range 5-4000ng/ml $Ga(NO_3)_3$ was linear with time. Transfer of gallium either to the serosal compartment of the everted sac or into the blood and tissues of the perfused rat was also linear with time over the examined concentration range. Addition of the metabolic inhibitor 2,4-dinitrophenol (10^{-4}M) to either the *in-vivo* or *in-vitro* system did not affect tissue uptake or transport of gallium.

It is possible to compare the mucosal uptake results at 500ng/ml $Ga(NO_3)_3$ in both systems (both preparations incubated for a set 20 minute period). It is obviously impossible to measure initial wet weight of jejunal tissue in the *in-vivo* system so both sets of results were expressed as ng Gallium absorbed per mg of protein per 20 minute incubation period (table 4.1)

TABLE 4.1. COMPARISON OF *IN-VIVO* AND *IN-VITRO* TISSUE UPTAKE OF GALLIUM.

	TISSUE UPTAKE (ng Ga/mg protein/20minutes)
EVERTED SAC	8.37 ±1.08
PERFUSION	6.12 ±0.77

Notes.

1). There was no significant difference in gallium uptake between *in-vivo* and *in-vitro* techniques (±SEM, n=4).

Extracellular fluid was also measured in the above experiments the ECF being significantly reduced in the *in-vivo* system (ECF *in-vivo* , 10.57% ±0.57, n=4;

in-vitro 15.83% ±0.51, n=4, P<0.1%). It is possible that the act of everting the intestinal sacs created an artificially high extracellular space caused by fluid adhering to the outstretched villi.

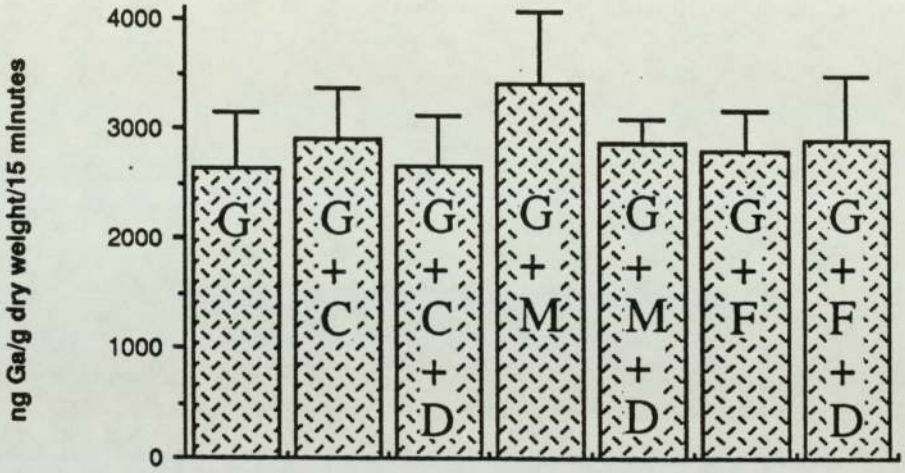
4.3.5. EFFECT OF CITRATE, MALTOL AND FLUORIDE ON THE *IN-VIVO* UPTAKE AND TRANSFER OF GALLIUM.

The addition of citrate, maltol and fluoride did not affect tissue uptake of gallium in the *in-vivo* perfusion system (figure 4.9). Similarly the addition of 2,4-dinitrophenol in conjunction with citrate, maltol or fluoride did not significantly alter gallium uptake (figure 4.9). The citrate results contrast with those from the everted sac experiments. The *in-vitro* tissue uptake of gallium was reduced with the addition of citrate to the incubating buffer, whereas no affect was observed *in-vivo*.

The *in-vivo* transfer of gallium has been divided into tissue uptake (liver, kidney and spleen) and blood uptake. Although transfer of gallium to the tissues was enhanced in the presence of citrate and maltol, the results were not significant (figure 4.10a). The presence of gallium in the blood however, was significantly greater when citrate and maltol was added to the incubating buffer (figure 4.10b). Contrary to the effects of citrate and maltol, fluoride inhibited the transport of gallium to the blood (P<5%) and possibly to the tissues (P<10%)(figure 4.10a and 4.10b).

Significant levels may have been achieved in blood due to the strong affinity of the plasma protein transferrin for gallium (Fe^{3+} is the normal metal ion binding to transferrin, but Ga^{3+} and Al^{3+} binding have also been observed, Larson et al. 1981; Martin et al. 1987). Is is possible that after the incubation periods had finished and the tissues and blood were collected, the majority of gallium was still plasma bound having not yet been transferred to the tissue reservoirs.

FIGURE 4.9. EFFECT OF CITRATE, MALTOL AND FLUORIDE ON THE TISSUE UPTAKE OF GALLIUM AT 500ng/ml.



Notes

1).Intestinal uptake of gallium measured by luminal loss (and therefore includes transported gallium).

2).Legend-

G = Gallium (500ng/ml) only in Krebs buffer (control)
G + C = Gallium + citrate (1mM)
G + C + D = Gallium + citrate + DNP (0.1mM)
G + M = Gallium + maltol (20μM)
G + M + D = Gallium + maltol + DNP
G + F = Gallium + fluoride (0.5mM)
G + F + D = Gallium + fluoride + DNP

3). The effects of DNP on uptake and transfer of gallium have previously been recorded in figures 4.7 and 4.8.

4). Each result is the mean of four observations (±SEM).

FIGURE 4.10. EFFECT OF CITRATE, MALTOL AND FLUORIDE ON THE TRANSPORT OF GALLIUM AT 500ng/ml.

FIGURE 4.10A. TRANSFER OF GALLIUM TO LIVER, KIDNEY AND SPLEEN.

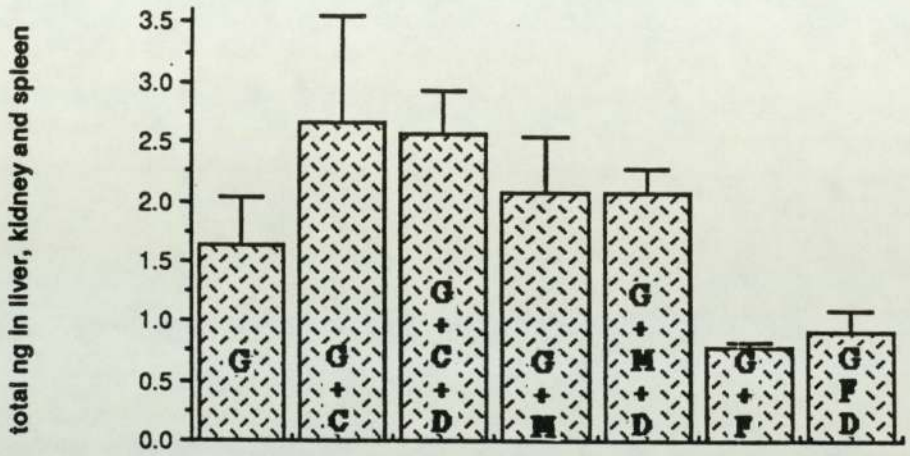
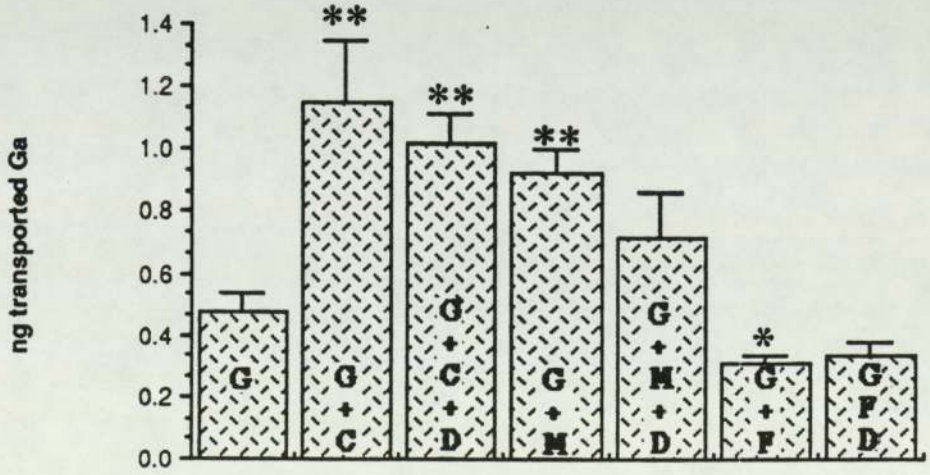


FIGURE 4.10B. TRANSFER OF GALLIUM TO BLOOD (ml^{-1}).



Notes
1). For legend see previous diagram (figure 4.9)
2). *P<5%, **P<1%, indicates a significant difference in the amount of transported gallium from the control (gallium only) value (n=4, ±SEM).

The reasons for the enhancement of gallium transfer in the presence of citrate and maltol (formation of membrane soluble species) and inhibition in the presence of fluoride (formation of insoluble GaF_3 compounds) have been discussed in the previous chapters (see 3.3.7).

4.4. CONCLUSIONS.

The tissue uptake and transfer of gallium in an *in-vivo* perfusion system has been assessed. Preliminary viability studies ensured that the technique was suitable for further absorption studies. The tissue uptake and transfer of gallium was found to be linear over the concentration range 5-4000ng/ml of $Ga(NO_3)_3$. Addition of the metabolic inhibitor 2,4-dinitrophenol did not affect either tissue uptake or transfer of gallium when measured at the lowest and highest of buffer gallium concentrations examined. The results (comparing well with those of the everted sac system) indicate gallium transport to be via a passive diffusive pathway. The tight-junctional complex (zona occludens) is the most likely route for the transport of the hydrated gallium ion.

Gallium is likely to be transferred to the blood and immediately bound to a transferrin-like protein after transport through the epithelial cell barrier. Unlike the hydrated gallium ion, gallium-citrate and gallium-maltol are likely to be transported through the apical cell membrane into the cell and out via the basal cell membrane. These species however, are thought not to exist in the blood plasma at low gallium concentrations, any gallium (either in complex or 'free') being sequestered by transferrin. A further discussion of transportable species is given in Chapter 5 and in the general discussion (Chapter 6).

These results are consistent with a two-step mechanism of uptake which has similarily been proposed for iron by Manis and Schacter (1962). An initial rapid

binding of gallium to the mucosal surface (already observed for aluminium, scandium and gallium in *in-vitro* everted sacs) is followed by a slower (and qualitatively lower) transport of gallium into the body.

CHAPTER FIVE

SPECIATION OF GALLIUM IN THE SMALL INTESTINE

CHAPTER 5. SPECIATION OF GALLIUM IN THE SMALL INTESTINE.

5.1. INTRODUCTION

Aluminium has a relatively small ionic radius (0.54Å) and a high oxidation potential (+1.66V). It is therefore highly reactive, strongly attracting anions and forming stable complexes (Skalsky and Carchman, 1983). Aluminium is expected to bind especially with oxygen donor ligands for example phosphates and hydroxides.

In the lumen of the gastrointestinal tract there is potential for numerous aluminium-ligand complexes. With the high reactivity of aluminium, there is the possibility for ligand formation with both the large variety of dietary components and intestinal secretions.

If complexes with aluminium form which promote uptake by the epithelial cells of the small intestine there may be increased neurotoxic implications. Conversely aluminium complexes which form, but are not absorbed to any appreciable extent may be useful as a binding agent preventing uptake in people who are exposed to unusually large quantities of aluminium.

Possible dietary components which are known to affect the bioavailability of other trace metals such as zinc, copper and iron and which may affect the bioavailability of aluminium are amino acids, tannins (found particularly in tea and coffee) and phytates (an inositol compound found naturally in many plants).

Phytic acid is a good example of a dietary component which influences trace metal absorption. It has six phosphates, these oxygen donor ligands forming complexes with many trace metal cations (Kratzer and Vohra, 1986) thus reducing absorption of the metal. Although iron binds to phytates and dietary fibre (Rossander, 1987), bioavailability is thought to depend upon ligands simultaneously present in the small bowel fluids (Leigh and Millar, 1983). Complexation between aluminium and phytic acid has been little mentioned, although the formation of a complex between the two is highly likely.

The previous results chapters have outlined the importance of nutritional status and dietary ligands in influencing the bioavailability of gallium. This chapter attempts to define the actual speciation within the lumen of the small intestine and to explain further the interactions between gut contents and soluble gallium-ligand complexes. It is these soluble gallium species which are potentially available for transport and therefore are of interest.

5.2 MATERIALS AND METHODS

5.2.1. ANIMALS. See section 2.2.1.

5.2.2. CHEMICALS. See section 2.2.2.

5.2.3. DOSING REGIME AND DISSECTION

Animals, either fed or fasted were dosed with 0.3mls 10^{-3}M $Ga(NO_3)_3$ + 10-50μCi ^{67}Ga (see individual results tables for exact composition of dosing solution) under light ether anaesthesia for restraint. The rats were sacrificed 2 hours after the oral dose (this time being when maximal amounts of radioisotope were located within the small intestine). The body was opened by a small midline incision in the abdomen and the small intestine excised. The stomach, caecum, large intestine, liver, kidney and spleen were also removed to give an indication of recovery of the dose.

The small intestine was flushed with 20mls 0.9% NaCl to remove digestia and faecal matter. The gut was divided into duodenum, jejunum and ileum and counted for ^{67}Ga content. The gut washings were centrifuged at 100000G for 1 hour in a MSE Superspeed 50 (MSE Ltd. Crawley, UK) at 4°C. The results of centrifugation, a pellet designated the insoluble phase, and the supernatant (the soluble phase) were separated and counted for ^{67}Ga content.

5.2.4. ANALYSIS OF THE SOLUBLE PHASE BY GEL-FILTRATION CHROMATOGRAPHY.

The speciation of gallium in the soluble phase of the intestinal gut washings was analyzed by gel-filtration chromatography using Sephadex G15 (Pharmacia Ltd, Milton Keynes, UK) in a 60cm x 1.5cm glass column (Amicon Wright Ltd. Stowehouse, UK). The dry Sephadex beads were swollen in excess elution buffer (25mM Tris-HCl, 100mM NaCl, pH 7.4, Cochran et al. 1984) for 24 hours. The swollen gel was degassed and packed into the column at a flow rate of 3.5 ml/minute. The column was equilibriated by running 3 bed volumes of elution buffer and calibrated using Dextran Blue (void volume) and tritiated water (exclusion volume). 5mls of standard/sample were loaded and thirty-five 6ml fractions were collected from all columns using an LKB Redirac 2112 fraction collector (LKB-Pharmacia Ltd, Milton Keynes, UK) at a flow rate of 3mls/minute. The void volume of the G15 column was measured at fraction 9, whilst the exclusion volume was found at fraction 17.

The soluble phase of the gut washings was applied to the column and eluted using the above conditions. Fractions were counted by γ or ß counting as required.

Samples eluting in the void volume of the G15 column (maximum molecular weight detection 1500) were reeluted on Sephadex G75 (maximum molecular weight detection 80000) using similar column conditions except for a reduction in flow rate to 1ml min^{-1}.

The G75 column was calibrated using a commercial molecular weight marker marker kit (Sigma Chemicals) consisting of bovine serum albumin (66KD), ovalbumin (43KD), carbonic anhydrase (29KD), cytochrome C (12.4KD) and insulin (5.4KD)(D = Dalton, unit of mass approximately equivalent to that of a hydrogen atom).

Standard elution profiles of $Ga(NO_3)_3$ only and gallium with EDTA, maltol and citrate were also obtained using a similar procedure.

5.2.5. ION EXCHANGE CHROMATOGRAPHY (I.E.C.)

I.E.C. was used to separate ionic gallium from the gallium-maltol complex. Gel filtration chromatography was not suited to the separation of these species because of the lack of resolution between the two species.

An anion exchanger DEAE Sephadex was used to separate ionic gallium from gallium-maltol. The gel was swollen in a similar fashion to Sephadex G15. In order to exchange the counter ion from the supplied chloride ion, the slurry was suspended in a 0.5M solution of maleic acid for 6 hours. The maleate anion was required as the counter ion as the Tris-Maleate buffering system is one of the few to work over the pH range 5-8 (Biochemists Handbook, 1961). The gel was washed with 25mM Tris, 10mM maleic acid, pH 8 until the slurry became alkaline. The gel was packed into a Perspex Amicon Wright column (45cm x 1cm) and 3 bed volumes of elution buffer pumped through the column to allow for equilibration.

Gallium only (2mls 10^{-3}M $Ga(NO_3)_3$ + 1μCi ^{67}Ga) or gallium-maltol (2mls 10^{-3}M $Ga(NO_3)_3$, 10^{-2}M maltol + 1μCi ^{67}Ga) was applied to the column and a desending pH gradient eluted through the column (LKB 11300 Ultrograd gradient mixer and mixer driver). The pH gradient ran between 25mM Tris, 10mM maleic acid, pH 8 to 10mM Tris, 20mM maleic acid, pH 5. The gradient was ran over a 4 hour period and eighty 3ml fractions were collected (LKB 7000 Ultrorac fraction collector). Fractions were counted for ^{67}Ga by γ-counting as needed.

The soluble phase of gut washings of animals which had been dosed with gallium-maltol were also applied to the column for speciation analysis.

5.2.6. ORAL DOSING/REDOSING EXPERIMENTS AND CHROMATOGRAPHY OF DOSED/REDOSED GUT WASHINGS.

Fed animals were dosed and the soluble phase of the gut washings chromatographed as in 5.2.3 and 5.2.4.

A large molecular weight complex binding to gallium was identified in fraction 9 (figure 5.3a). The 6ml fraction was counted for ^{67}Ga activity and immediately frozen at -70°C for 45minutes. It was then freeze dried overnight and the resulting powder reconstituted to 400µl with distilled water. 0.3mls of the solution was dosed to either a fed or fasted rat and the procedures for 5.2.3 and 5.2.4 repeated as before.

5.2.7. INCUBATION OF JEJUNAL MUCOSAL TISSUE WITH GALLIUM FOR SPECIATION ANALYSIS.

Mucosal tissue from upper and mid jejunal regions of rat small intestine was opened into strips and incubated for 20 minutes in 20mls of HEPES physiological buffer (see 3.2.3) at 37°C in a shaking water bath. $Ga(NO_3)_3$ was added to give a final concentration of 10^{-6}M (radiotracer, 10µCi ^{67}Ga). Mucosal scrapes were obtained using glass microscope slides and the tissue homogenized in eluent buffer to give a 25% w/v solution. After centrifugation (100000G for 1 hour) the supernatant was applied to G15 and G75 columns for speciation analysis. G75 fractions were kept for protein measurement.

5.2.8. SPECIATION ANALYSIS OF INTESTINAL FLUIDS

5.2.8.1. PERFUSION FLUIDS (*IN-VIVO* INTESTINAL TECHNIQUE) (INCLUDING ANALYSIS OF PLASMA BOUND GALLIUM).

The jejunum of a rat was perfused as previously described (see 4.2.4 and 4.2.5) with Krebs Henseleit bicarbonate buffer and $Ga(NO_3)_3$ at a concentration of 500ng/ml, (radiotracer, 10µCi ^{67}Ga). After the fourth, 15 minute incubation period 5mls of the perfusate solution was applied to the G15 column for analysis. In an effort to measure transported species blood was drawn from the rat at the end of the experiment whilst the animal was still under anaesthetic. The blood sample was centrifuged in a microreaction vial for 3 minutes. The plasma was

drawn off and applied to a G75 column and eluted under the conditions previously described (5.2.4).

The blood in the perfused rats contained less than 0.5ng/ml of gallium and was designated at being a 'low' concentration. In order to introduce a 'high' concentration of gallium into the blood 0.3ml 1mM $Ga(NO_3)_3$ and 10μCi ^{67}Ga was injected intramuscularly. After 30 minutes the rat was anaesthetised and blood collected.

Plasma was applied to the G75 column and again eluted using the previously described procedures.

5.2.8.2. EVERTED SAC MUCOSAL AND SEROSAL FLUIDS (*IN-VITRO* INTESTINAL TECHNIQUES).

Mucosal and serosal fluids were collected fron jejunal everted sacs at the end of 20 minutes incubation and applied to G15 columns. They were eluted under the previously described conditions. Fractions were kept for further protein measurement (see 5.2.12).

5.2.9. EVERTED SAC STUDIES WITH LARGE MOLECULAR WEIGHT SPECIES ISOLATED FROM THE SOLUBLE PHASE OF GUT WASHINGS.

The large molecular weight gallium binding species identified in the soluble phase of gut washings from fed rats was isolated using the procedures of 5.2.3 and 5.2.4. Fractions from several columns were collected to a total of 60mls (6 x 10ml incubations). The eluent buffer (25mM Tris-HCl, 100mM NaCl, pH 7.4) was used as an incubating buffer with the addition of 28mM glucose. Jejunal everted sacs were prepared (see 3.2.4) and incubated at 37°C for 20 minutes. The mucosa and serosal fluids were counted for ^{67}Ga content, and the mucosal fluids were applied to the G15 column for speciation analysis.

A control experiment was performed where the incubating buffer contained gallium only instead of the large molecular weight gallium binding species.

5.2.10. INCUBATION OF HUMAN SMALL INTESTINAL FLUIDS FOR SPECIATION ANALYSIS.

Ileal fluids were collected from a 30 year old man who had undergone a total colectomy operation as a result of ulcerative colitis. The patient had an ileoanal pouch inserted and had a temporary colostomy bag. After a cereal breakfast ileal fluids were collected over a four hour period from 8.30 to 12.30am.

