THE INTESTINAL ABSORPTION OF LEAD: THE IMPORTANCE OF LEAD SPECIATION

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Clinically evident lead poisoning often results from the absorption of lead across the gastrointestinal tract.

The possibility of an active transport mechanism for lead in the duodenum was examined by studying the transport of lead in the everted sac against a concentration gradient. No active transport of lead was demonstrated.

The effect of the presence of food in the rat gut on the absorption of an oral lead dose was studied *in vivo*. Fasted rats absorbed substantially more lead than fed rats. More of the administered lead dose was recovered in the blood four hours after dosing than in any other internal organ. Total recovery of lead was not achieved, suggesting that in both fed and fasted rats, an important lead reservoir had still not been taken into account. Reasons for the reduced lead absorption following feeding were examined by studying the lead species formed in the gut of fed and fasted rats. Gut washings from fed and fasted rats were incubated with lead *in vitro*, and the species formed were separated by gel filtration chromatography. Besides soluble lead carbonate and lead phosphate, lead was present as a number of lead-protein, lead-peptide and lead-amino acid complexes in both fed and fasted animals. In the fed animal the identity of the lead-protein complexes remains unknown. In fasted animals one of the proteins is thought to be metallothionein.

The transport of the lead species elucidated was investigated *in vivo*. Lead, when administered orally with metallothionein, showed a slight although non-significant reduction in absorption. The presence of lead in the gut lumen as carbonate and phosphate ion pairs was also investigated using an *in vivo* perfusion technique. The presence of ion pairs reduced lead absorption.

These studies indicate that the majority of lead is transported as the hydrated cation, $(Pb(H_2O)_4^{2+})$, and the formation of strongly associated soluble lead complexes reduces cation availability and therefore reduces lead absorption.

Key words: intestinal lead absorption
speciation
metallothionein
soluble ion pairs

To my parents

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List of Abbreviations

A.B.T.S.	2,2'-azino di-(3 ethyl benzthiazoline sulphonic acid)
ATP	adenosine triphosphate
c.p.m.	counts per minute
D.T.P.A.	diethylenetriaminepentaacetic acid
E.C.	Enzyme Commission
E.D.T.A.	ethylenediaminetetraacetic acid
E.E.C.	European Economic Community
E.P.A.	Environmental Protection Agency
H.P.L.C.	high performance liquid chromatography
i.d.	inner diameter
I-125 H.S.A.	human serum albumin labelled with iodine-125
K.H.B.	Krebs Henseleit Buffer
M.W.	molecular weight
n	sample number
N.T.A.	nitriloacetic acid
o.d.	outer diameter
[S]:[M]	ratio of serosal concentration to mucosal concentration
T.C.A.	trichloroacetic acid
% T.D.A.	percentage of the total dose administered
Tris	tris-(hydroxymethyl)-aminomethane

Chapter 1

INTRODUCTION

Lead is considered to be the most abundant non-essential trace metal in the human body (Rose, 1983). It has no known essential or useful biochemical function in man. All documented effects seem to be adverse, and it is therefore of considerable concern as a potential health hazard.

1.1 Lead in the environment

Lead is a natural constituent of the environment and thus has always been found in food, water and air. However, present day environmental levels are very much elevated compared to those existing prior to the technological revolution of the eighteenth and nineteenth centuries, which saw the increased production, use and deposition of lead-containing materials (Southwood, 1983). Consequently, over the last two hundred years, the potential for exposure of the general population to lead has increased and controversy remains as to whether levels of lead found in the modern environment are safe. Of particular importance to man is the fact that lead, contaminating environmental water and air, enters the biological cycle by incorporation into animals and plants used for food, and thereby eventually becomes available for absorption by man. Apart from environmental exposure, a large section of the population is exposed to lead in the home in the form of lead containing paints, glazes, dyes and cosmetics (Hilburn, 1979; Lawther, 1980). An extensive review, detailing the exposure of man to lead, can be found in the report of the Royal Commission on Environmental Pollution (Southwood, 1983).