The ileal fluids were immediately diluted 1:1 with simple physiological buffer (NaCl/KCl/glucose; see 3.2.3). $Ga(NO_3)_3$ was added to a final concentration of 10^{-6}M (radiotracer, 10μCi ^{67}Ga). The fluids were incubated for 1 hour in a shaking water bath at 37°C and centrifuged at 100000G for a further hour. The soluble phase (i.e. supernatant) was applied to the G15 and G75 columns for speciation analysis. All fractions were frozen and analysed for protein content (5.2.12).

5.2.11. GEL ELECTROPHORESIS.

Fractions eluted from the columns which were of interest for analysis by gel electrophoresis were freeze dried overnight and the remains dissolved in 300μl distilled water. 200μl of the sample was diluted 1:1 with sample buffer (consisting of 5mls 0.5M Tris, pH 6.8, 10ml 10% w/v sodium dodecyl sulphate (SDS) or units thereof), 0.5ml mercaptoethanol, 5ml glycerol, 10ml water, 0.4ml 5% w/v bromophenol blue solution) and boiled for 5 minutes prior to the running of the gel.

The gel electrophoresis equipment (Mini-Protean gel electrophoresis equipment, Biorad Ltd. Watford, UK) was cleaned using methanol and the bottom of the plates sealed with 1% agarose solution. The separating gel (6ml stock 1 (44% w/v

acrylamide, 0.8% w/v bisacrlyamide or units thereof), 7.5ml Tris, pH 8.8, 9.5ml water, 0.6ml SDS, 56µl NNN'N'-Tetramethylethylene-diamine (TEMED), 80µl 10% ammonium persulphate solution (AMPS)) was poured between the plates leaving a gap of 2cm at the top and left to set for 15 minutes. The stacking gel (2ml stock 2 (30% w/v acrylamide, 0.8% w/v bisacrylamide), 3mls 0.5M Tris, pH 6.8, 6.4ml water, 120µl SDS, 32µl TEMED, 40µl AMPS or units thereof) was poured between the plates on top of the separating gel and a plastic 10mm comb inserted to leave tracks for the application of samples. The gel was left for a further 15 minutes to set and the comb removed. 5,10, and 20µl of sample was applied to each of the tracts using a Hamilton syringe. The plates were clamped together and placed in a bath filled with electrode buffer (6g Tris, 28.8g glycine, 20ml SDS, 10% w/v, pH 8.3). A 15mA current was applied and the gel ran for 45 minutes.

After the current was switched off the gel was prised from the glass plates and fixed in Coomassie blue stain (0.1% w/v Coomassie blue, 50% w/v methanol, 10% w/v glacial acetic acid for 2-4 hours, detection limit 0.3 to 1µg/ protein band). The gel was finally washed in destain (10% methanol, 7.5% glacial acetic acid) until the sample bands were clearly visible..

If the protein content was below that of the sensitivity of the Coomassie blue technique, alternatively a silver staining technique could be used (detection limit 2-5ng/ protein band). The polyacrylamide gel was fixed with 'fixing solution' (50% methanol, 10% acetic acid, 40% water) for 30 minutes, destained using the above Coomassie blue destainer for 60 minutes, fixed in 10% gluteraldehyde for 30 minutes, washed in water for 2 hours, stained with silver nitrate solution (3.5ml concentrated NH_4OH (30%) added to 42ml of 0.36% NaOH made up to 200ml with water, mixed carefully with 8mls 19.4% silver nitrate) for 15 minutes, washed in water for 5 minutes and developed with 25mls of developing solution (0.5g sodium citrate, 0.5ml 37% formaldehyde, water up to 100mls) in 500mls

water. This solution was added until the protein bands appeared. Finally the gel was fixed with 'Kodak Rapid Fix' for 5 minutes and washed with excess water.

5.2.12. MEASUREMENT OF PROTEIN IN GEL FILTRATION FRACTIONS.

Proteins were measured by the method of Lowry et al. (1951).

0.4ml sample was added to 2ml reagent. (reagent consisting of 50ml 2% Na_2CO_3 in 0.1M NaOH and 1ml of 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate or units thereof). After a 10 minute incubation at room temperature 0.2ml Folins reagent (diluted 1:1) was added and again mixed thoroughly. After 30 minutes standing at room temperature the samples were read at 750nm against a reagent blank (Shimadzu UV spectrophotometer, Japan). The calibration was from 25-200µg/ml bovine serum albumin. Samples were diluted accordingly to lie within this range.

5.3 RESULTS AND DISCUSSION

5.3.1. STANDARD ELUTION PROFILES.

Sephadex G15 was calibrated with Dextran blue 2000 (void volume, V_o) and tritiated water (exclusion volume, V_i). These points were eluted at fractions 9 and 17 respectively.

A simple solution of 10^{-3}M $Ga(NO_3)_3$ + 1µCi ^{67}Ga eluted at fraction 17 (figure 5.1a). A characteristic 'tail' was observed as the gallium gradually eluted out. This was possibly caused by the hydrolyzed gallium species reversibly binding to the Sephadex gel. Recoveries of samples were acceptable, generally being over 70% in most cases. Variability in recoveries was thought to be due to the amount of radioactivity or to the nature of the samples applied to the column.

FIGURE 5.1A. STANDARD ELUTION PROFILE OF GALLIUM NITRATE (SEPHADEX G15).

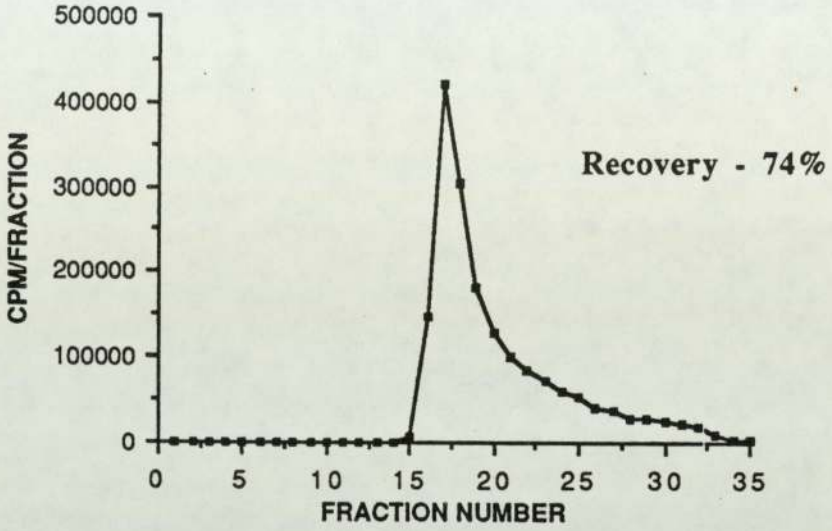
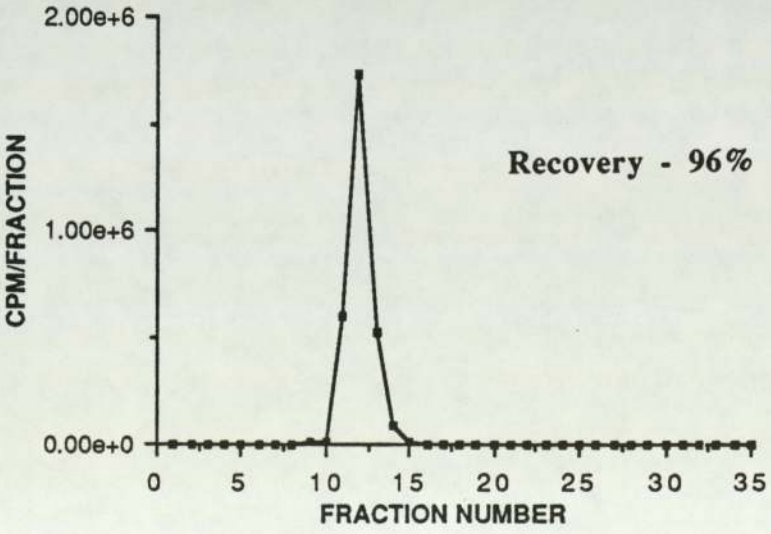


FIGURE 5.1B. STANDARD ELUTION PROFILE OF GALLIUM NITRATE AND EDTA (SEPHADEX G15).



A strong Ga-EDTA complex rapidly formed when 10^{-3}M $Ga(NO_3)_3$ and 10^{-2}M EDTA + 1μCi ^{67}Ga were incubated at room temperature. The complex solely elutes at fraction 12 (figure 5.1b) and recovery approached 100%. EDTA can also be applied to the column to remove excess gallium, particularly when recoveries were at the lower end of the recovery range. This is essential to prevent contamination from one column to the next if the same gel is to be reused.

A similar incubation of gallium and citrate produced a single peak at fraction 10 (chromatograph not shown). Recovery of this species approached 95% suggesting that the majority of gallium in solution had complexed with citrate.

10^{-2}M maltol and 10^{-3}M $Ga(NO_3)_3$ + 1μCi ^{67}Ga eluted at a similar position to $Ga(NO_3)_3$ alone (figure 5.2a) using gel filtration chromatography (i.e. separation by molecular size). When applied to an ion exchange column however, under a descending pH gradient the neutrally charged gallium-maltol complex eluted at a fraction equivalent to the void volume. The hydrated gallium cation eluted when the pH gradient reached acidity (figure 5.2b). The addition of ferric ions (10^{-2}M $FeCl_3$) displaced the majority of gallium from the gallium-maltol complex leaving gallium in the 'free' ionic form (figure 5.2c).

5.3.2. THE ORAL DOSING OF GALLIUM IN FED AND FASTED RATS.

Chromatographs of the soluble phase of gut washings after the oral administration of gallium have identified both a large and low molecular weight species, peaks 1 and 2 respectively (figures 5.3a and 5.4a). Peak 1 was significantly greater in fed rats than fasted rats (table 5.1). Peak 2 is thought to be associated with the hydrated gallium ion which alone eluted at fraction 17 (figure 5.1a)

FIGURE 5.2A. STANDARD ELUTION PROFILE OF GALLIUM NITRATE AND MALTOL (SEPHADEX G15).

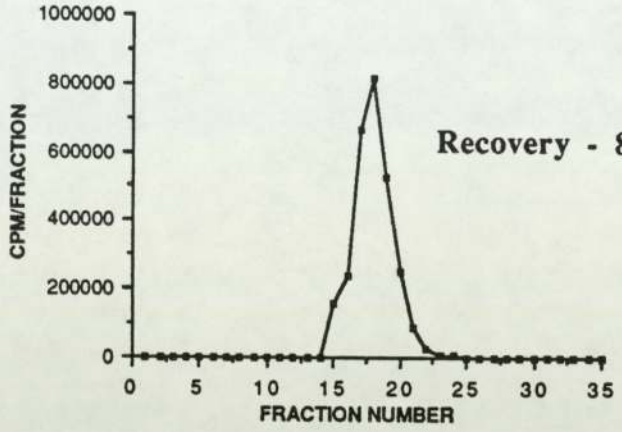


FIGURE 5.2B. SEPARATION OF GALLIUM NITRATE FROM GALLIUM-MALTOL BY DEAE SEPHADEX.

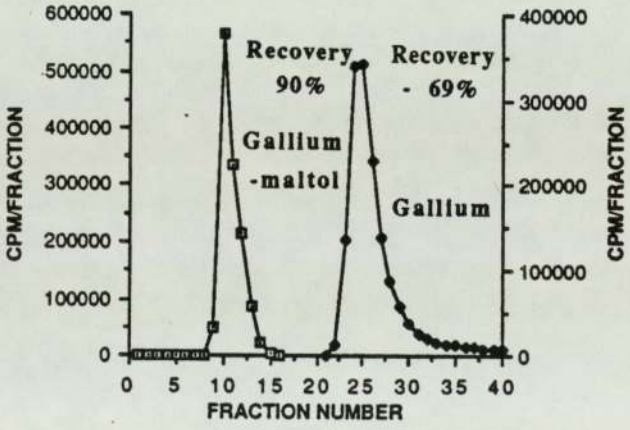


FIGURE 5.2C. DISPLACEMENT OF GALLIUM FROM GALLIUM-MALTOL BY FERRIC IONS (DEAE SEPHADEX).

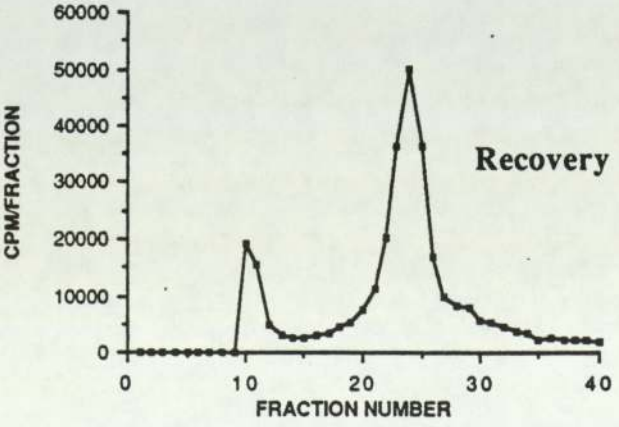


FIGURE 5.3A. CHROMATOGRAPH OF SOLUBLE GUT WASHINGS FROM FED RAT ORALLY DOSED WITH GALLIUM (SEPHADEX G15).

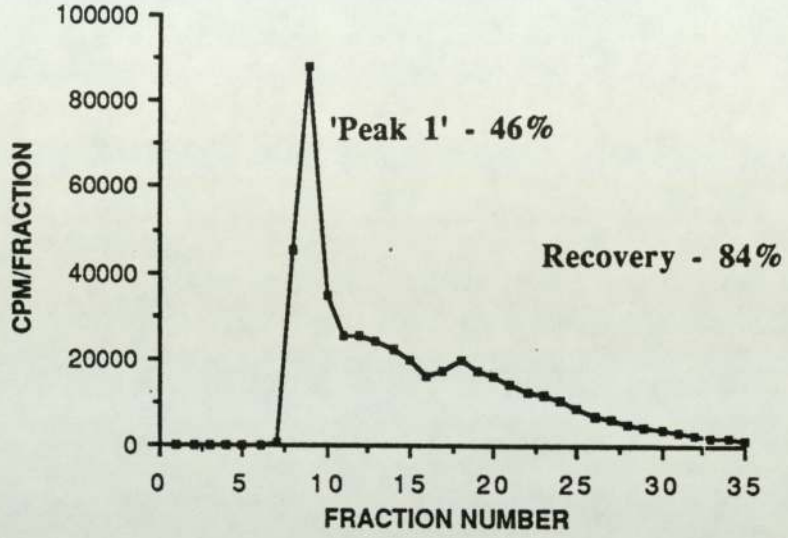


FIGURE 5.3B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.

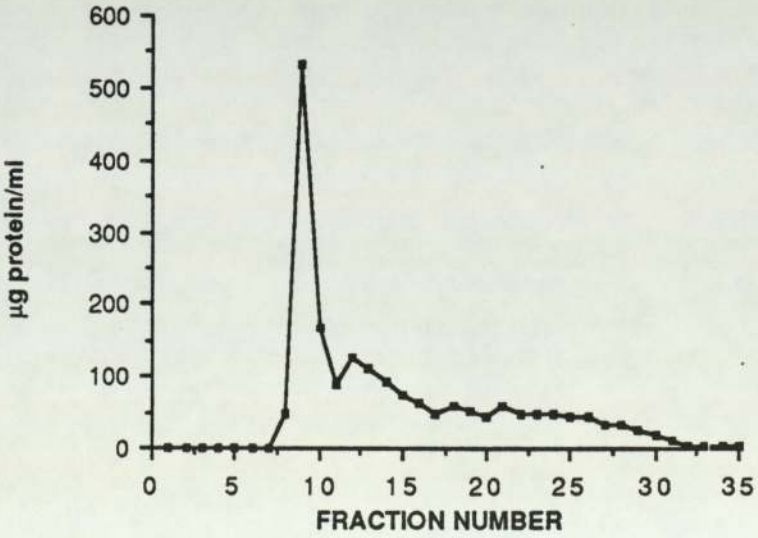


FIGURE 5.4A. CHROMATOGRAPH OF SOLUBLE GUT WASHINGS FROM FASTED RAT ORALLY DOSED WITH GALLIUM (SEPHADEX G15).

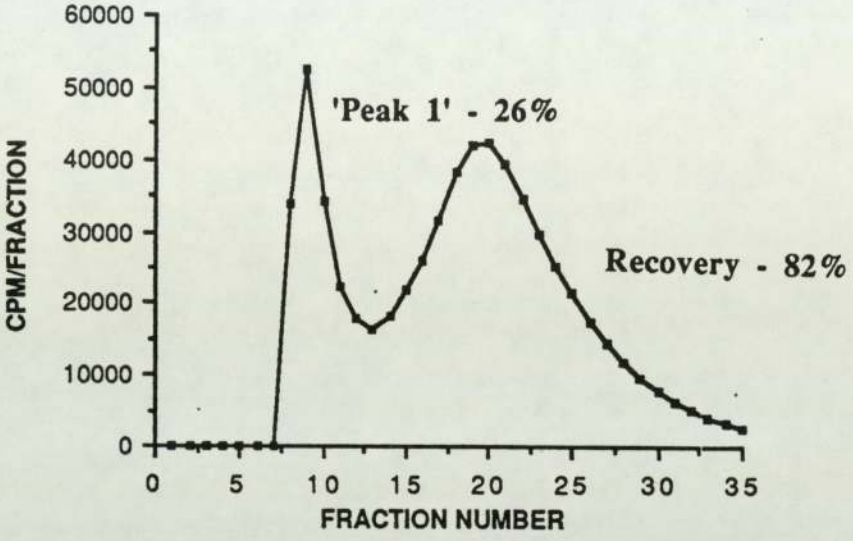


FIGURE 5.4B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.

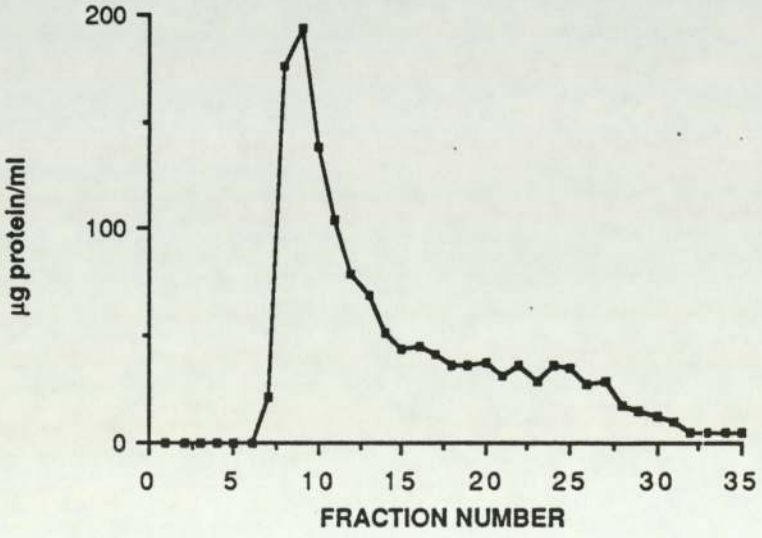


TABLE 5.1. PEAK 1 COMPARISONS IN FED AND FASTED RATS ORALLY DOSED WITH GALLIUM.

	FED (n=5)	FASTED (n=6)
Average % of chromatograph associated with Peak 1(±SEM)	42.4% ±1.59	26.3% ±2.70

Fed Peak 1 > fasted Peak 1, P< 0.1%, df=9.

Analysis of the thirty-five eluted fractions for protein content showed there to be a co-eluting of protein at the fraction where Peak 1 was identified (figures 5.3b and 5.4b). In the supernatant phase of gut washings it appears that gallium forms a soluble gallium-protein species (M.W. > 1500) which is prevalent in the washings from fed rats.

If Peak 1 is reeluted on Sephadex G75, two peaks are observed in both fed and fasted washings (figures 5.5a and 5.6a). The first peak corresponds to a molecular weight of approximately 80KD, whilst the second is less than 4KD. It is possible that some of the gallium attributed to Peak 2 is 'free' gallium having dissociated from the gallium complex. A similar dissociation (accounting for less than 10% of bound gallium) was observed whilst eluting on Sephadex G15. This small dissociation of gallium must always be considered when interpreting chromatographs to prevent wrong conclusions being drawn from the data.

Protein analysis identified the presence of a 80KD protein eluting at a similar position to that of gallium (figures 5.5b and 5.6b). Peak 2 however, did not seem to be associated with any particular protein suggesting that gallium was associated with soluble inorganic ligands for example bile complexes or was the $Ga(H_2O)_6^{3+}$ cation if dissociation had occurred.

FIGURE 5.5A. RE-ELUTION OF 'PEAK 1' FROM FED RAT (SEE FIG. 5.3A) ON SEPHADEX G75.

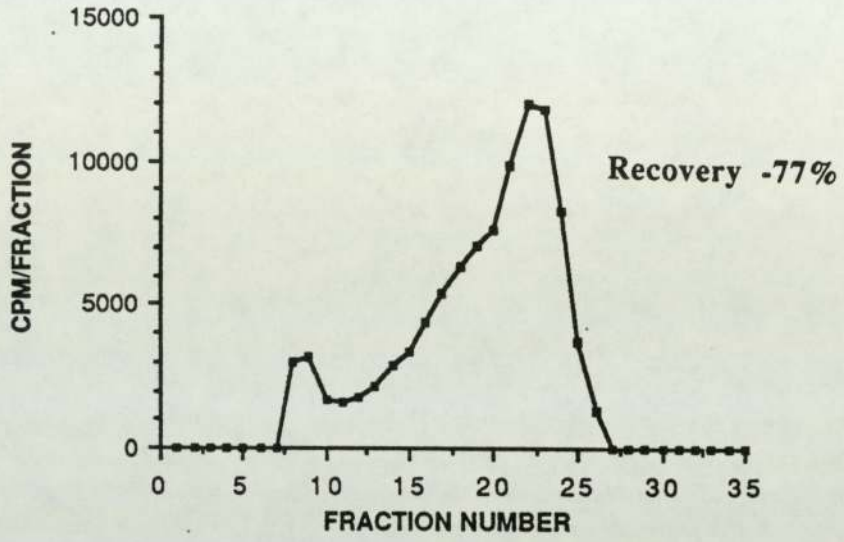


FIGURE 5.5B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.

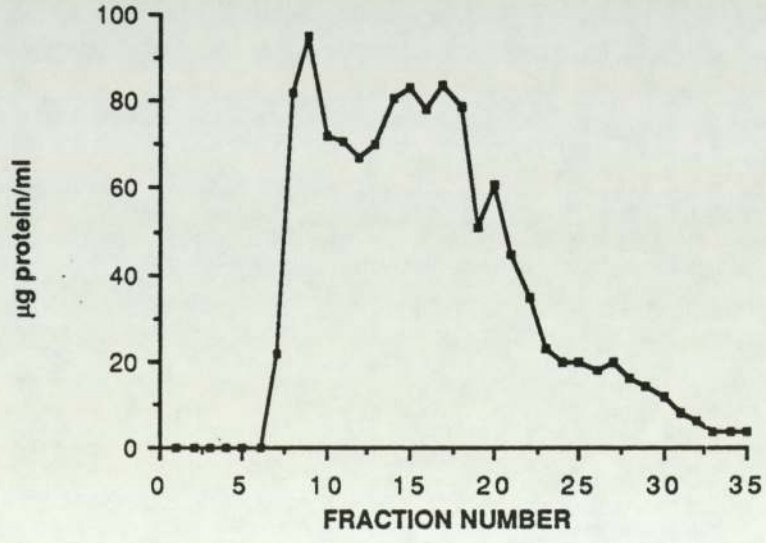


FIGURE 5.6A. RE-ELUTION OF 'PEAK 1' FROM FASTED RAT (SEE FIG. 5.4A) ON SEPHADEX G75.

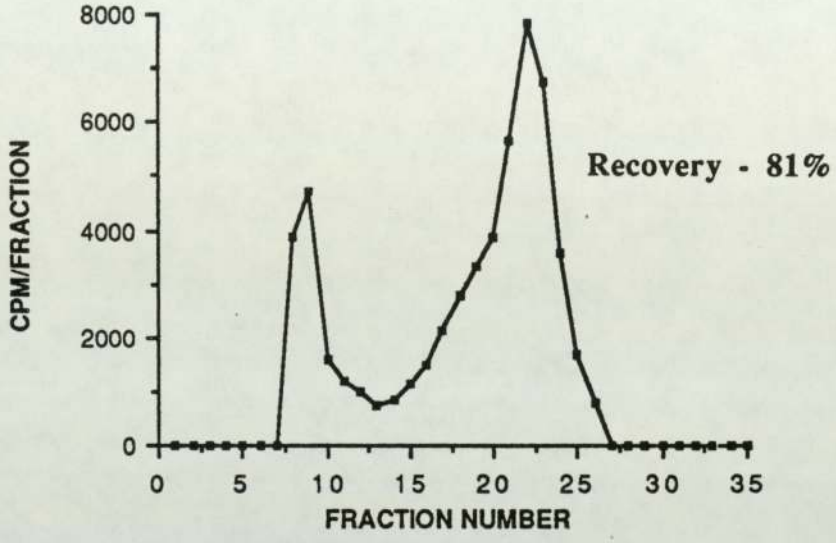
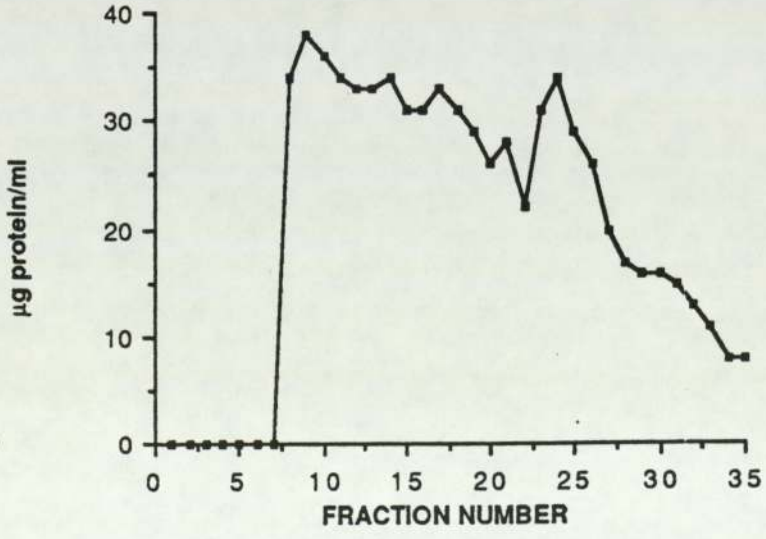


FIGURE 5.6B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.



Analysis of Peak 1 (from the G15 gel) by SDS gel electrophoresis identified 10 proteins with molecular weights ranging from 6KD-130KD (figure 5.7). Electrophoresis also revealed that similar proteins were present in the soluble phase of gut washings from both fed and fasted rats, the proportions of some (judged qualitatively by the density of the bands) differed when nutritional status was altered.

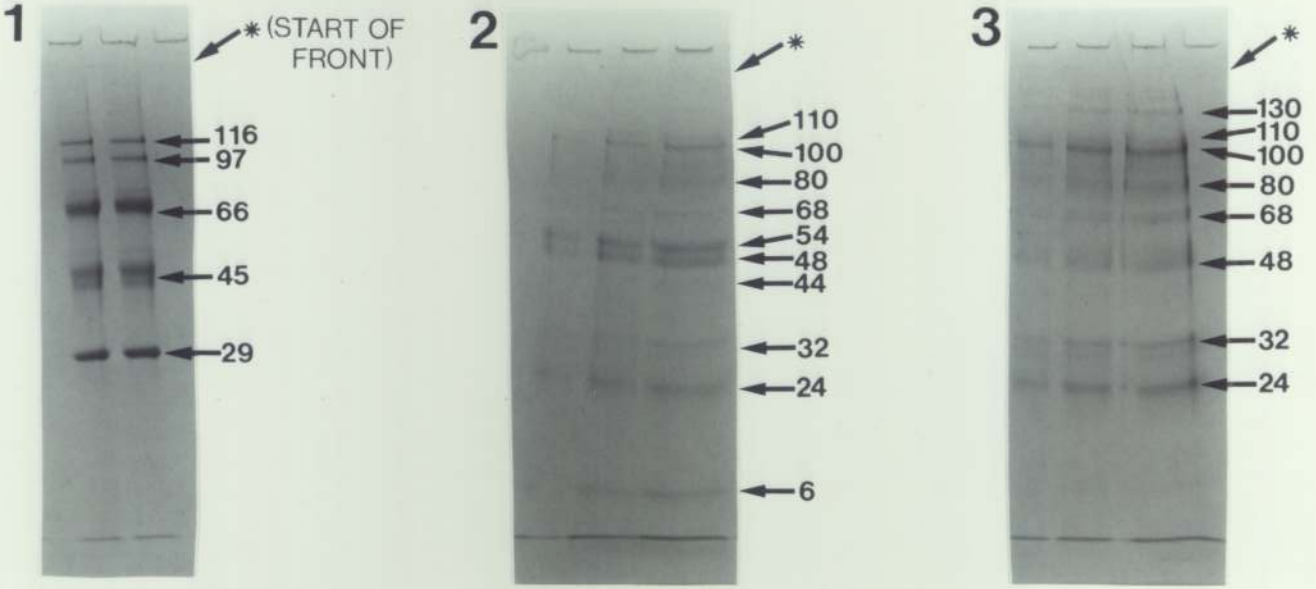
Nutritional status (i.e. whether the rat was fed or fasted) affects the distribution of gallium within the gastrointestinal tract (table 5.2). Significant amounts of gallium were retained in the stomach after 2 hours in the fed rat. In the fasted rat the majority of gallium has passed through the stomach and is potentially available for absorption. The fact that there is increased concentrations of gallium in the lumen as a result of gastric emptying in the fasted rat may be another factor accounting for the increased absorption of gallium in this state (see chapter 2).

In both fed and fasted rats binding of gallium to the duodenum, jejunum and ileum is observed even after a thorough flushing of the gut with isotonic saline (table 5.2). A previous non-specific binding of gallium has been observed in both *in-vivo* and *in-vitro* intestinal experiments (see chapters 3 &4). The actual amounts of transported gallium however, are quantitatively lower. In the *in-vitro* everted sac experiments less than 1% of the gallium bound to the mucosal surface was actually transported to the serosal compartment (see table 3.1).

2 hours after the oral administration of gallium to the fed rat, the distribution of gallium between the pellet and the supernatant (soluble phase) fraction was approximately 8:1 (table 5.2). The gallium associated with the pellet would be insoluble unabsorbable material (e.g. inorganic gallium salts and gallium bound to undigested cell fragments). The higher ratio of the pellet, and therefore more non-transportable species in the fed animal may account in part for the reduced absorption of gallium in the fed state (see chapter 2).

FIGURE 5.7. SDS-GEL ELECTROPHORESIS OF 'PEAK 1' ISOLATED FROM SOLUBLE PHASE OF GUT WASHINGS FROM FED (SEE FIG. 5.3A) AND FASTED (SEE FIG. 5.4A) RATS ORALLY DOSED WITH GALLIUM (*IN-VIVO* INCUBATION) (GEL - COOMASSIE BLUE STAINED).

(*INDICATES START OF GEL FRONT)



MOLECULAR WEIGHT MARKERS

'PEAK 1' FROM FED ANIMAL

'PEAK 1' FROM FASTED ANIMAL

All results expressed in KD (Kilo-Dalton ≈ molecular weight of 1000)

TABLE 5.2. TISSUE DISTRIBUTION OF ^{67}Ga IN FED AND FASTED RATS 2 HOURS AFTER THE ORAL ADMINISTRATION OF GALLIUM NITRATE.

ORGAN	FED		FASTED	
STOMACH	9.32	±2.80	0.53	±0.18*
DUODENUM	1.05	±0.59	0.12	±0.04
JEJUNUM	3.16	±1.14	1.04	±0.43
ILEUM	5.08	±0.86	4.43	±1.58
CAECUM	0.35	±0.20	0.89	±0.82
LARGE INTESTINE	0.03	±0.02	1.97	±1.09
LIVER	0.0072	±0.0018	0.079	±0.048
KIDNEY	0.0031	±0.0013	0.028	±0.014
SPLEEN	0.0010	±0.00014	0.0108	±0.0034*
SOLUBLE	8.29	±1.20	19.62	±2.68**
PELLET	65.92	±3.87	52.54	±4.68
TOTAL	93.21	±5.81	81.26	±7.68

Notes.

1) Results expressed as a % total dose administered (%TDA), n=5, ±SEM.

2) Fed and fasted rats orally dosed with 0.3mls 10^{-3} $Ga(NO_3)_3$ + 10μCi ^{67}Ga.

3) 'Soluble' and 'pellet' indicates the separation of small intestinal fluids by centrifugation.

4) *P<0.5, **P<0.1, indicate a significant difference between fed and fasted animals (unpaired students t-test).

5.3.3. THE REDOSING OF PEAK 1 ISOLATED FROM THE SOLUBLE PHASE OF GUT WASHINGS FROM FED RATS.

Peak 1 isolated from the soluble phase of gut washings in rats orally dosed with gallium is thought to be associated with a soluble protein (see previous section). The formation of this as yet unidentified species in combination with a reduction in the concentration of free hydrated gallium ions (see figures 5.3a and 5.4a) may be responsible for the reduced absorption of gallium in the fed state (see chapter 2 and table 5.2, where significantly less gallium was observed in the tissues of fed rats than in fasted after oral administration of gallium).

As the species is significantly greater in fed rats than fasted rats (table 5.1) it is possible that it has a protective nature, its formation preventing the absorption of gallium to a certain extent.

When Peak 1 (isolated from fed rats) was redosed to both fed and fasted rats, gallium was not detected in either the liver, kidney or spleen in any of the experimental animals. Enough radiotracer ^{67}Ga (approx. 50μCi, 5 x the normal dose) was introduced into the isolation procedure to ensure that if any transport did occur the detection of ^{67}Ga would not be a problem. Chromatographs of the gut washings from the redosed animals indicate that the majority of gallium was retained as the high molecular species (figures 5.8a and b). It appears therefore that the formation of this species plays some role in preventing the absorption of gallium possibly to a greater extent in the fed rat (see also 5.3.7. 'Transport of Peak 1 in jejunal everted sacs').

5.3.4. THE ORAL DOSING OF GALLIUM AND MALTOL IN FED AND FASTED RATS.

The oral dosing of gallium and maltol ($10^{-3}M/10^{-2}M$) produced similar chromatographs of the soluble gut washings in fed and fasted rats to those of the gallium only dosed (figures 5.9a to 5.10b). Both a large and small molecular

FIGURE 5.8A. CHROMATOGRAPH OF SOLUBLE GUT WASHINGS FROM FED RAT ORALLY DOSED WITH 'PEAK 1' (SEE FIG.5.3A) (SEPHADEX G15).

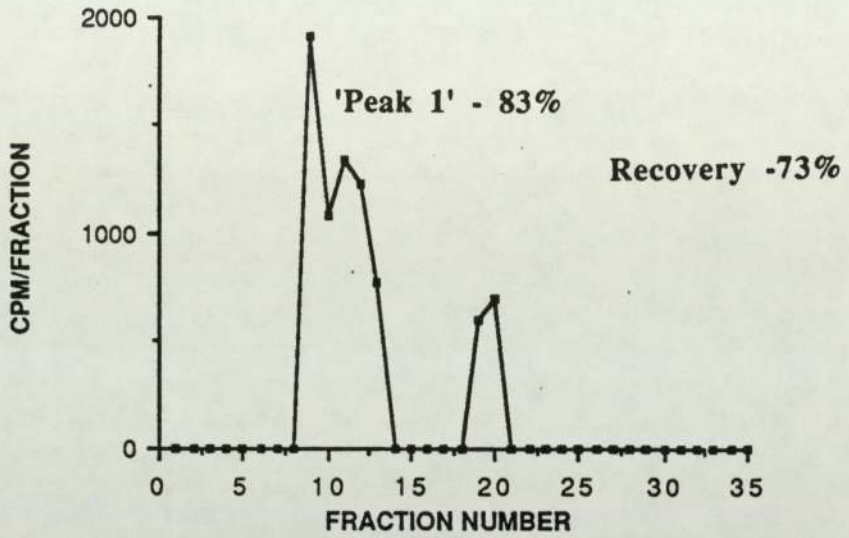


FIGURE 5.8B. CHROMATOGRAPH OF SOLUBLE GUT WASHINGS FROM FASTED RAT ORALLY DOSED WITH 'PEAK 1' (SEE FIG. 5.4A) (SEPHADEX G15).

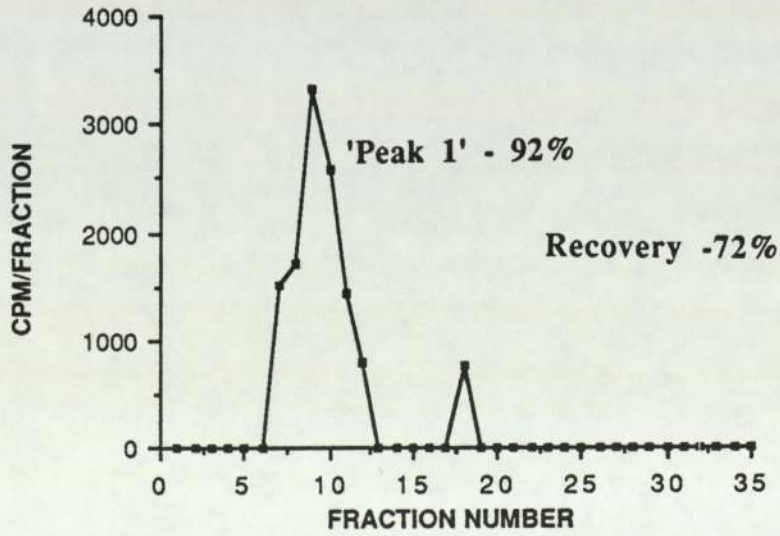


FIGURE 5.9A. CHROMATOGRAPH OF SOLUBLE GUT WASHINGS FROM FED RAT ORALLY DOSED WITH GALLIUM AND MALTOL (SEPHADEX G15).

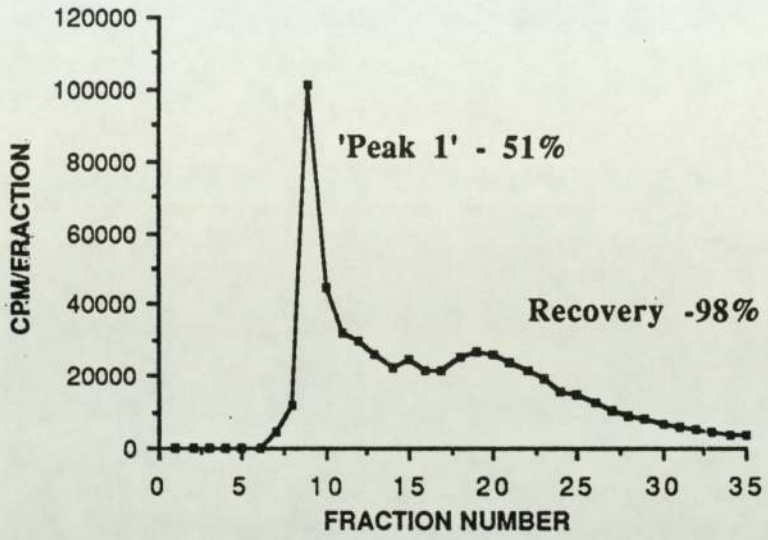


FIGURE 5.9B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.

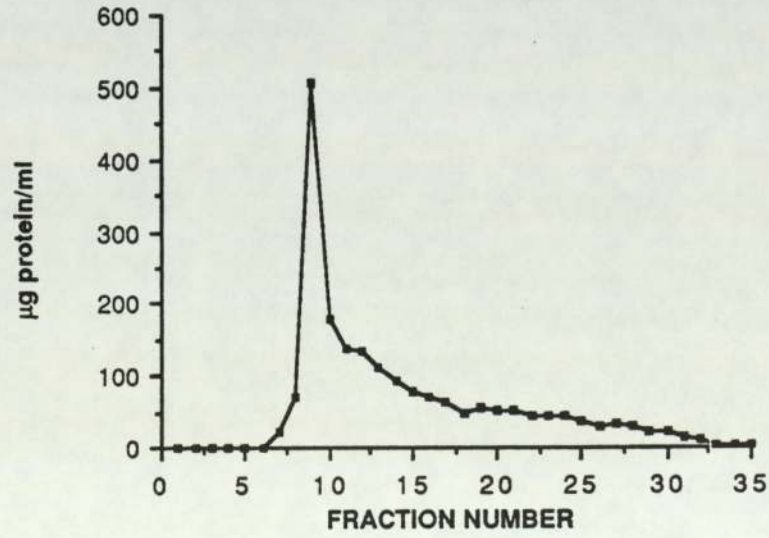


FIGURE 5.10A. CHROMATOGRAPH OF SOLUBLE GUT WASHINGS FROM FASTED RAT ORALLY DOSED WITH GALLIUM AND MALTOL (SEPHADEX G15).

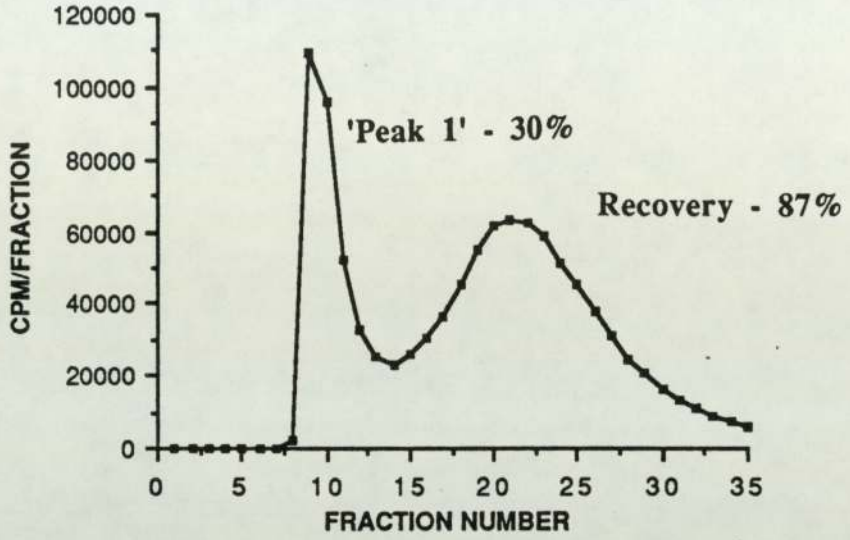
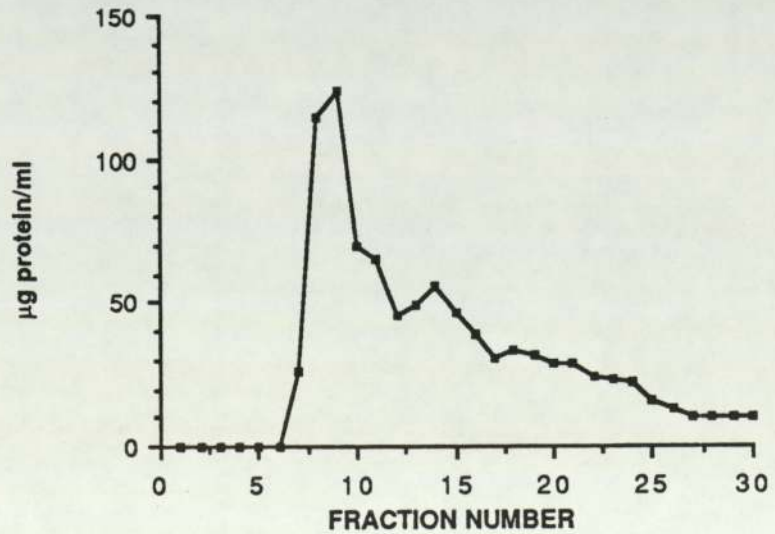


FIGURE 5.10B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.



weight species were identified, the large molecular weight complex (gallium-protein species) increasing in proportions in the fed rat (table 5.3)

TABLE 5.3. PEAK 1 COMPARISONS IN FED AND FASTED RATS ORALLY DOSED WITH GALLIUM AND MALTOL

	FED (n=6)	FASTED (n=6)
Average % of chromatograph associated with Peak 1 (±SEM)	46.2% ±3.3	22.0% ±3.5

Fed Peak 1 > fasted Peak 1, P< 0.1%, df=10.