1.2 Lead and health

The effects of lead on health have been extensively studied and reviewed (Goyer and Rhyne,1973; Damstra,1977; W.H.O., 1977; Lawther,1980; Waldron, 1980; Rose,1983; Southwood, 1983; DeMichele,1984; Boeckx, 1986; Goyer, 1988). Initially, lead poisoning manifests itself as a series of non-specific symptoms, such as anorexia, constipation, nausea and vomiting, muscle and joint pains, irritability, confusion, lethargy, tremors and colic (DeMichele, 1984; Goyer, 1988). The best recognised toxic effects are principally directed against the blood, nervous system and kidneys. A classical sign of lead poisoning is microcytic hypochromic anaemia (Petering, 1980), characterised by a decrease in haem biosynthesis, largely due to the inhibition by lead of

delta-aminolaevulinic acid dehydratase and ferrochelatase, and a reduction in erythrocyte survival (Hernberg *et al.*, 1967). The nervous system is particularly sensitive to the toxic effects of lead. In adults, who usually experience chronic exposure to lead, the peripheral nervous system is affected (Goyer and Rhyne, 1973), lead causing demyelination and/or degeneration of axons and impaired nerve conducting velocity (Waldron, 1980; Mahaffey, 1981). In children, the central nervous system is affected causing disturbances in intellegence and behaviour (Rutter, 1983). Accumulation of lead in the central nervous system, seen more often in children than adults (Goyer and Rhyne, 1973), ultimately leads to potentially fatal encephalopathy (Mahaffey, 1981; Rose, 1983). The toxicological effects of lead on the kidney are twofold; reversible renal tubular dysfunction, usually seen in children after acute lead exposure (Goyer, 1988), resulting in aminoaciduria, phosphaturia and glucosuria (Waldron, 1980), and irreversible chronic interstitial nephropathy which causes the tubular and glomerular atrophy and interstitial fibrosis seen in adults chronically exposed to lead (Waldron, 1980; Goyer, 1988). Lead also adversely affects the liver, immune system, cardiovascular system, reproductive system (Rose, 1983) and auditory and visual pathways (Schwartz and Otto, 1987; Lilienthal *et al.*, 1987).

1.3 The uptake of lead into the body

There are three routes by which lead can enter the body; skin, lungs and intestine. According to the Royal Commission on Environmental Pollution (Southwood, 1983), absorption through the skin is usually negligible. The daily lead intake from air for adults ranges from 1-15μg and the average daily dietary intake from food and water is 100μg. The principle route of lead entry into the body is therefore via the gastrointestinal tract (table 1.1).

1.4 Sources of ingested lead

1.4.1 Food

Food becomes contaminated with lead either at the source, during storage or during preparation. Cereals, fruit and vegetables absorb some lead from soil contaminated with lead from atmospheric fallout or lead-rich sewage sludges currently used as fertilisers (Southwood, 1983). This lead may then be transferred from plants to

Table 1.1 The uptake of lead into the body (% of daily lead uptake) (Southwood, 1983)

Rural/small town	Inhalation	Ingestion
Adult	8	92
2 year old child	1	99
Inner city		
Adult	36	64
2 year old child	3	97

All values are approximate and the data show considerable variation.

animals. Animals can concentrate lead to a certain degree, although the absorption of ingested lead by ruminants is low and much of that absorbed is found in bone, and therefore unavailable for human consumption. Fish, however, can accumulate lead to a much greater extent (Harrison and Laxen, 1981).

More lead is found in canned food than in corresponding fresh food, due to the use of lead solder. The solder on cans is thought to contribute on average about 15µg of lead to the total daily dietary intake of adults, (table 1.2). Increasing use of welded as opposed to soldered cans, however, is reducing this means of dietary contamination. Present legislation dictates that no infant food be contained in soldered cans (Goyer, 1988).

Some lead in food may have originated from the water in which the food was cooked, or in the case of dried food, rehydrated (Moore *et al.*, 1979; Little *et al.*, 1981). About 3µg of the total lead ingested by adults per day is thought to come from glaze on ordinary crockery, (table 1.2).