Peak 2 in the gallium only dosed animals was thought to be associated with the 'free' hydrated gallium ion. Peak 2 however, in the gallium-maltol dosed animals may either be gallium only or gallium-maltol since gallium and gallium-maltol eluted at the same fraction on Sephadex G15 (figures 5.1a and 5.2a). Analysis of Peak 2, therefore is impossible without reeluting this fracton on an ion exchange column (figure 5.2b).

In the fed rat the gallium maltol species did not exist (figures 5.2b and 5.11a), gallium being present only in the hydrated ionic form. In the fasted rat however both the gallium-maltol species and ionic gallium were present (figures 5.2b and 5.11b).

The enhanced uptake of gallium observed only in fasted rats dosed with gallium-maltol is probably due to the lack of decomposition of this lipid soluble species in the lumen of the small intestine (see chapter 2 for tissue distribution details). Ferric ions have been observed *in-vitro* to displace gallium from the gallium-maltol complex (figure 5.2c). In the lumen of fed rats it is possible that

FIGURE 5.11A. RE-ELUTION OF 'PEAK 2' FROM FED RAT DOSED WITH GALLIUM AND MALTOL (SEE FIG.5.9A) BY DEAE SEPHADEX.

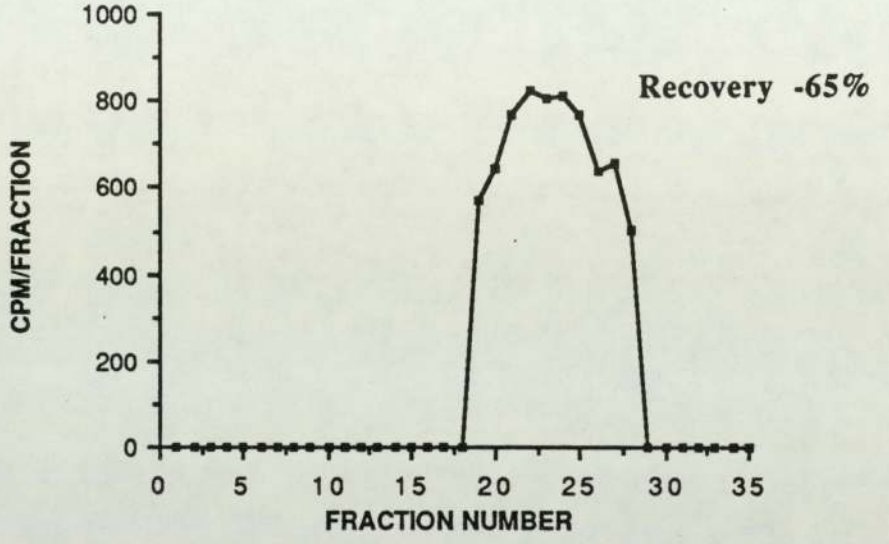
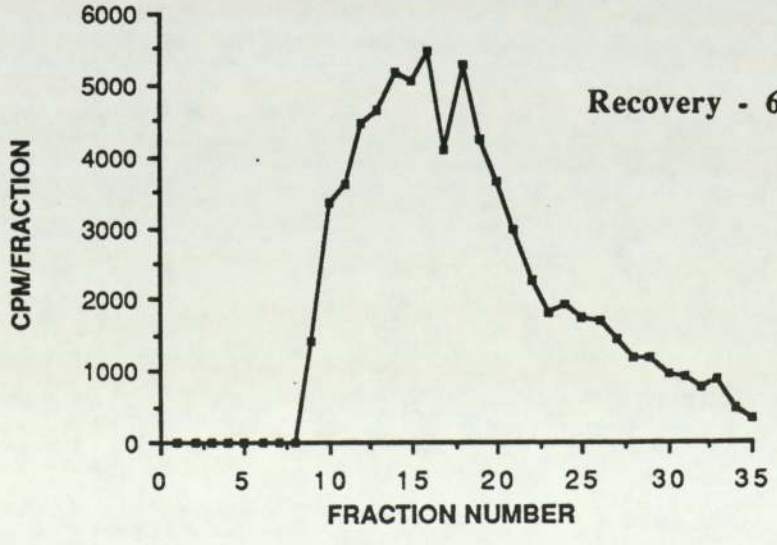


FIGURE 5.11B. RE-ELUTION OF 'PEAK 2' FROM FASTED RAT DOSED WITH GALLIUM AND MALTOL (SEE FIG. 5.10A) BY DEAE SEPHADEX.



dietary iron successfully competed with gallium for maltol leaving gallium to form other insoluble inorganic species (e.g. with hydroxides or phosphates).

5.3.5. INCUBATION OF JEJUNAL TISSUE.

Jejunal tissue was incubated with gallium in order to determine the origins of Peak 1 previously identified as an unabsorbable gallium-protein complex. It is not evident whether this species is dietary derived or is released from the intestinal mucosa. Although present to a greater extent in the gut washings of fed rats (perhaps as a result of increased dietary intake) the protein may be released from the epithelia as a result of the actual presence of food.

The soluble phase of jejunal tissue was applied to Sephadex G15 and produced a chromatograph which was similar to that of the fasted rat (the greatest proportion of gallium coeluting at a position similar to that of ionic gallium) with a large molecular weight peak eluting in the void volume (figures 5.4a and 5.12a). When this fraction was reeluted on G75, 2 peaks were observed (figure 5.12b). The first peak eluted in the 80KD range but was present to a smaller extent than the first peak of the reresolved G75 chromatographs previously mentioned (figures 5.5a and 5.6a, G75 chromatographs of Peak 1 obtained from the soluble phase of fed and fasted rats dosed with gallium only).

Protein analysis (figure 5.12c) indicated there to be a large protein presence in the 80KD fraction where gallium eluted. The second peak however, contained the majority of gallium and eluted in the region < 4KD. Again it is possible that some dissociation of a gallium-protein complex occurred whilst the column was running leaving ionic gallium to be eluted in the exclusion volume region. This phenomena however, would not solely account for the presence of gallium in peak 2. Figure 5.12c (protein in G75 fractions) indicated there to be a small peak of protein which co-eluted with peak 2. It is possible that the formation of a second gallium-protein complex could have formed during the incubation of gallium with the jejunal mucosa.

FIGURE 5.12A. CHROMATOGRAPH OF SOLUBLE PHASE OF JEJUNAL MUCOSA INCUBATED WITH GALLIUM (SEPHADEX G15).

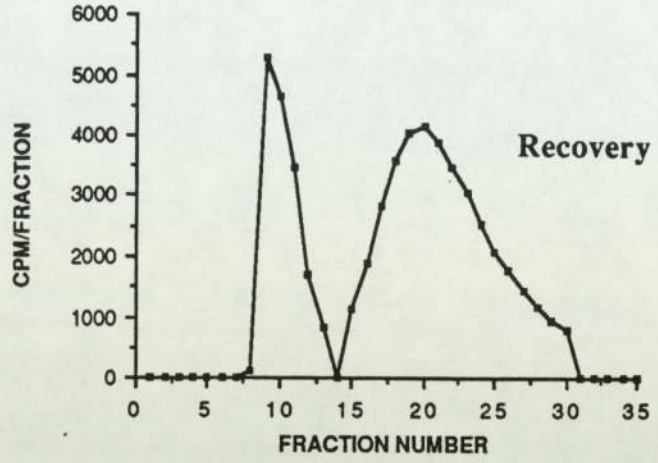


FIGURE 5.12B. RE-ELUTION OF 'PEAK 1' FROM ABOVE CHROMATOGRAPH BY SEPHADEX G75.

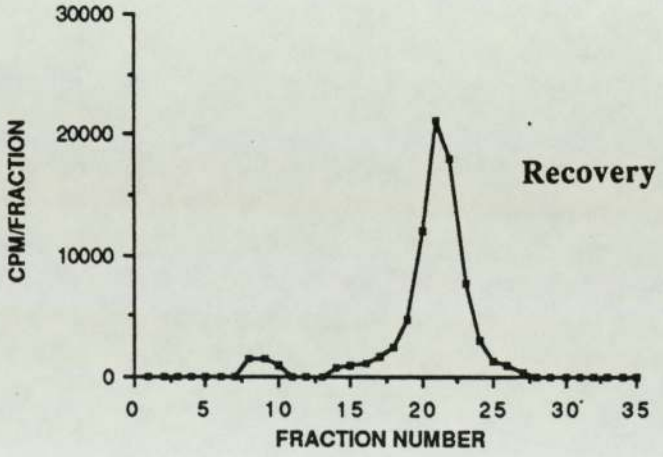
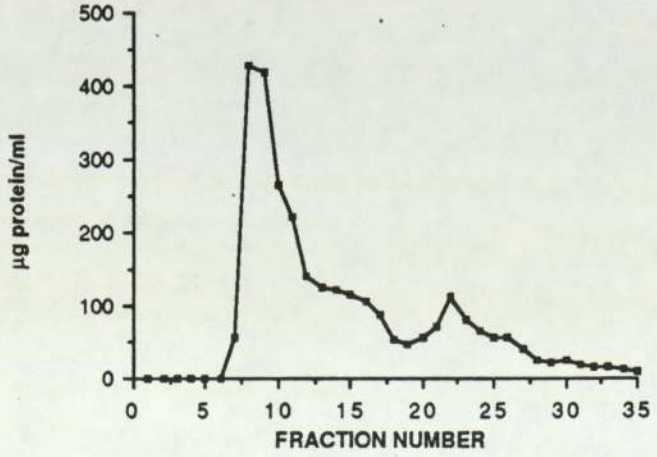


FIGURE 5.12C. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH (G75).



Gel electrophoresis of peak 1 isolated from the G15 chromatograph (figure 5.12a) identified a large number of proteins ranging from 12-73KD (figure 5.14, lane 1). It is possible that the 80KD protein binding to gallium identified by gel-filtration chromatography (figure 5.12b) corresponded with the 73KD protein isolated by electrophoresis. Autoradiography would more successfully identify gallium binding to specific proteins.

5.3.6. SPECIATION ANALYSIS OF INTESTINAL BUFFER FLUIDS.

5.3.6.1. *IN-VIVO* PERFUSION FLUIDS AND BLOOD PLASMA.

Chromatography of the perfusion fluids indicated the majority of gallium to be present in the ionic form (figure 5.13a). It is also possible (although not able to distinguish on gel-filtration systems) that gallium formed low molecular weight soluble inorganic ion pairs with the constituents of the Krebs buffer for example chloride, phosphate, carbonate or sulphate.

The remaining gallium was associated with a large molecular weight species (M.W. >1500) which appears to be mucosally derived as any soluble inorganic species would not attain a molecular weight of 1500 or over. Analysis of protein in the G15 fractions indicated there to be a protein coinciding with the larger molecular weight gallium species (figure 5.13b). It cannot be assumed however, that a gallium-protein complex has formed. During the *in-vivo* perfusion process epithelial cells would be expected to desquamate as they would in any situation where lateral movement of substances (e.g. food, liquids, perfusion fluids) is occurring. It was also noticed during the course of *in-vivo* experimentation that increasing amounts of mucous was released into the lumen and incorporated into the perfusion fluids. Any of the above factors might account for the presence of protein in the void volume.

The large molecular weight gallium fraction (i.e. peak 1) was reeluted on a G75 column, there was insufficient radioactivity however, for ^{67}Ga to be attributed to a specific molecular weight.

FIGURE 5.13A. CHROMATOGRAPH OF MUCOSAL INCUBATION FLUIDS AFTER *IN-VIVO* PERFUSION (SEPHADEX G15).

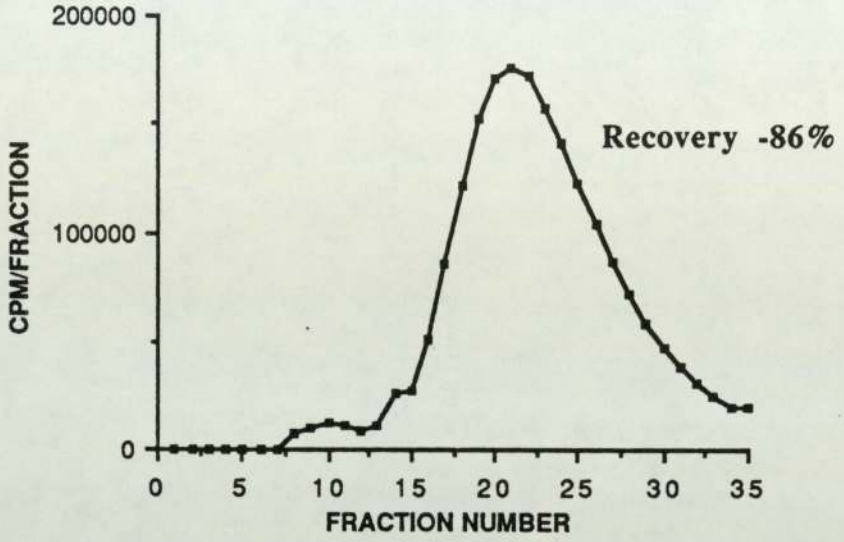
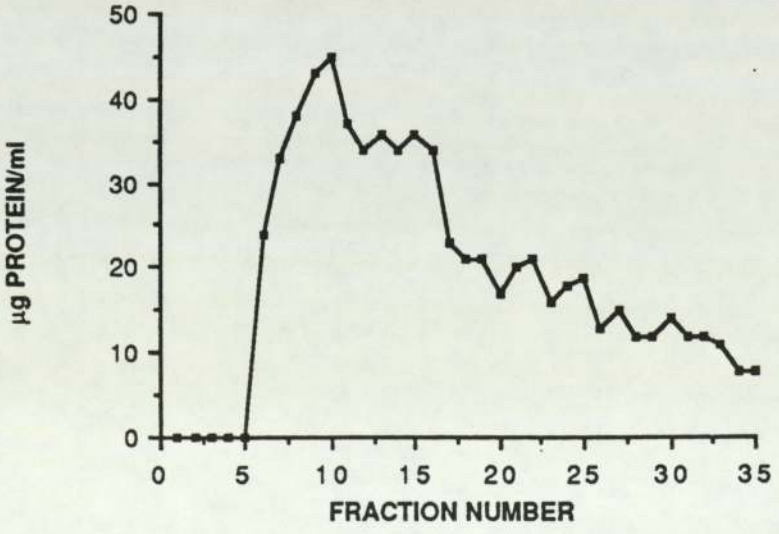


FIGURE 5.13B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.



Gel electrophoresis with silver staining identified 6 proteins in the first peak isolated by G15 chromatography (figure 5.14, lane 2). One of these was a 73KD protein, which was similarly identified when the jejunal mucosa was incubated with gallium. Although binding of gallium to a large molecular weight protein may occur, there is no evidence to suggest that a metallo-type transport protein is involved in absorption mechanisms. Kinetic studies from chapters 3 and 4 indicate that gallium is transported via a passive energy-independent process.

Although this *in-vivo* technique does not allow a quantitative measure of transported gallium, it is possible to sample blood and make comparative estimates of gallium transfered into the body compartment. In addition it is possible to spin the blood down and elute the plasma for subsequent speciation analysis.

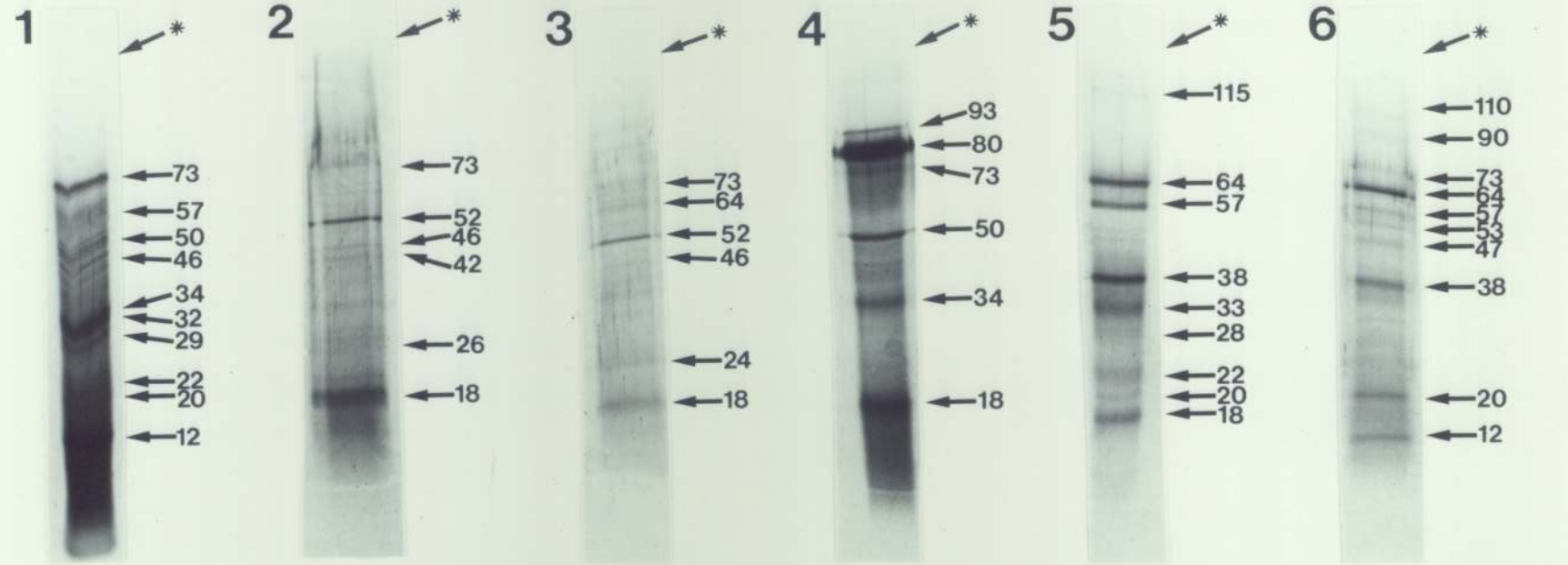
When the jejunum was perfused with 25mls 500ng/ml $Ga(NO_3)_3$ over four, 15 minute periods the blood contained less than 0.5ng Ga/ml. The plasma was eluted on Sephadex G75 and a single peak was identified in the range 70-80KD (figure 5.15a).

Although aluminium has been suggested to bind to albumin (Van der Voet et al. 1987) most workers agree that gallium (and aluminium) are more likely to follow the path of the similarily sized ferric (Fe^{3+}) ion and bind to transferrin (human blood concentation ≈40mg/100mls,M.W. = 86KD, Ganrot, 1986) (Larson et al. 1981; Martin et al. 1987).

At a blood concentration of < 0.5ng/ml, gallium was arbitarily designated to be at a 'low' concentration. As the gastrointestinal tract has proved to be a formidable barrier to the entry of gallium (see chapter 2) a 'high' concentration was parenterally administered (by IM injection) to overcome the problem of low levels of absorption in acute periods. 30 minutes after the IM injection of 0.3mls 1mM $Ga(NO_3)_3$ + 10μCi ^{67}Ga, the plasma contained 0.65 μg Ga/ml. When 3mls of plasma was eluted from the G75 column 2 peaks approximately equal in

FIGURE 5.14. SDS-GEL ELECTROPHORESIS OF RAT AND HUMAN SAMPLES ISOLATED BY GEL-FILTRATION CHROMATOGRAPHY. SEE INDIVIDUAL LANES FOR FURTHER DETAILS (GEL - SILVER STAINED).

(*INDICATES START OF GEL FRONT)



'PEAK 1' ISOLATED FROM JEJUNAL INCUBATION (FIG. 5.12A)

'PEAK 1' ISOLATED FROM MUCOSAL PERFUSION FLUIDS (FIG. 5.13A)

'PEAK 1' ISOLATED FROM MUCOSAL EVERTED SAC FLUIDS (FIG. 5.16A)

'PEAK 1' ISOLATED FROM SEROSAL TRANSFER FLUIDS (FIG. 5.17A)

'PEAK 1' FROM HUMAN SMALL BOWEL FLUIDS (*IN-VITRO* INCUBATION) (FIG. 5.19A)

'PEAK 1' FROM RAT SMALL BOWEL FLUIDS (*IN-VITRO* INCUBATION) (FIG. 5.20C).

All results expressed as KD (Kilo-Dalton ≈ molecular weight of 1000)

FIGURE 5.15A. CHROMATOGRAPH OF GALLIUM IN BLOOD PLASMA AT A 'LOW' CONCENTRATION (SEPHADEX G75).