1.4.2. Drink

Liquids are consumed as drinking water, as milk and as prepared beverages. The majority of households in Great Britain have a lead concentration in daytime samples of tap water of less than 10µg/l (Department of the Environment, 1977), well below the 50µg/l recommended by the E.E.C. (Pocock, 1980) and the E.P.A. (Levin, 1987) and the proposed E.P.A. standard of 20µg/l currently under consideration (Hileman, 1987). The median lead content of tap water in Great Britain of about 10µg/l is thought to contribute approximately 9µg to the daily lead intake of adults (Southwood, 1983). However, many tap water samples show significantly elevated lead levels, 20% of households in Great Britain having water lead levels in excess of those recommended by the E.E.C. (Pocock, 1980). The highest concentrations of lead are found where lead pipes, or pipes joined by lead-containing solder, are used to channel corrosive, soft, demineralised or acidic water. However in non-corrosive water, lead levels at the tap of 160-250µg/l can be produced on overnight standing, due to the galvanic corrosion of the solder which occurs when two metals with different electro-chemical potential are in the same environment (Levin, 1987). As a result of this plumbosolvency, both the Netherlands and Germany have banned the use of lead solder in water systems, and a

Table 1.2 A model scheme for the intake of lead by ingestion (Southwood, 1983)

Source of lead intake	Adults µg/day	Children µg/day
food	85.0	40.0
solder used in cans	15.0	6.9
tap water beverage	8.5	4.4
cooking water	0.8	0.4
tableware glaze	3.0	-
Total lead intake by ingestion	112	52

These values are averages for data with considerable variance, and apply to non-smoking, non-drinking adults and two year old children in a rural environment.

1986 amendment to the "Safe Drinking Water Act" of 1974 has prevented the use of materials containing lead in public water systems in the U.S.A. since June 1988 (Levin,1987).

Lead is present at low concentrations in cows' milk and is likely to contribute 5-20μg/day to lead intake. A pint of beer may add 10μg of lead to the daily intake, a canned fruit juice, 20μg/day, or 500mls of wine, as much as 60μg/day (Harrison and Laxen, 1981).

1.4.3. Other sources of gastrointestinal lead

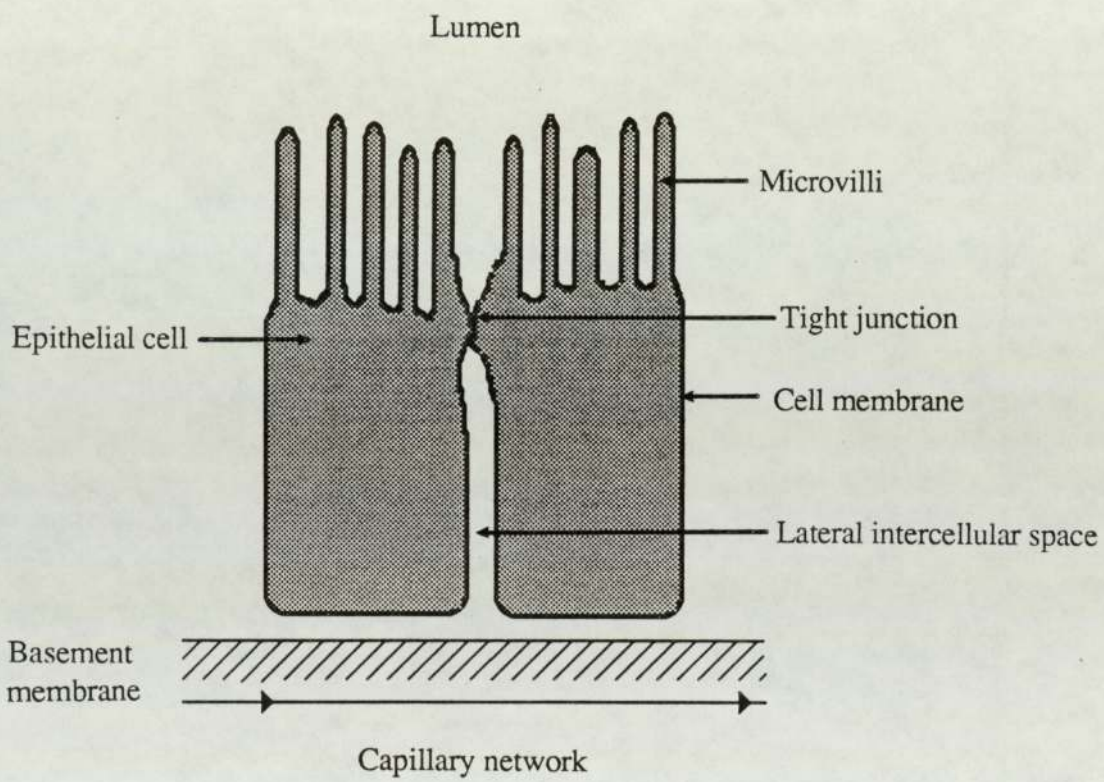
Dust and dirt may represent an important source of ingested lead. Lead contaminated dust taken into the body by inhalation may be filtered in the upper nasopharyngeal region and cleared by the mucociliary escalator into the gastrointestinal tract (Rose, 1983), putting people who live or work in areas of high airborne lead at risk. Also at risk from this type of lead exposure are professional and amateur decorators who inadvertently inhale dust when dry-sanding leaded paint. Children may also inadvertently ingest dust and dirt by licking or sucking fingers or contaminated objects in their environment. Those children displaying pica are especially at risk (Southwood, 1983).