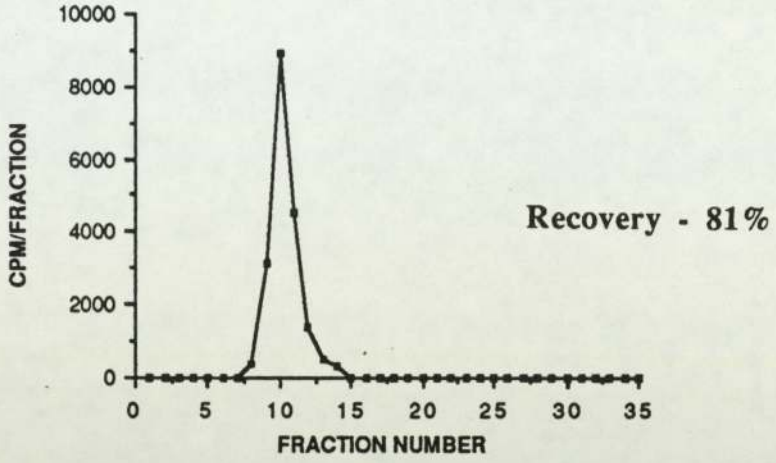


FIGURE 5.15B. CHROMATOGRAPH OF GALLIUM IN BLOOD PLASMA AT A 'HIGH' CONCENTRATION (SEPHADEX G75).

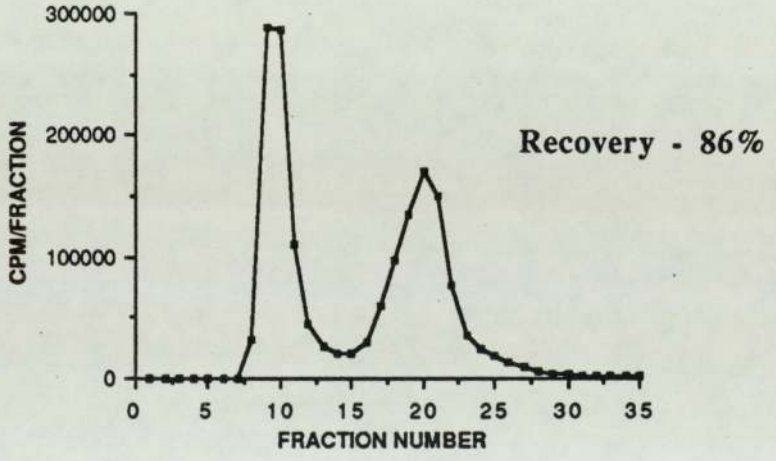
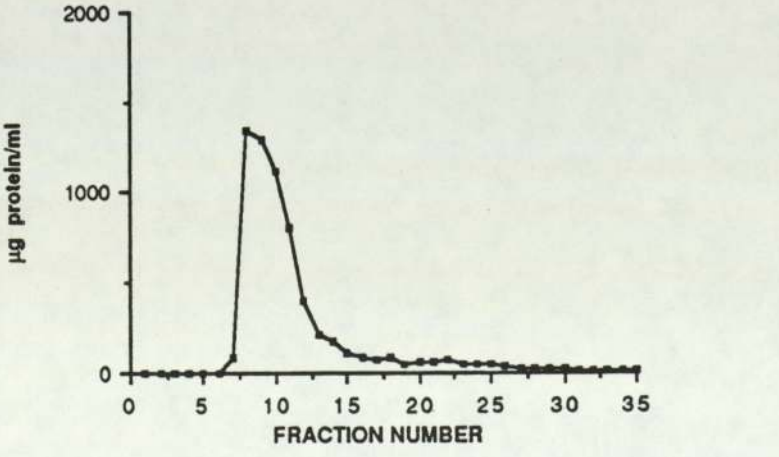


FIGURE 5.15C. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.



dimensions were observed (figure 5.15b). The first peak coelutes in the same position that was identified when the low gallium concentration plasma was eluted (i.e. equivalent to the formation of a gallium-transferrin complex). Analysis of protein in the fractions indicates a simultaneous high concentration of protein at this position (figure 5.15c). Although the fraction most probably contains albumin (the most prevalent plasma protein at a concentration of 3-4.5g/100ml, [M.W. = 68KD], Lehninger, 1975), transferrin is also likely to be present. The resolving power of Sephadex G75 is limited in the regions after the void volume (see Sephadex manual (Pharmacia Chemicals) for further explanation of column properties). The protein was found to saturate at approximately 325ng/ml gallium (≈ 4.64μmol/l). The level of human transferrin bound to aluminium in haemodialysis patients has been found to be approximately 7.4μmol/l (Kaehny et al. 1977).

The gallium attributed to peak 2 does not coincide with a plasma protein (in clinical terms this might be described as the ultrafiltrable fraction). The most likeliest low molecular weight gallium binder is citrate which has a relatively high concentration in plasma (citrate =[0.1mM/l]) and forms strong complexes with gallium and aluminium (Martin, 1986). At higher plasma gallium concentrations it appears that after the saturation of transferrin any remaining gallium forms a second complex with the low molecular weight citrate complex. The balance of this equilibrium however, might be altered if a transported gallium complex had an affinity constant greater than that of gallium-transferrin or gallium-citrate.

5.3.6.2. *IN-VITRO* EVERTED SAC FLUIDS

A). INCUBATION WITH GALLIUM ALONE.

Analysis of the *in-vitro* everted sac mucosal fluids revealed a similar pattern of speciation to the *in-vivo* perfusion fluids (figures 5.13a and 5.16a). The protein content of these fractions was also similar with a peak coinciding with the first

FIGURE 5.16A. CHROMATOGRAPH OF MUCOSAL FLUIDS AFTER *IN-VITRO* (EVERTED SAC) INCUBATION (SEPHADEX G15).

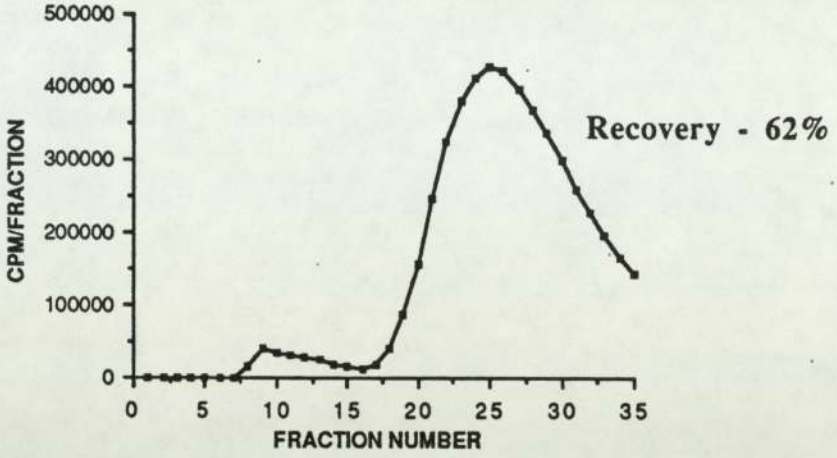


FIGURE 5.16B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH

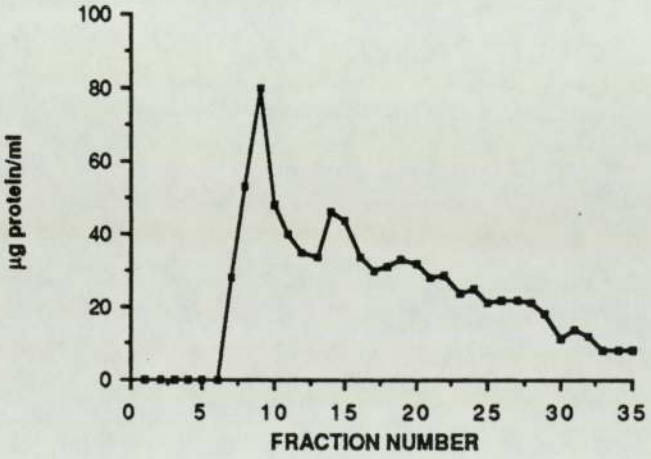
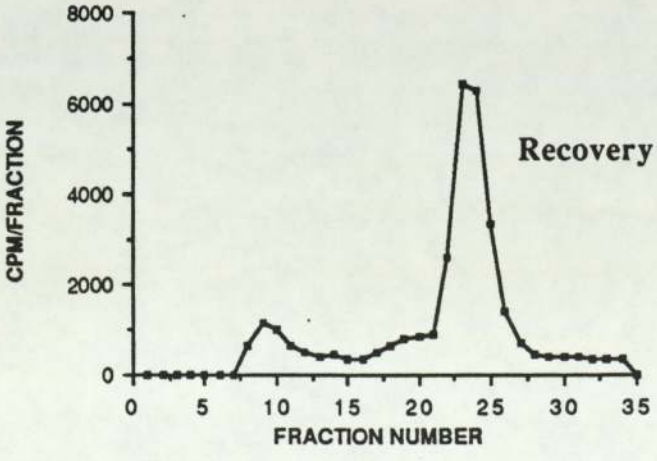


FIGURE 5.16C. RE-ELUTION OF 'PEAK 1' FROM ABOVE CHROMATOGRAPH (FIG. 5.16A) BY SEPHADEX G15.



smaller gallium peak identified in the void volume (i.e. molecular weight > 1500)(figure 5.16b). The majority of eluted gallium was found in the exclusion region and purported to be the soluble ionic gallium or soluble ionic pair complexes (see 5.3.6.1).

The 2nd peak of gallium occurred several fractions later than at the previously noted exclusion volume (fraction 17). Both mucosal and serosal fluids followed this trend. At the end of 20 minutes incubation both the mucosal and serosal fluids were noted to be more viscous in nature than the original Krebs buffer. As the flow rate and elution buffer remained unaltered, it is possible that the viscosity of the samples delayed the elution of the lowest molecular weight species present by a loose reversible binding action.

Reelution of peak 1 on Sephadex G75 indicated there to be the presence of a gallium complex at approximately 80KD (figure 5.16c). There was insufficient protein in the fractions to be identified even by the sensitive method of Lowry. The nature of this complex therefore can only be suggested, although previous experiments would point to a gallium-protein complex. It is possible that the identity of the peak is similar to that of the reeluted (G75) jejunal mucosa peak 1 (figure 5.12b).

The more sensitive technique of electrophoresis however, identified 6 proteins (figure 5.14, lane 3). It appears that similar proteins are released into the mucosal incubating fluids during both *in-vivo* and *in-vitro* experimentation.

Again the majority of the reeluted gallium is attributed to a low molecular weight complex the identity of which is unknown. Although it is likely that some dissociation of gallium-complexes has occurred leaving ionic gallium, the magnitude of the peak suggests that a low molecular weight species has formed.

G15 analysis of the serosal fluids after 20 minutes of incubation (figure 5.17a) follows a similar pattern of speciation to the G15 mucosal fluid elution profile

FIGURE 5.17A. CHROMATOGRAPH OF SEROSAL FLUIDS AFTER *IN-VITRO* (EVERTED SAC) INCUBATION (SEPHADEX G15).

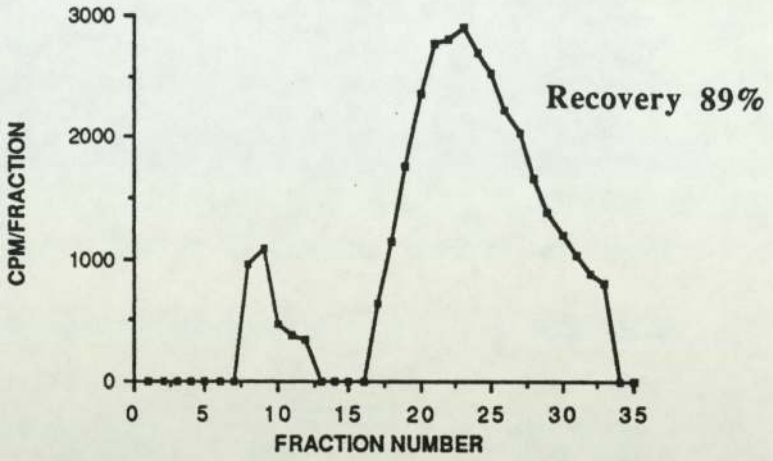


FIGURE 5.17B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.

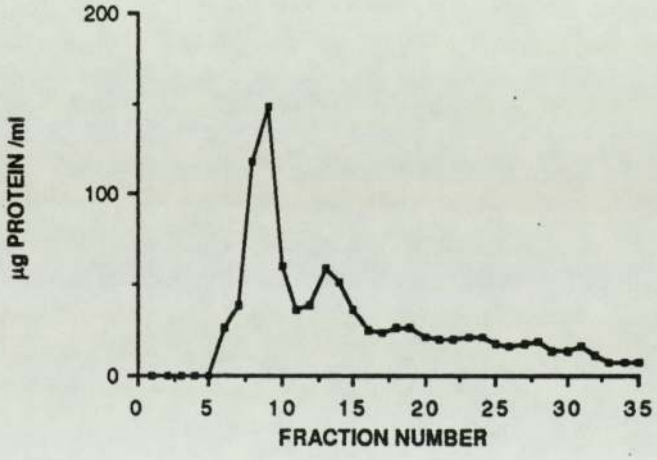
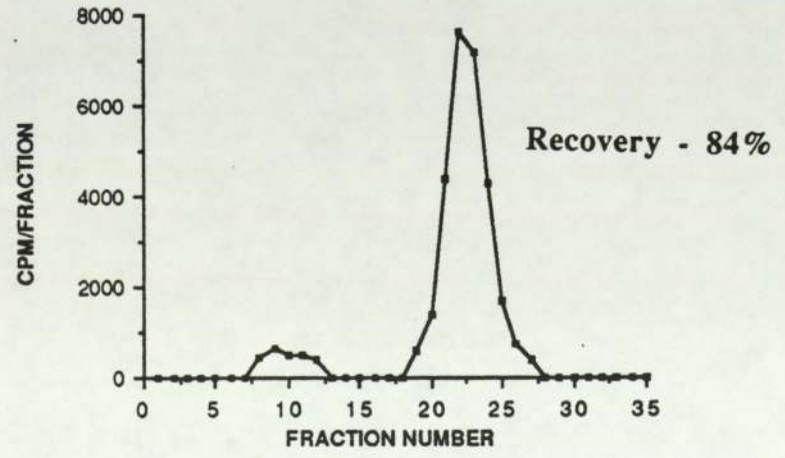


FIGURE 5.17C. RE-ELUTION OF 'PEAK 1' FROM ABOVE CHROMATOGRAPH (FIG. 5.17A) BY SEPHADEX G75.



(figure 5.16a). A small gallium peak was identified in the void volume again corresponding to a large protein presence at this position (figure 5.17b). The major gallium peak was the second, thought to be associated with 'free' ionic gallium. Relution of this G15 peak 1 on G75 again gave a bimodal distribution, the smaller of the peaks eluting at a position equivalent to approximately 80KD.

It is not possible to clarify whether this large molecular weight gallium complex (probably of protein origin) is the transported species. Having been identified in both mucosal and serosal fluids it could be suggested that gallium is transported to a certain extent in complex with this large molecular weight protein. Electrophoresis of the serosal fluids identified the large presence of an 80KD protein aswell as other proteins (figure 5.14, lane 4). This particular protein was not identified in the *in-vivo* or *in-vitro* mucosal fluids. The origins of this protein however, remain unknown. It could possibly be released independently from the serosal membrane, or alternatively may have been transported (either bound or unbound to gallium) from within the intestinal mucosa.

Kinetic evidence however, (see Chapters 3 and 4) does not point to the presence of any specific metallo-transport proteins acting in an energy-dependent fashion. The majority of gallium in both the mucosal and serosal fluids is present as the 'free' hydrated form and collective evidence (from this and previous chapters) indicates that this is the major transported species when gallium is present alone in the incubating buffers.

Although the possibility of the formation of a large metallo-transport protein cannot be discounted, it is more likely that some of the gallium ions have bound to proteins extruded by both the mucosal and serosal membranes. As aluminium (and gallium) are non-physiological it is unlikely that a specific transporter protein exists. Although aluminium and gallium have been likened to the ferric ion, evidence from this thesis does not imply that the three ions are transported across the intestinal mucosa by similar mechanisms.

Aluminium and gallium have been observed to traverse the intestinal membrane via a passive paracellular route (i.e. through the tight junctions). Transport is energy independent being unaffected by the addition of the metabolic inhibitor 2,4-dinitrophenol (see chapters 3 & 4). The *in-vitro* tissue uptake and subsequent serosal transfer of gallium was not significantly different in duodenum, jejunum or ileum.

The absorption of iron is believed to be based upon the maintenance of a constant body iron concentration (normal adult body load = 3-5g iron, Spivey-Fox and Rader, 1988). The mechanisms are regulated by intraluminal factors (the chemical nature and concentration of iron in the gut), mucosal factors (iron status of the proxmal small intestinal mucosa) and corporeal factors (rate of red blood cell production and tissue iron stores). A combination of these three factors regulates iron absorption so that when iron stores are depleted absorption is stimulated and vice versa (Conrad, 1987).

B) INCUBATION WITH GALLIUM AND MALTOL

The incorporation of maltol into the Krebs buffer does little to alter gallium speciation when the mucosal and serosal fluids are eluted by gel-filtration chromatography (chromatographs not shown being similar to figures 5.16a to 5.17b). There is the small presence of a possible gallium-protein complex on both sides of the mucosal membrane, the majority of gallium however, being eluted in the exclusion volume regions as the hydrated gallium ion or in complex with maltol.

From the elution profile studies with gallium alone and gallium-maltol (figures 5.1a, 5.2a, 5.2b) it was observed that the two species could not be separated by gel-filtration alone. Ion-exchange chromatography however, separating on the basis of charge can differentiate the species. It is therefore possible to discover the nature of the transported species. The mucosal incubating fluid chromatograph

was similar to that of 5.16a, peak 2 including both the hydrated gallium ion and the gallium-maltol species. When peak 2 was reeluted by ion-exchange chromatography the majority of the gallium was attributed to the gallium-maltol species (figure 5.18a). Speciation analysis of the serosal fluids indicated there to be a combination of both gallium-maltol and gallium alone, the former mentioned species being the most prevalent (figure 5.18b).

The enhanced *in-vitro* transport of gallium observed in the presence of maltol (gallium only, serosal transfer = 0.0070 (±0.0013) ng Ga/mg wwt; gallium and maltol, serosal transfer = 0.011 (±0.00073) ng Ga/mg wwt, $P<5\%$) may be explained by the partial presence of the neutral lipid soluble species in the serosal compartment (see chapter 3). Maltol also enhanced the absorption of gallium in the fasted rat and in the *in-vivo* perfused animal (see chapters 2 & 4). The rate of transport of the lipid-soluble gallium maltol complex appears to be greater than that of the 'free' hydrated gallium ion crossing the epithelial membrane by the rate-limiting tight-junctions.

5.3.7. EVERTED SAC STUDIES WITH THE LARGE MOLECULAR WEIGHT SPECIES ISOLATED FROM SOLUBLE GUT WASHINGS OF RATS ORALLY DOSED WITH GALLIUM.

The formation of a large molecular weight species binding to gallium in the soluble phase of gut washings (figure 5.3a) was thought to be partially responsible for the diminished absorption of gallium in the fed rat when compared to the fasted (see chapter 2 and 5.3.3). The large molecular weight species (peak 1) was isolated and jejunal everted sac experiments performed (see 5.2.9). The results are described in table 5.4.

FIGURE 5.18A. CHROMATOGRAPH OF MUCOSAL FLUIDS AFTER *IN-VITRO* INCUBATION WITH GALLIUM AND MALTOL (DEAE SEPHADEX).

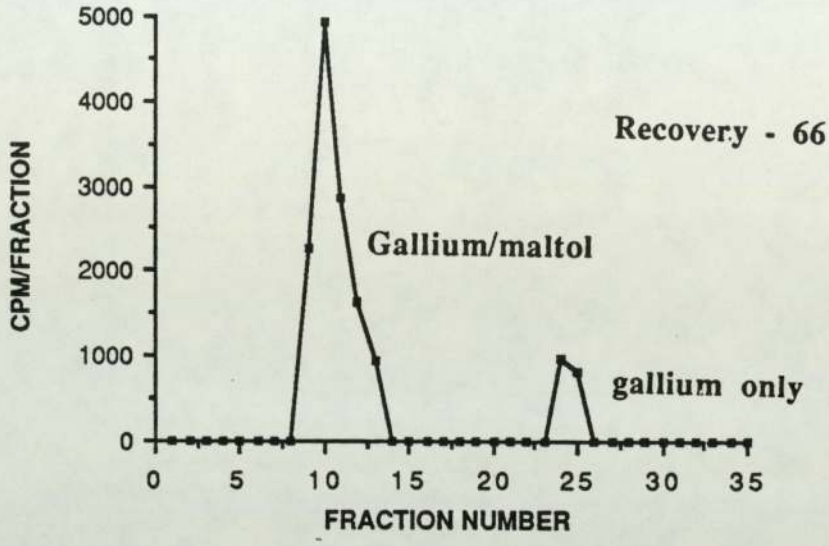


FIGURE 5.18B. CHROMATOGRAPH OF SEROSAL FLUIDS AFTER *IN-VITRO* INCUBATION WITH GALLIUM AND MALTOL (DEAE SEPHADEX).

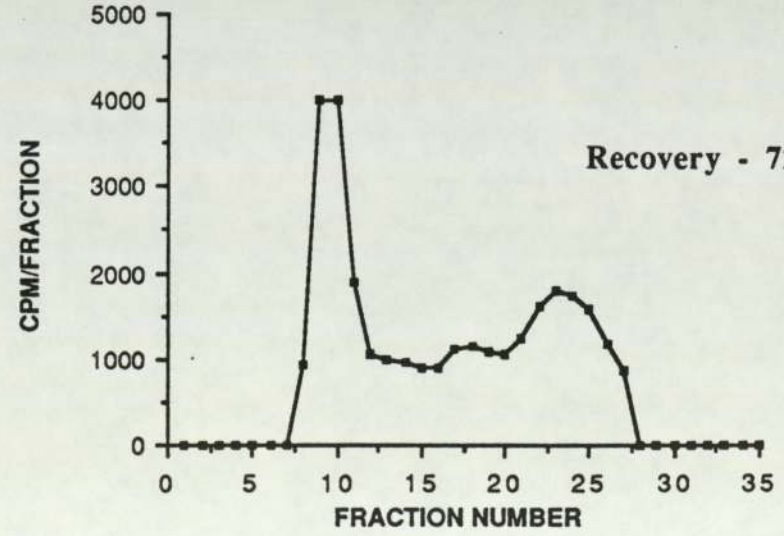


TABLE 5.4. *IN-VITRO* EVERTED SAC EXPERIMENTS: THE MUCOSAL UPTAKE AND SEROSAL TRANSFER OF 'PEAK 1'

MUCOSAL UPTAKE

PEAK 1 = 11.91% (±1.52) GALLIUM ONLY = 20.55% (±2.59)*

SEROSAL TRANSFER

PEAK 1 = NOT OBSERVED GALLIUM ONLY = 0.68% (±0.17)

Notes.
1). The concentration of gallium in both test (Peak 1) and control (gallium only) incubating fluids was ≈ 10ng/ml.

2). Mucosal uptake results expressed as % of incubating gallium taken up by the mucosa per gram wet weight tissue (n=5,±SEM), *P<5% indicates a significant increase in gallium tissue uptake in the gallium only experiments compared to Peak 1.

3). Serosal transfer results expressed as % of mucosally bound gallium transported per gram wet weight tissue (n=5,±SEM).

Sufficient activity of gallium was introduced into the experiment to ensure that if transport occurred the detection of ^{67}Ga would not be a problem. The mucosal uptake of gallium was significantly lowered in the test case (incubation with 'Peak 1') when compared to the control (gallium alone) whist transport of gallium was not observed when incubating with the large molecular weight species (table 5.4). The results of this *in-vitro* experiment support the findings of the oral dosing/redosing experiments (5.3.3) where the readministered 'Peak 1' was not identified in the tissues.

Chromatography of the mucosal washings (not shown) from the test case indicate the majority (>75%) of gallium to remain in association with the large molecular weight species.

5.3.8. SPECIATION ANALYSIS OF HUMAN SMALL BOWEL FLUIDS.

Speciation analysis of human intestinal washings revealed a similar pattern to that of the rat with the exception of a small third peak eluting between the large molecular weight species (probably of protein origins) and the exclusion volume

species (either soluble ion pair complexes or free hydrated gallium) (figure 5.19a).

Analysis of protein in the fractions of this G15 chromatograph identified 3 protein peaks coeluting with the gallium peaks (figure 5.19b). It is likely that the last two protein peaks represent the elution of polypeptide fragments possibly being the products of digestion. It is not possible to specify however, whether gallium is binding to these proteins particularly in the lower molecular weight range because of the formation of other expected inorganic complexes.

If the first large molecular weight peak is reresolved on Sephdex G75, 2 peaks are observed (figure 5.20a). The first is identified in the range 80KD and coelutes with a protein (figure 5.20b). The second gallium peak is observed in the exclusion volume region (<4KD) and corresponds to a smaller protein peak. Again it is not possible to state whether this peak is a gallium-protein complex or is the result of dissociation on the column leaving (in part) hydrated gallium.

The *in-vitro* incubation of gallium with rat small intestinal fluids (rather than the *in-vivo* oral administration of gallium) produced a similar chromatographic result both to the oral dosing of gallium and the incubation of human fluids with gallium. Gel electrophoresis of 'Peak 1' from both human and rat *in-vitro* experiments identified a range of proteins from 12 to 115KD (figure 5.14, lanes 5 & 6). Several of the proteins coincided in both human and rat samples. Although gel-filtration cannot reproduce the resolution of electrophoresis techniques, neither can electrophoresis follow the pathway of the radioisotope. The determination of gallium binding to specific proteins could be more accurately achieved with autoradiography methods.

The speciation of gallium in human small bowel fluids was largely similar to to both the *in-vivo* (orally dosed animals, see 5.3.2) and *in-vitro* species formed in rat small intesinal fluids. Due the difficulty in obtaining human small bowel fluids and performing *in-vivo* human speciation studies, it appears that the rat is a

FIGURE 5.19A. CHROMATOGRAPH OF HUMAN SMALL BOWEL FLUIDS AFTER INCUBATION WITH GALLIUM (SEPHADEX G15)

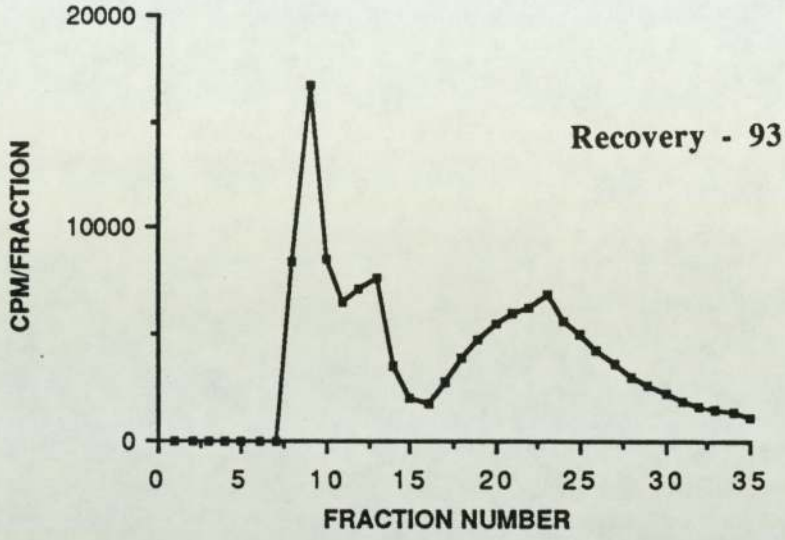


FIGURE 5.19B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.

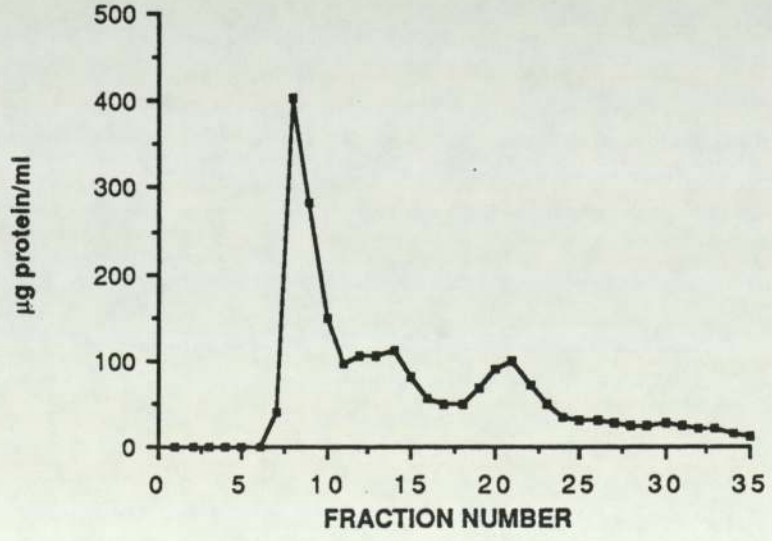


FIGURE 5.20A. RE-ELUTION OF 'PEAK 1' FROM HUMAN FLUIDS (SEE FIG.5.19A) BY SEPHADEX G75.

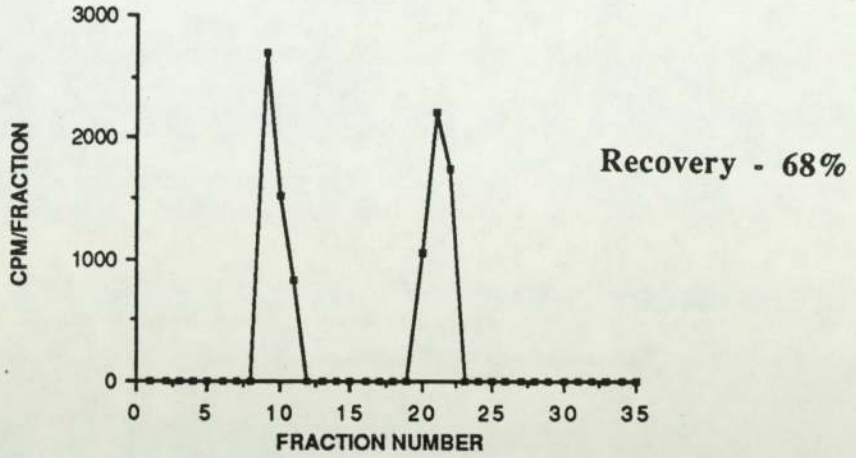


FIGURE 5.20B. PROTEIN CONTENT IN FRACTIONS FROM ABOVE CHROMATOGRAPH.

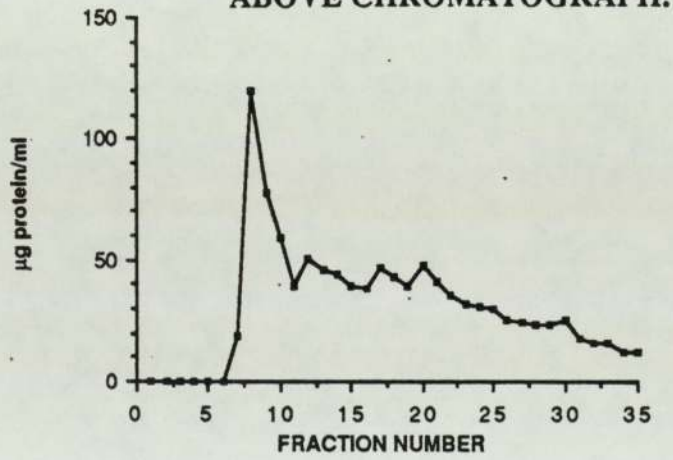
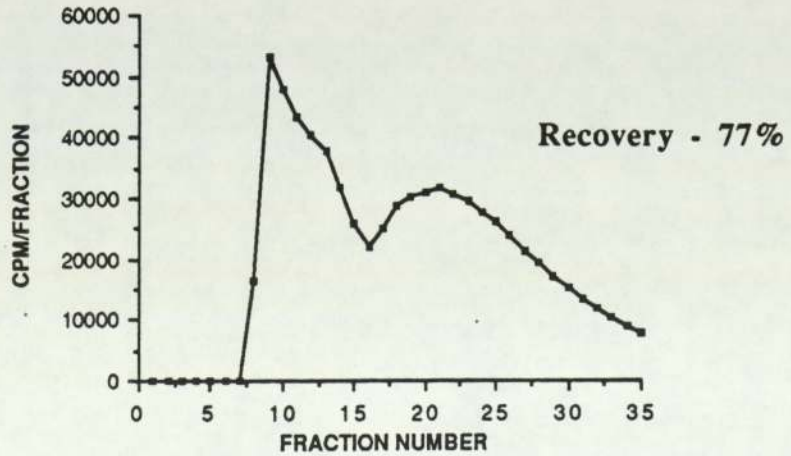


FIGURE 5.20C. CHROMATOGRAPH OF RAT SMALL BOWEL FLUIDS AFTER INCUBATION WITH GALLIUM (SEPHADEX G15)



suitable model to study gallium and aluminium speciation and ligand interaction (ultimately leading to bioavailability differences and possible neurotoxicolgical consequences).

5.4 CONCLUSIONS.

Standard solutions of $Ga(NO_3)_3$ with EDTA, citrate, maltol and ferric ions were characterised by gel-filtration and ion-exchange chromatography.

When the soluble phase of gut washings of fed and fasted rats orally dosed with gallium were applied to Sephadex G15 gel both a large and small molecular weight species were identified. The large molecular weight gallium binding species ('Peak 1') was significantly greater in proportion in fed rats compared to fasted. Peak 1 corresponded to a large presence of protein in those fractions. Analysis of Peak 1 by SDS gel electrophoresis identified 10 proteins ranging from 6KD to 130KD, comparative proteins being identified from both fed and fasted 'Peak 1'.

The second low molecular weight peak corresponded to the region where ionic gallium was eluted. This peak was proportionally greater in fasted rats compared to fed. Gallium absorption was enhanced in fasted rats possibly as a result of the increase in proportions of 'free' hydrated gallium present in the small bowel fluids. The large molecular weight gallium binding protein in the fed rat (in combination with a reduction in the concentration of hydrated gallium) may be responsible for lowering gallium absorption in the fed state. This hypothesis is supported by the inverse correlation observed between % Peak 1 size and the % TDA observed in the largest organ measured, liver ($r=0.93$, $P<0.1\%$, $df=9$).

When Peak 1 was isolated and readministered to both fed and fasted animals, no absorption into any of the body organs was observed. The soluble phase of gut washings from these redosed rats was chromatographed and the majority of

gallium was retained as this high molecular weight species. *In-vitro* everted sac experiments were also performed with the isolated Peak 1. Results of these studies confirmed the *in-vivo* redosing experiments where no gallium (bound in the form of the molecular weight complex) was transported to the serosal compartment. It is possible therefore, that the formation of this gallium binding protein is protective in nature, certainly to a greater extent in the fed state.

The origins of this gallium-binding protein is as yet unidentified, either being dietary or mucosally derived. When jejunal tissue was incubated with gallium again a large molecular weight peak was observed in the void volume region, similar in proportions to G15 'Peak 1' identified in the soluble phase of gut washings. When this peak was reresolved on G75 a small peak in the region 80KD (coinciding with the presence of protein) was present. A similar peak at 80KD was observed when 'Peak 1' from the gut washings was reresolved. Although it is possible to compare the peaks from the jejunal incubation and the soluble gut washing chromatographs results must be interpreted with caution. The resolving power of Sephadex G75 gel at the high molecular weight regions is limited. In addition SDS gel electrophoresis identified several proteins above the resolution limits (≈ 80KD) and the 80KD gallium binding protein could be attributed to any one of these.

When the mucosal fluids of *in-vitro* and *in-vivo* intestinal absorption experiments were eluted on Sephadex G15, a large molecular weight gallium binding complex (co-eluting with the presence of protein) was identified as a minor peak. The major peak was associated with free hydrated gallium ion which is thought in these experiments to be the transported species. When this large molecular weight species was reeluted on Sephadex G75 again a gallium binding complex at approximately 80KD was present (and again corresponding to the presence of protein). In the everted sac mucosal fluids however, this species represented only 1/250 of the gallium identified in the hydrated ionic form.

When gallium and maltol were simultaneously dosed, again the large molecular weight species binding to gallium was observed ('Peak 1') after analysis by gel filtration chromatography. This species was predominant in the fed rat. The second peak possibly contained both gallium alone and/or the gallium-maltol complex and was therefore reresolved by ion-exchange chromatography. In fed rats dosed with both gallium and maltol, the gallium-maltol complex was not present suggesting that the species had been decomposed by other dietary components. In the fasted rats, however both the gallium-maltol species and gallium alone were present in the gastrointestinal contents. The presence of both species in the gut lumen explains the enhanced absorption of gallium in the presence of maltol (the resulting species being neutral, resistant to hydrolysis and lipid soluble) in the fasted as opposed to the fed rat (see chapter 2). Ion exchange chromatography also identified the gallium-maltol species in both mucosal and serosal fluids from *in-vitro* everted sacs.

At a low concentration of perfused gallium (i.e. *in-vivo* intestinal experiments) the gallium-binding species present in blood was a single large molecular protein, probably a gallium-transferrin complex. At a high concentration however, gallium was present in combination with transferrin and a low molecular species, probably gallium-citrate. When transferrin is saturated with gallium, any remaining gallium possibly reverted to the low molecular weight citrate species.

When human small bowel fluids were incubated with gallium, a similar pattern of G15 speciation was observed to the soluble phase of the fasted rats orally dosed with gallium (i.e. a bimodal distribution representing a large molecular weight gallium binding species and a low molecular weight peak probably representing hydrated gallium). A similar 80KD peak (corresponding to the presence of protein) was observed when the large molecular weight species was reeluted on G75.

Unfortunately human mucosal tissue was not available and therefore speciation after incubation with mucosa could not be compared to that of small bowel fluid speciation.

CHAPTER SIX

GENERAL DISCUSSION

CHAPTER 6. GENERAL DISCUSSION.

6.1 INTRODUCTION

Although aluminium is abundant in the environment, the body burden within humans is limited (<35mg) due to the formidable barriers which aluminium must overcome upon entry.

During the past 15 years however, toxicity has been observed. The aetiology of dialysis dementia (Wills and Savory, 1983) and dialysis osteomalacia (Malluche and Faugere, 1985) has been attributed to aluminium poisoning, whilst there is the possibility that aluminium is involved in the pathogenesis of senile dementia of the Alzheimer type (Perl and Good, 1987) and parkinsonian dementia of Guam (Garruto et al. 1986). Since water borne exposure to aluminium in dialysate fluids has largely been eliminated by haemofiltration methods (Ward et al. 1978) it is thought that the major route to aluminium in kidney failure is via the gastrointestinal tract. This is due to the ingestion of large quantities of $Al(OH)_3$, used to bind intestinal phosphate thus preventing hyperphosphataemia.

The general public at large may also be at risk to aluminium exposure. As aluminium is the most common metal in the earth's surface it is not possible to prevent it from being included in the diet. Aluminium salts are commonly used as food additives, particularly in baking and pickling processes. Aluminium cookware is also a source of dietary aluminium especially when food is cooked under acidic conditions (Lione, 1985). Aluminium is also known to accumulate in certain plants. The tea bush is known to contain high concentrations of aluminium, infused Indian and Chinese tea containing up to 60ppm aluminium (Coriat and Gillard, 1986). The addition of aluminium sulphates to aid water purification, a practice adopted by many water authorities, is probably the greatest general source of aluminium. Water aluminium content has been measured up to 3500µg/l (country wide range - <10 to 3500µg/l) (Davison et al. 1982).

Recent epidemiological evidence has correlated the incidence of Alzheimers disease with an increase in drinking water aluminium concentration (Martyn, 1988).

If oral aluminium is the major contributor to the body burden of aluminium, an understanding of absorption mechanisms, intestinal speciation and bioavailability is important. Intestinal research to date has been limited because of difficulties in the quantification of aluminium. Although there are radioisotopes of aluminium (^{26}Al, $t_{1/2} = 7 \times 10^5$ years; ^{28}Al, $t_{1/2}$ = 2.27minutes, both ß-emitting), none of them are suitable for studying physiological and biochemical systems. This thesis assessed the suitability of two chemically similar Group 3 γ-emitting radioisotopes, ^{46}Scandium ($t_{1/2}$ = 84 days) and ^{67}Gallium ($t_{1/2}$ = 78 hours) for furthering the understanding of aluminium metabolism and proposed that these isotopes could be utilized as possible markers for aluminium in both animal and clinical experimentation.

6.2 GALLIUM AND SCANDIUM AS MODELS FOR ALUMINIUM

6.2.1. TISSUE DISTRIBUTION EXPERIMENTS

4 hours after the oral administration of aluminium, gallium and scandium, less than 1% of the total dose administered was identified in the tissues. This restricted absorption is thought to be due to the formation of insoluble, unabsorbable phosphate and hydroxide complexes (table 2.2).

Nutritional status (i.e. either fed or fasted rat) was also important in determining levels of uptake. Absorption of aluminium, gallium and scandium was significantly enhanced in fasted rats when compared to fed (table 6.1). In fasted rats, scandium levels were significantly lower than those of aluminium (both indigenous and exogenous aluminium) and gallium (table 2.2).

TABLE 6.1. SUMMARY OF TRENDS OBSERVED AFTER THE ACUTE ORAL ADMINISTRATION OF ALUMINIUM, SCANDIUM AND GALLIUM AND THE EFFECTS OF CITRATE, MALTOL AND FLUORIDE ON BIOAVAILABILITY OF GALLIUM.

Element/additive	**Fed rat**	**Fasted rat**
Aluminium	-	Increased absorption compared to fed rat *
Scandium	-	Increased absorption compared to fed rat*
Gallium	-	Increased absorption compared to fed rat***
Gallium/citrate	No effect	Increased absorption compared to fasted gallium only dosed rats**
Gallium/maltol	No effect	Increased absorption compared to fasted gallium only dosed rats**
Gallium/fluoride	Decreased absorption compared to fed gallium only dosed rats**	Decreased absorption compared to fasted gallium only dosed rats***

Notes.
1). Results taken from table 2.2. and table 2.4.
2). See individual tables for exact figures and dosing regimes.
3). *P<5%, **P<1% and ***P<0.1% indicates a significant difference from the control value of that group.

Studies of the acute tissue distribution of aluminium, gallium and scandium indicated that all three showed a particular retention (per unit weight of tissue) in skeletal tissue. Retention into spleen, heart and muscle tissue was also noted (figures 2.7 and 2.8).

Clinically the retention of aluminium in the skeletal tissue of uremics taking $Al(OH)_3$ as a phosphate binder is a serious problem. The symptoms (bone softening and fracturing) are however, reversible in the early stages after chelation with desferrioxamine (Seyfert et al. 1987).

Nutritional status did not affect the relative tissue distribution of aluminium, gallium and scandium, only to lower the presence of the elements in the fed tissues.

The long half-life of ^{46}Scandium (84 days) allows long term experiments to be performed. After a 12 day chronic dosing program significantly greater concentrations of scandium were observed in all tissues measured except spleen (figure 2.5). This accumulation may be explained by the model proposed in section 6.8. Again when the results were converted to activity per unit weight, accumulation of scandium was most pronounced in skeletal tissue. Similar observations had been made in the acute aluminium, gallium and scandium experiments. Binding occurs predominantly in the metabolically active regions of bone tissue (Koo et al. 1988). This is due to the high specificity of all Group 3 elements for phosphates. Iwata (1979) has observed that aluminium can substitute into the fine crystals of calcium hydroxyapatite which are active at the osteoid and mineralisation front. It is possible that scandium and gallium act in a similar fashion. The severity of osteomalacia is thought to correlate with the bone aluminium content (Goodman et al. 1984).

The chronic scandium experiments of this thesis confirm the noted accumulation of Group 3 elements (in particular the clinical observations of aluminium accumulation in renal disease) in skeletal tissue.

In addition to the scandium observed in the femur, retention was also noted in spleen, heart and muscle. Similarly aluminium, gallium and scandium accumulation was noted in these tissues in the acute experiments.

After the 12 day oral dosing program had ended, a second group of rats were kept for a further 5 days to allow for excretion mechanisms to take place. Minimal amounts of scandium were recorded in liver, kidney and spleen, whilst no scandium was detectable in heart or brain (table 2.3). A similar 'washout' of aluminium from the brain may be occurring in dialysis dementia patients who stop taking the phosphate binding aluminium hydroxide. Preliminary encephalopathic symptoms have shown to be reversible when aluminium administration ceased (Masselot et al. 1978).

After this 5 day period however, the amounts of scandium retained in muscle and femur tissue did not significantly differ from the levels which were recorded at 12 days. The retention mechanisms (high specificity for the phosphate groups at the mineralisation front) which were thought to be responsible for aluminium, gallium and scandium accumulation in skeletal tissue after acute exposure, were likely to be responsible for the accumulation and lack of excretion in the chronically dosed experiments.

^{67}Gallium was used in short term experiments to study the effects of some food and water additives on the bioavailability of gallium. Nutritional status was found to play an important role in affecting bioavailability of gallium when simultaneously dosed with citrate, maltol or fluoride.

Citrate and maltol enhanced the absorption of gallium in the fasted rat only whilst fluoride inhibited the bioavailability of gallium in both fed and fasted rats. (table 6.1). The neutral-lipid soluble gallium-citrate or maltol complex is thought to persist only in the fasted intestinal lumen. In the fed state gallium is thought preferentially to bind to insoluble inorganic ligands (see also discussion on intestinal speciation). Fluoride however, maintains its complex with gallium in

both fed and fasted animals, at least to a certain extent resisting the formation of insoluble complexes in the fed state.

Addition of citrate, maltol and fluoride, did not however, affect the relative tissue distribution of gallium compared to control (gallium only dosed) in either fed or fasted animals.

The intestinal interactions of aluminium with citrate and fluoride in humans have been previously discussed, whilst maltol has been postulated to be a potential neurotoxic complexing agent (Slanina et al. 1984, 1986; Spencer et al. 1981; Finnegan et al. 1986, 1987).

6.2.2. *IN-VITRO* INTESTINAL EXPERIMENTS

In addition to the *in-vivo* tissue distribution studies, comparative *in-vitro* everted sac experiments were undertaken with aluminium, scandium and gallium to determine mechanisms of uptake.

The *in-vitro* mucosal uptake of aluminium, scandium and gallium saturated with time after 30 minutes of incubation (figures 3.1A-3.3A). The saturation of mucosal binding sites however, was less pronounced for gallium when compared to aluminium and scandium. Serosal transfer of all three elements was quantitatively lower than mucosal uptake, but was linear with time (figure 3.1B-3.3B). Addition of the metabolic inhibitor 2,4-dinitrophenol did not affect mucosal uptake or serosal transfer in any of the three elements measured. The results indicate that aluminium, scandium and gallium were transported by a similar energy independent mechanism.

The percentage of mucosally bound element transported to the serosal compartment was less than 1% for aluminium, gallium and scandium (table 3.1). Similar levels of permeation were observed in the *in-vivo* tissue distribution experiments. The percentage of transported scandium was significantly less than that of aluminium or gallium in both fasted animals and in *in-vitro* everted sacs.

These qualitative *in-vivo* tissue distribution and *in-vitro* everted sac experiments, which have comparisons with clinical situations indicate that both gallium and scandium are to a certain extent suitable markers for studying aluminium in physiological situations. As scandium showed a lower tendency for absorption, gallium was considered preferential and utilised for the majority of experimentation in this thesis.

6.3 THE *IN-VITRO* AND *IN-VIVO* INTESTINAL ABSORPTION OF GALLIUM.

The *in-vitro* everted sac incubation indicated that there was an avid binding of gallium to the mucosal surface in the first few minutes of incubation followed by a slower uptake over the rest of the 30 minute incubation period. Gallium transport to the serosal compartment was approximately one hundreth of the mucosally bound gallium, movement being linear over the incubation period. The concentration based uptake and transport of gallium was linear over the range 5 to 4000ng/ml gallium, whilst addition of the metabolic inhibitor 2,4-dinitrophenol did not affect either mucosal uptake or serosal transfer of gallium (figures 3.4/3.6).

The results are indicative of mucosal uptake and serosal transfer of a passive energy-independent nature. Transfer is probably via the cation specific paracellular (i.e. tight-junctional) route.

Gel-filtration chromatography of the *in-vitro* mucosal and serosal fluids indicated that the major species in solution was the 'free' hydrated gallium ion (figure 5.16A/5.17A). It is likely that this species was the transported species. Chromatography also identified that gallium was bound to a minor species approximately 80KD in size. Gel electrophoresis of this minor fraction identified several proteins in both the mucosal and serosal fluids. The results of the kinetic

studies however, did not suggest that gallium was actively transported and any binding to proteins appears to be irrelevant in affecting the passive transport mechanisms.

The effect of pH upon the absorption of gallium was also studied in the *in-vitro* system. Although there was a significant reduction in fluid transport, both mucosal uptake and serosal transfer of gallium increased when the pH of the Krebs incubating buffer was reduced from 7.4 to 3.4 (table 3.2). With increasing acidity there is an increase in the concentration of the hexahydrated $Ga(H_2O)_6^{3+}$ species. This species is not hydrolysed (unlike those species at neutral pH e.g. $Ga(H_2O)_3(OH)_3$, see also figure 1.1) and is therefore more likely to be transported.

The *in-vivo* intestinal perfusion experiments revealed a similar pattern of results to the *in-vitro* experiments. Again there was a rapid binding of gallium in the first period of incubation (figure 4.5). At all concentrations measured gallium absorption was significantly greater in the first 15 minutes compared to the subsequent three,15 minute incubation periods. The concentration based uptake of gallium (as measured by the loss of gallium from the perfusate, and thereore including transport) was linear as was the transfer of gallium to liver, kidney, spleen and blood system (figures 4.4 and 4.6). Addition of the metabolic inhibitor 2,4-dinitrophenol again did not affect either mucosal uptake or serosal transport of gallium (figure 4.7/4.8).

A similar analysis of the mucosal incubation fluids by gel-filtration chromatography indicated that 'free' hydrated gallium was the major species available for transport. Like the *in-vitro* studies, a large molecular weight gallium binding species was identified, this complex probably being protein derived. Again the *in-vivo* results indicate that gallium was transported passively and binding of gallium to this as yet unidentified protein (a minor species compared to

the proportion of gallium in the hydrated form) is not thought to be part of the transport process in the normal disease-free rat.

A comparison of the *in-vitro* and *in-vivo* mucosal uptake of gallium indicated there to be no significant difference between the two methods of experimentation (table 4.1).

To complement the oral dosing experiments with gallium and citrate, maltol or fluoride (table 6.1), similar experiments were performed in both the *in-vitro* and *in-vivo* intestinal situations. In these studies there was not the confounding presence of dietary and intestinally secreted ligands (secreted in response to the presence of food) affecting the formation of the complexes under investigation and therefore specific metal-ligand interactions can be studied.

Similar results to the fasted oral dosing experiments were recorded in the *in-vitro* intestinal experiments where citrate and maltol enhanced the serosal transfer of gallium whilst fluoride inhibited the movement of gallium (figures 3.9/10/11). Addition of the metabolic inhibitor 2,4-dinitrophenol did not affect the transfer of gallium in the presence of citrate, maltol or fluoride.

Ion-exchange chromatography indicated that the gallium-maltol complex was present in both mucosal and serosal fluids (figures 5.18A/B). The enhanced transport of gallium in the presence of maltol is thought to be due to the formation of a neutral lipid soluble complex which persists even after transport. Transfer across the epithelial barrier is likely to be via an intracellular route (rather than the paracellular route taken by the 'free' hydrated gallium ion). Studies with inhibitors suggest that even in a ligand complex, gallium is transferred in a passive fashion.

Although chromatography experiments did not study gallium-citrate complexes in detail, a similar situation is thought to occur when compared to the gallium-maltol complex.

Gallium formed an insoluble complex with fluoride, resulting in a decreased transport of gallium to the serosal compartment. In some metabolic situations

sodium fluoride can act as as inhibitor of glycolysis (Cox and Peters, 1979) with a resulting decrease in metal ion absorption. The decreased transport of gallium is not however, thought to be an inhibitory effect since gallium absorption was not reduced in the presence of 2,4-dinitrophenol or phloridzin.

Similar observations with gallium and citrate, maltol or fluoride were recorded with the *in-vivo* perfusion system (figures 4.9 and 4.10). Gallium absorption into the blood system was significantly enhanced in the presence of citrate and maltol, whilst fluoride inhibited the transfer and subsequent appearance of gallium in the blood. Unlike the tissue distribution experiments (4 hours duration), there was not a significant enhancement (with citrate and maltol) or inhibition (with fluoride) in the liver, kidney or spleen tissues taken out of the animal after the perfusion experiment had finished. It is likely that, since the perfusion experiments were for the duration of approximately 2 hours only, that blood.bound gallium had not passed into the tissue reservoirs.

6.4. THE INTESTINAL SPECIATION OF GALLIUM.

The intraluminal status of aluminium and gallium is often a forgotten aspect of bioavailability studies. The previous section has outlined the mechanisms of gallium absorption and mucosal interactions which may affect the transport of body to the body compartment. Gel-filtration chromatography has indicated that in both *in-vitro* everted sac and *in-vivo* perfusion experiments, the hydrated gallium ion is the predominant species in the incubating buffers. Therefore in these experiments it was the kinetic behaviour of only this species which was considered. In reality (i.e. in the *in-situ* human or rat small intestine) this would not be the case. Gel-filtration chromatography of the soluble phase of small intestinal fluids after the oral administration of gallium has shown that concentrations of 'free' hydrated gallium are limited. A similar situation is likely to occur with aluminium.

Nutritional status is probably the greatest factor in influencing the availability of gallium. Chromatography experiments indicated that the proportion of 'free' gallium was significantly reduced in the fed state compared to the fasted. These observations in part explain the reduced absorption of aluminium, gallium and scandium in the orally dosed fed rats compared to the fasted.

Gel-filtration chromatography has also identified a large molecular weight protein binding to gallium in the soluble phase of gut washings after oral administration (figures 5.3A/5.4A). The complex known as 'Peak 1' was significantly greater in proportion in fed rats compared to fasted, and may be in part (possibly in conjunction with a reduction in 'free' gallium concentration) be responsible for the reduced absorption of gallium observed in fed rats. When this species was isolated and readministered to both fed and fasted rats, transport to the tissues was not observed. In addition 'Peak 1' was incubated with *in-vitro* everted sacs, similarly no transport to the serosal compartment being observed (table 5.4). It is possible therefore that 'Peak 1' has a protective function.

The identity of this species, can as yet only be speculated upon. Peak 1 isolated from Sephadex G15 coincided with the large presence of protein. Upon re-elution by G75 however, Peak 1 gives a bi-modal distribution. The first gallium peak in the region ≈80KD coincided again with the large presence of protein, whilst the second (in the region <4KD) accounted for a lesser presence of protein and may possibly be associated with other ligands. It is likely that gallium was protein bound in the first peak, caution however, must be applied in designating the origins of the second.

Although it is possible that gallium may have bound to a low molecular weight protein other factors must be taken into account, notably that some dissociation occurs on the column, or that gallium was bound in a low molecular weight inorganic form e.g. with bile salts.

Gel electrophoresis has identified that Peak 1 consists of approximately 10 proteins ranging from 6-130KD (figure 5.7). Although in some cases gallium coincides with proteins, binding of the two can only be suggested particularly at low molecular weights, where other complexes might exist. Autoradiography of the gel (not done in this thesis) would determine more successfully whether gallium was bound to a particular protein.

It is possible that Peak 1 was either dietary or mucosally derived. Incubation of jejunal tissue however, indicated that gallium was bound in a similar way to the gallium identified in the soluble phase of gut washings (i.e. elution on G!5 gives both a large molecular weight gallium binding species and a second peak corresponding to the presence of the hydrated gallium ion. Re-elution of the first peak by G75 gives a bimodal distribution).

In addition to speciation analysis of the small bowel fluids of gallium only dosed animals, the speciation of animals dosed with gallium and maltol were also studied. In the tissue distribution experiments maltol enhanced the absorption of gallium in fasted rats only. Speciation analysis of the gut washings attempted to explain these observations.

In the gallium-maltol dosed animals, the large molecular weight gallium binding species ('Peak 1') existed, again increasing in proportion in the washings from the fed rats compared to the fasted. In addition to Peak 1, gallium was present in the fasted rat as the gallium-maltol complex and the free hydrated gallium ion(figure 5.11B). In the fed rat however, the only identifiable gallium species were Peak 1 and free gallium (figure 5.11A). Thus the gallium-maltol species persists only in the lumen of the fasted animal, significantly increasing transport of gallium by diffusing across the epithelial membrane intracellularly (free gallium being transported by the rate limiting paracellular route). In the fed lumen gallium-maltol had dissociated, possibly as a result of competition by other metal ions for the maltol binding sites. *In-vitro* incubation studies with ferric iron and

gallium-maltol indicated that iron could dissociate 95% of gallium from the gallium-maltol complex (figure 5.2C). It is possible that a similar situation occurs in the *in-vivo* fed lumen. It is also likely that gallium binds to other ligands in preference to maltol in the fed lumen, in particular phophates and hydroxides.

Although the citrate anion was not investigated in the detail that maltol was, similar interactions are likely to occur in the gut lumen to explain the enhanced absorption of gallium in the presence of citrate in the fasted animal but not the fed.

In the lumen of the small intestine there are numerous possibilities for ligand interactions with gallium and aluminium which have yet to be studied. It is these intraluminal factors which are thought to influence bioavailability. In the case of aluminium, ligands forming soluble transportable complexes (e.g. with citrate or maltol) may have increased neurotoxic implications.

6.5. THE SPECIATION OF GALLIUM IN BLOOD.

The immediate *in-vivo* consequence of gallium having been transported from the lumen of the small intestine and across the epithelial cell layer is entry into the capillary network. Gel-filtration chromatography (Sephadex G75) of blood plasma taken from a rat whose small intestine had been previously perfused with gallium indicated that a large molecular weight protein (≈80KD) bound 100% of the transported gallium (figure 5.15A).

The majority of evidence suggests that transferrin (the plasma protein responsible for the transport of iron) is the protein binding to blood aluminium (Trapp 1983; Martin et al. 1987). Cochran et al. (1983) has reported a binding of gallium to plasma transferrin as have Vallabhajosula et al. (1980). The results of this thesis suggest that at low concentrations (<300ng/ml) gallium remains protein bound with the non-existence of an ultra-filtrable fraction. At concentrations greater than approximately 300ng/ml gallium, the transferrin protein is saturated, and the remaining gallium becomes bound to a low molecular weight fraction (<4KD)

(figure 5.15B). Similar observations have been noted by Gardiner et, al., (1984). Martin (1986) has proposed citrate to be the pre-eminent low molecular weight binder. Gardiner et.al., (1984) were less definitive in their choice mentioning phosphates, carbonates, citrates, oxalates, fluorides and other organic ligands as possible low molecular weight binders.

6.6. GALLIUM SPECIATION OF HUMAN SMALL BOWEL FLUIDS.

In all of the studies previously mentioned the rat has been used as an animal model to explain the consequences of aluminium metabolism which may occur in humans. Difficulties in obtaining sufficient quantities of viable human tissue make the need for animal experimentation. The use of human fluids extracted by non-invasive measures however, can provide confirmatory evidence that the rat is an adequate model to study physiological and biochemical systems in man.

The collection of human small bowel fluids by means of emptying a colostomy bag provides such an opportunity to compare rat and human intestinal speciation after incubation with gallium.

Gel-filtration chromatography of the soluble phase of human small bowel fluids incubated for 1 hour at 37^{o}C with gallium indicated that a large molecular weight gallium binding complex ('Peak 1') had formed in addition to a peak coinciding with the presence of free hydrated gallium or other low molecular weight ligands. (figure 5.19A). Analysis of the fractions indicated the large presence of protein co-eluting with the large molecular weight gallium species. Similar results were recorded with the gut washings from fasted rats. Re-elution of the human 'Peak 1' on Sephadex G75 gave a bimodal distribution of gallium, a pattern which was similarly observed in the rat fasted gut washings.

Gel electrophoresis identified the presence of several proteins in the human 'Peak 1', some of which coincided with proteins identified from the rat 'Peak 1' (figure

5.14, lanes 5 & 6). Autoradiography however, was not performed and binding of gallium could not be assigned to an individual protein.

The only difference between the human and rat gut washings was the presence (on the shoulder of human 'Peak 1') of a small third minor peak, again corresponding to the presence of protein. The position of the peak indicated a complex of a complex approximately 1KD in size. The possibility exists of a binding of gallium to a protein fragment or polypeptide chain.

There are however, major similarities in these preliminary human incubation experiments to the behaviour of gallium in the rat intestinal lumen. Although rats are fed a purified diet consisting mainly of celluloses and fibres, it appears that dietary interactions which may be important in affecting gallium bioavailability in humans can be confidently studied in the rat model.

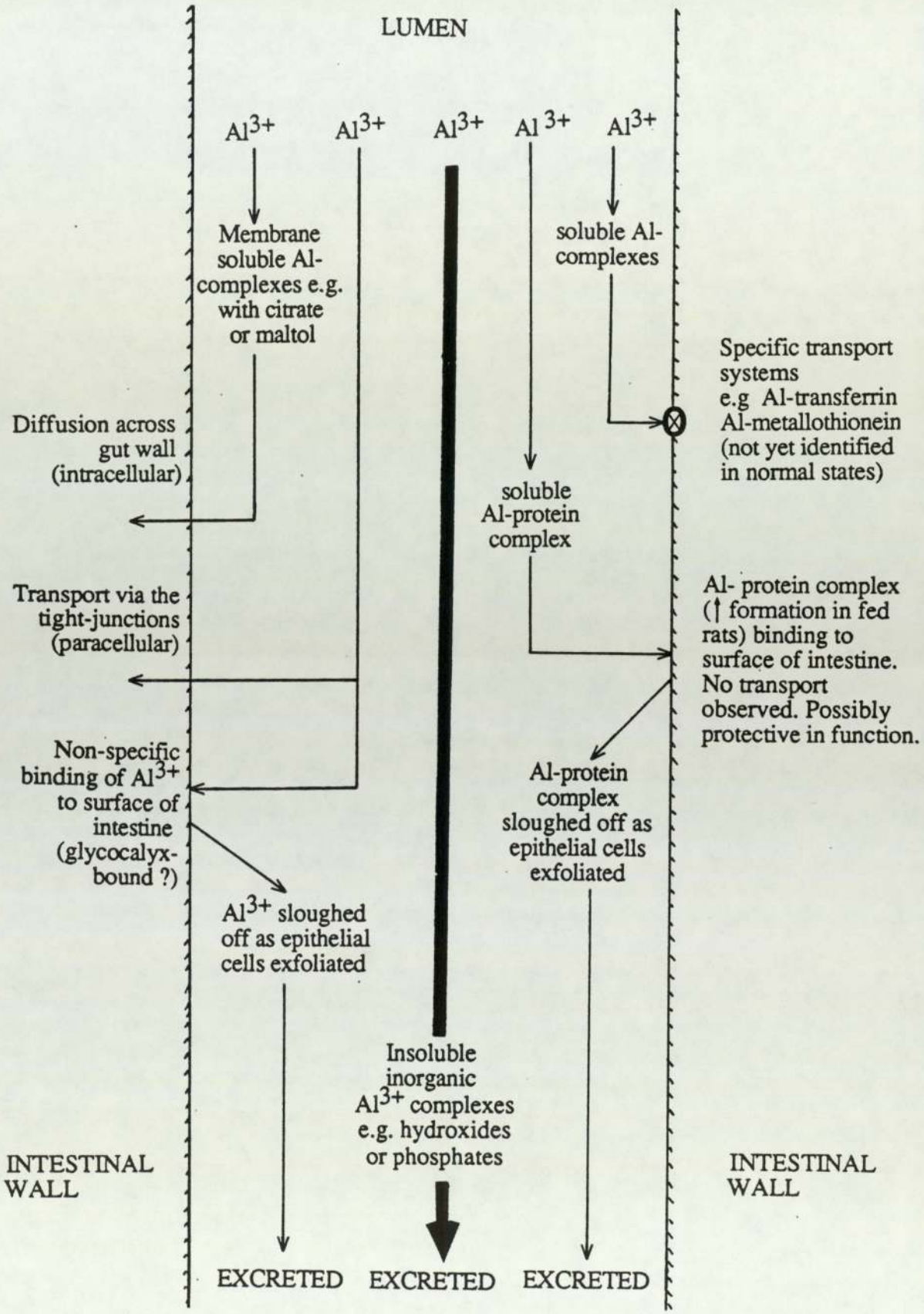
6.7. A PROPOSED MODEL FOR THE INTESTINAL SPECIATION AND ABSORPTION OF ALUMINIUM.

Figure 6.1 summarises the findings outlined in this discussion and proposes several intestinal mechanisms of aluminium which have been derived with the use of the radioisotope ^{67}Gallium as an analogue. From the diagram it can seen that several fates await gallium upon its entry to the small intestine. The majority of ingested gallium is bound to inorganic complexes for example hydroxides or phosphates. It is thus rendered unavailable for absorption and excreted in the faeces. A small percentage (≈1%) is thought to be bioavailable and is therefore of interest.

The immediate consequence of tissue incubation with gallium was a rapid binding to the mucosal surface in both *in-vitro* and *in-vivo* experiments. Similar observations with aluminium have been noted by Van der Voet and de Wolff (1984). The avidity of Group 3 metals may be due to the attraction that cations

FIGURE 6.1. POSSIBLE FATES OF ALUMINIUM

UPON ENTERING THE SMALL INTESTINE.



have for acid mucopolysaccharides (Ando et al. 1985). These structures form the basis of the glycocalyx, the fine fibres which form a 'fuzzy' coating on the brush border membrane and radiate out into the lumen of the gastrointestinal tract. Quaterman (1982) has proposed that the glycocalyx may play a role in the absorption of metals. In starvation, the glycocalyx was found to be increased and there was a concomitant increase in metal absorption. The binding of metals to the glycocalyx has been previously observed by Partridge (1986) and Farrar and Coleman (1988). It is possible that the glycocalyx acts as a 'net' capturing metallic cations from the lumen, a proportion of which are subsequently released for transport by the epithelial membrane (Farrar and Coleman, 1988). It is likely that any gallium ions bound to the mucosal surface but are not actually transported would be sloughed off as desquamation processes occur.

The route of transfer across the epithelial cell barrier depends upon the ligand status of gallium. In combination with such species as citrate or maltol, a membrane soluble complex is formed and transport is thought to take place intracellularly. Transport of these complexes was not inhibited by the presence of 2,4-dinitrophenol and were thought to occur by simple diffusive means.

When gallium existed as the hydrated ion, transport was thought to be via the cation specific paracellular route. Similar results have been obtained by both Van der Voet and de Wolff (1984, 1986) and Provan and Yokel (1988). They found aluminium to be transported by a passive energy-independent route and to be unaffected by the presence of 2,4-dinitrophenol. Further evidence to suggest that transport was via the tight-junctions is provided by Proven and Yokel (1988) who found reduced aluminium absorption in the presence of the paracellular blockers kinetin and triaminopyrimidine.

The transport of gallium therefore, appears to be a two-stage process with the rapid binding of gallium followed by a slow diffusion into the serosal compartment. A similar two-stage mechanism has been proposed for iron uptake by Manis and Schacter (1962).

Since gallium and iron are thought to be similarly transported in plasma, it is possible that the two metals may be intestinally transported via similar mechanisms. The results of this thesis, and of other workers (Van der Voet and de Wolff, 1984, 1986; Provan and Yokel, 1988) indicate that under normal conditions, gallium and aluminium were transported purely by an energy-independent route.

The intestinal absorption of iron possibly occurs by both active and passive mechanisms (May and Williams, 1980; Finch and Cook, 1984). Howard and Jacobs (1972) have identified both an active and passive component in the absorption of iron. The active system (thought to be mediated by intestinal transferrin, Huebers et al. 1983) was thought to operate in the lower dose range, at the higher dose range iron diffuses through the epithelial membrane with unlimited capacity (Narins, 1980). Cox and Peters (1979) observed iron uptake in human duodenal mucosa and found it to be energy dependent. The mucosal uptake of iron was thought to be the rate limiting step for the entry of iron into the human circulatory system. The existence of a energy independent mechanism was not however discounted .

Iron absorption is also thought to be regulated by corporeal factors (e.g. tissue iron stores)(Ragan,1983; Conrad,1987). Studies by Fernandez-Menendez et.al., (1988) however, have suggested there to be no negative feedback mechanism controlling aluminium absorption from the gastrointestinal tract. A previous chronic administration of aluminium did not reduce aluminium absorption, suggesting that the aluminium replete animal could not control further uptake.

Although dietary induced iron deficiency increased mucosal uptake and transfer of iron, there was no affect upon the uptake and transfer of gallium (Valberg et. al. 1981). From these finding it was suggested that gallium was not involved in competition for mucosal iron binding sites or for iron transport proteins. Van

der Voet and de Wolff (1987) found that ferric iron did not affect the *in vivo* uptake or transfer of aluminium. Ferrous iron however, increased tissue uptake of aluminium but reduced transfer to the portal blood side. Possible interactions between aluminium and ferrous iron may have involved transferrin and/or ferritin mediated systems. The authors however, gave no detailed explanation of their observations.

The existence of a specific intestinal aluminium binding protein to mediate transport seem unlikely in the disease free rat, since the majority of evidence (including that of this thesis) points to transfer by diffusion. In disease states, however, (e.g. vitamin D depleted, iron depleted, chronic kidney failure) it may be possible that a metallo-transport protein is expressed and an active component is introduced in addition to the passive mechanisms. This hypothesis may explain the enhanced absorption of aluminium observed in the uremic state (Alfrey, 1987).

The formation of an unabsorbed large molecular weight gallium binding protein is of interest because it may exert a protective effect against gallium absorption. The identity of the species which also existed in human small bowel fluids incubated with gallium, can as yet only be speculated upon.

The possibility exists that gallium has bound to a metallotype protein, transport of which was not observed. Similar speciation studies with lead have been undertaken, Holt, (1988) observing a similar large molecular weight lead binding protein in the soluble phase of gut washings after the oral administration of lead acetate. Partridge (1986) found absorption of this species to be significantly reduced upon readministration. Holt (1988) has proposed this unknown lead species to be a lead metallothionein complex. Results from this thesis however, do not suggest that gallium bound to metallothionein, as gallium was recovered only in the regions ≈80KD and <4KD. Metallothionein with a molecular weight of ≈7KD would have eluted in a position 5 fractions to the left of the second (<4KD peak.

Jacobs and Miles (1970) have identified a large molecular weight protein binding ferric iron, from biliary secretions. It was thought not however, to play an important role in iron absorption and may possibly bind to the similarly sized gallium ion.

There is the possibility that gallium binds to mucosal transferrin. Gel electrophoresis of the large molecular weight peaks isolated from Sephadex G15 chromatography has identified a protein approximately 80KD (similar to the molecular weight of transferrin) in the human gut fluids, the rat gut fluids and the rat mucosa incubation experiments. The paradox exists however, that this gallium-protein complex restricts the intestinal absorption of gallium, whilst transferrin plays an important role in the absorption of iron (Huebers et al. 1983; Conrad, 1987).

Pollack and Lasky (1976) have identified a gut iron binding protein (GIBP) which has a molecular weight of ≈78KD (and may therefore be the ≈80KD proteins identified by gel electrophoresis). The molecule was immunologically different from both transferrin and ferritin. As the protein was thought to slowly transfer iron to transferrin, it is possible that binding to gallium may have been responsible for the non-absorption observed in both *in-vitro* and *in-vivo* experiments.

Adler, Lee and Berlyne (1986) observed aluminium to bind to a protein ≈9-10KD in the soluble mucosal fraction from canine duodenum. These fractions corresponded to a large calcium binding activity; the aluminium binding protein was therefore thought to be a soluble vitamin D dependent calcium binding protein. A binding of gallium to proteins in the 9-10KD region was not identified in any of the studies (rat small bowel fluids, human small bowel fluids, rat jejunal mucosa, *in-vitro* and *in-vivo* intestinal buffers).

The gallium-binding protein, binds less avidly to the mucosal surface than the free hydrated gallium ion and no subsequent transfer was observed. In a similar way to the hydrated gallium ion being removed from the body, the mucosally bound

gallium-protein is expected to be sloughed off as epithelial cells are exfoliated and excreted with the majority of ingested gallium.

6.8. A PROPOSED MECHANISM TO ACCOUNT FOR THE ACCUMULATION OF ALUMINIUM IN THE BRAIN.

Evidence from this thesis, using the radioisotope ^{67}Gallium as a marker for aluminium, has been collated to propose a hypothesis for the accumulation of aluminium in the brain of Alzheimer's (or other neurodegenerative diseases associated with aluminium) patients.

The intraluminal speciation of gallium was an important contributor to the presence of gallium in the body compartment. In fasted animals the food additives citrate and maltol were found to enhance gallium absorption increasing its presence in various organs including brain tissue. Slanina et al. (1984) has also noted the increased presence of aluminium in rat brain after the simultaneous ingestion of aluminium and citrate. On the other hand certain complexes of gallium were less well absorbed and may be protective in nature. The large molecular weight gallium-binding protein was not absorbed at all in either *in-vivo* or *in-vitro* systems, whilst fluoride inhibited the uptake of gallium in both fed and fasted rats. The free hydrated gallium ion was absorbed to a certain extent, but was significantly lower when compared to the transport of the lipid soluble gallium complexes.

Any transported gallium at a low concentration was found to be wholly bound to the iron binding transport protein transferrin. Complexation of gallium to transferrin is strong and binding is thought to restrict accumulation within tissue reservoirs. It is likely therefore, that the binding of gallium to transferrin is a protective device, possibly in a similar manner in which the large molecular weight gallium binding protein prevented absorption from the small intestine.

At higher blood concentrations of gallium, transferrin binding is saturated, and the excess gallium is bound as a low molecular weight species (less than 4KD in size). It is possible that this low molecular weight species is 'mobile' resulting in increased absorption of gallium into the brain and other organs (figure 6.2). Citrate has been suggested as the pre-eminent low-molecular weight aluminium binder, (Martin, 1986).

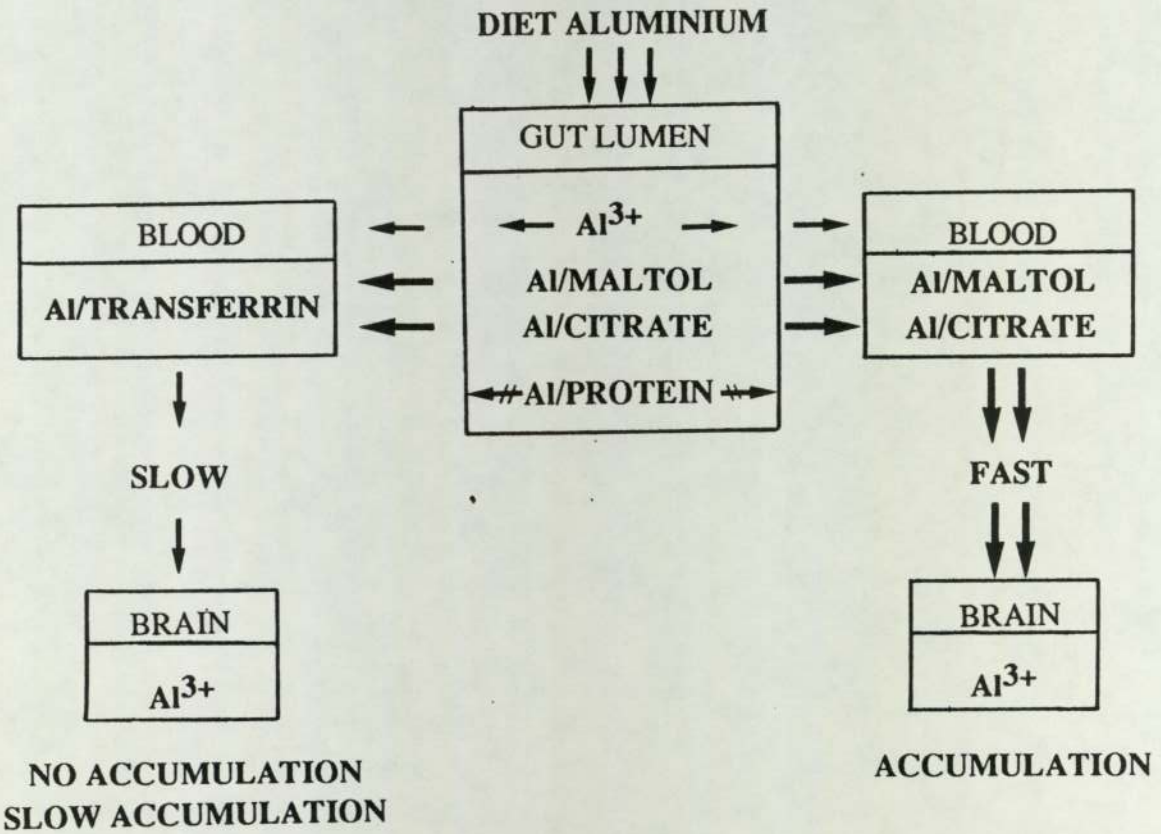
This idea may explain the significant accumulation of scandium in the chronically dosed animals compared to the acutely dosed animals (figure 2.5). If this hypothesis were true, then the presence of aluminium in a low molecular weight mobile form would depend upon a variety of factors.

The increased presence of suitable ligands in the small intestine can markedly increase the absorption of aluminium, resulting in greater plasma aluminium concentrations. The possibility also exists that aluminium could be absorbed as a low molecular weight, lipid soluble ligand which is not dissociated upon entering the circulatory system. If such a complex had a greater stability constant greater than that of aluminium transferrin, then the low molecular weight species would prevail in the plasma. The enhancement of tissue gallium levels in the presence of citrate and maltol could be explained in two ways. Initially the gallium-ligand complex showed enhanced absorption from the intestinal mucosa leading to greater presence in blood. Either the transferrin binding capacity for gallium had been saturated, leaving excess gallium to form a 'mobile' low molecular weight complex, or that transferrin was by-passed and the gallium was retained in the transported ligand form.

The binding capacity of transferrin for aluminium, is however probably the major factor in controlling the presence of these transportable low molecular weight complexes.

Ferric iron does not wholly saturate the transferrin binding sites (2 sites per molecule) leaving a certain number of sites unoccupied. This is known as the

FIGURE 6.2. A PROPOSED MECHANISM TO ACCOUNT FOR THE ACCUMULATION OF BRAIN ALUMINIUM IN ALZHEIMERS OR OTHER ALUMINIUM LINKED NEURODEGENERATIVE DISEASES.



unused iron binding capacity (UIBC)(Ganrot, 1985). In the healthy human the UIBC is thought to be between 40-50 μmole/l (Ganrot, 1986; Martin, 1986). In haemodialysis patients however, a transferrin binding capacity of 7.4μmole/l of aluminium has been observed (Kaehny et al. 1977). This value represents a significant decrease in the capacity observed in the healthy individual. The UIBC may therefore, hold the key for the accumulation of aluminium in the brain and the development of neurotoxicity. The UIBC is influenced by the release of ferric iron from the reticuloendothelial system and subsequent absorption into the bone marrow. If any of these factors were altered so that transferrin had less binding sites for aluminium, then the presence of the aluminium in the potent low molecular weight potent form would increase. This possibly happens in old age with a decreased transferrin production lowering the UIBC (Huebers and Finch, 1987). Other situations involving a decrease (or variation) in transferrin concentration include the genetic disorder atransferrinaemia (low levels of transferrin produced), protein calorie malnutrition (particularly important in children), inflammation, iron deficiency, loss of transferrin from kidneys or gastrointestinal tract and menstruating or pregnant women (Huebers and Finch, 1987).

The accumulation of aluminium solely in brain tissue by SDAT patients may be (in addition to the presence of 'mobile' aluminium species) due to a blood brain barrier (BBB) defect. Kim et al. (1986) found the intraperitoneal injection of aluminium lactate to increase the BBB permeability to [^{14}C] sucrose for up to 4 hours after the injection. The changes in permeability had reversed to normal by 24 hours. Kim et al. considered that increased BBB permeability in response to aluminium may be another important factor in the aetiology of SDAT, particularly after the chronic presence of aluminium in the body.

6.9. CONCLUSIONS.

1). Gallium and scandium proved to be adequate markers for aluminium in studies of intestinal absorption and tissue distribution.

2). Less than 1% of an administered dose of aluminium, gallium and scandium was identified in tissues 4 hours after dosing.

3). Aluminium, gallium and scandium showed a particular accumulation in skeletal tissue.

4). Nutritional status (i.e. whether the rat had been fed or fasted) was an important contributor to the bioavailability of aluminium, gallium and scandium.

5). Nutritional status was also important in gallium-ligand intestinal interactions. Citrate and maltol enhanced gallium absorption in fasted rats only whilst fluoride inhibited gallium bioavailability in both fed and fasted rats.

6). The *in-vitro* intestinal absorption of aluminium, gallium and scandium was via a 2-phase mechanism. A large non-specific mucosal uptake was followed by a slow passive diffusion into the serosal compartment. Transport was found to be unaffected by the metabolic inhibitor 2,4-dinitrophenol. Similar results were recorded for the *in-vivo* absorption of gallium.

7). Gallium was found to be bound to an intestinal protein in both *in-vitro* and *in vivo* experiments. Binding to this protein however, did not affect the passive transport of gallium in the normal disease-free rat.

8). Gallium was also bound to a unidentified large molecular weight species in the soluble phase of gut washings after the oral administration of gallium. The complex was significantly greater in the fed rat compared to the fasted. Isolation of this species and incorporation into both *in-vitro* and *in-vivo* experiments indicated that the species was not transported and may therefore have a protective function.

9). Gallium was thought to be transported in plasma by the iron binding protein transferrin particularly at low concentrations of gallium. At higher concentrations however, gallium is both transferrin bound and present as a low molecular weight complex.

10). It is in the form of these low molecular species that aluminium may be transported into the brain, perhaps accounting for the increase in tissue levels observed in Alzheimers disease and other aluminium related disease states.

CHAPTER 7. FURTHER WORK.

1). The further development of methods to identify and separate chemical species in the lumen of the gastrointestinal tract is needed. Possible techniques could include ion-exchange chromatography, gel-electrophoresis (coupled with autoradiography), fast protein liquid chromatography (FPLC), high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). Computer modelling techniques are also being developed to predict metal-ligand formation in biological environments.

2). The importance of food additives and how they affect the bioavailability of gallium and aluminium is an area requiring further study.

3). Further identification (using the above mentioned techniques) of the large molecular weight gallium binding protein identified in the soluble phase of gut washings from both fed and fasted rats is needed since this species may be protective in function.

4). A further study of the transportable gallium species and tissue gallium species is needed. This of particular importance in brain tissue since increased aluminium levels have been implicated in the aetiology of SDAT and other neurodegenerative disorders.

5). Since gallium is transported in the circulatory system via the iron transporting protein transferrin, it is possible that intestinal interactions may occur between the two elements. Further experiments might include *in-vivo* and *in-vitro* intestinal competitions studies with gallium and iron (using the radioisotope ^{59}Fe), the effect of iron on the formation of 'Peak 1', monitoring gallium and iron competition for food additives and dietary components, and comparing the relative tissue distributions of the two elements after oral administration.

6).The intestinal studies of this thesis have concentrated wholly on the healthy disease free rat. Future studies might involve the study of gallium absorption in disease or other states, e.g. after partial/total nephrectomy, partial/total parathyroidectomy, vitamin D deplete/replete, iron deplete/ replete, or in weanling, pregnant or senescent rats.

7). Further work analysing the speciation of gallium in human fluids could be attempted using gastric juices (identification of species in the acid-rich medium of the stomach); small bowel fluids (similar work to the rat studies, further identifying the large molecular weight gallium binding protein); plasma samples (to ascertain the nature of transportable gallium species) and autopsy tissue samples (to identify the speciation of gallium within tissue cytosol).

APPENDIX

STATISTICAL ANALYSIS

1). The statistical tables used were those of Murdoch J and Barnes J.A., Macmillan Press, London, (1978).

2).Statistical significant in all tests was taken at 5% or less. In general *P<5%, **P<1%, ***P<0.1%.

3). All error bars were quoted as standard error of the mean (±SEM).

$$\text{SEM} = \frac{\text{Standard Deviation (SD)}}{\sqrt{\text{number of observations (n)}}}$$

4).The unpaired students t-test was the statistical test used in the majority of cases in this thesis. It is primarily used to test for differences between two sets of means drawn from independent populations.

$$t = \frac{X_1 - X_2}{\sqrt{\left(\{SD_1^2/n_1\} + \{SD_1^2/n_2\}\right)}}$$

t has $(n_1 + n_2 - 2)$ degrees of freedom.

5). A one-way analysis of variance (see chapter 4) is used calculate whether differences among more than two sample populations are due to chance or whether there are real differences between the means. The calculations are based upon 'sums of squares' and are clearly described in Freund (1988).

6). To test whether a value is significantly different from unity (see chapter 2) the test

$$t = \frac{X - \mu}{\text{SEM of X}}$$

is applied, where X = test mean, μ = unity value, t has n-1 degrees of freedom. This is a one-tailed test since an increase or decrease (not both) from unity is being investigated.

7). The correlation coefficient (r) is a measure of the strength of the linear relationship between two variables. This test (based upon the total, regression and residual sum of squares) was performed using the Apple Macintosh 'Cricketgraph' program.

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