1.5 The morphology of the gastrointestinal tract

The small intestine may be divided into three regions; duodenum, jejunum and ileum. The inner surface of the small intestine is covered by a layer of absorptive epithelial cells thrown into folds, each fold being covered by projections named villi. The surface of the villi are characterised by a brush border comprised of numerous microvilli, which themselves are covered by a loose meshwork of mucopolysaccharide filaments referred to as the glycocalyx. The glycocalyx serves to maintain the region of high acidity between the apical cell membrane and the luminal contents known as the acid microclimate (Blair and Matty, 1974).

The intestinal epithelial cells are joined at their apical surface to form tight junctions (figure 1.1), cation specific channels (Armstrong, 1987) 10-16 Å in diameter (Moreno and Diamond, 1975). Below these tight junctions are spaces (lateral intercellular spaces) bound by the two cell membranes and the basement membrane. Molecules and ions may cross the brush border via the tight junctions and lateral intercellular space (the

Figure 1.1 A schematic representation of two adjacent intestinal epithelial cells



paracellular route) or by crossing the cell membrane (the transcellular route).

1.6 Models of gastrointestinal absorption

Data indicates that lead absorption may occur at any site in the small intestine (Blair *et al.*, 1979). The mechanism of lead absorption is however a matter of some controversy. Transport of lead by active mechanisms (Barton, 1984), passive mechanisms (Blair *et al.*, 1979) or a mixture of both (Flanagan *et al.*, 1979; Aungst and Fung, 1981b) has been suggested.

1.7 Factors affecting gastrointestinal lead absorption

The presence or absence of food in the intestine has a profound effect on lead absorption. In humans fasted for 18 hours, about 60% of a lead dose is absorbed through the intestine (Flanagan *et al.*, 1982; Heard and Chamberlain, 1982; Blake *et al.*, 1983; Blake and Mann, 1983; James *et al.*, 1985), compared to about 4% in fed humans (Heard and Chamberlain, 1982; Blake *et al.*, 1983; James *et al.*, 1985). A similar trend is observed in rats (Garber and Wei, 1974; Aungst and Fung, 1981a; Heaven, 1985; Partridge, 1986) which suggests they may be used as a reliable model for lead absorption in humans.

The effect of dietary constituents has been studied by James *et al.*, (1985). They suggested that the reduction in lead retention observed when administered with a meal was probably largely due to its content of calcium and phosphate salts and possibly the presence of cereal phytate which is thought to reduce intestinal lead absorption by the precipitation of lead in the presence of calcium (Wise and Gilburt, 1981; Rose and Quarterman, 1984). It was also suggested that some food with low mineral and phytate content reduces lead uptake possibly by stimulating digestive secretions of calcium and phosphate salts and acid. It appears therefore that protection against ingested lead does not only depend on the physical presence of food in the gut, but also on the type of food present.

1.8 The effect of various dietary components on gastrointestinal lead absorption

The effect of dietary composition on lead absorption has been studied

extensively and it is now generally considered that protein deficiency, (Mylroie *et al.*, 1977) and fat excess (Barltrop and Khoo, 1976) enhance lead absorption, as does low dietary calcium, iron, magnesium, zinc and phosphate (reviewed by Coleman *et al.*, 1983 and Flanagan *et al.*, 1982). The effects of various vitamins (Smith *et al.*, 1978; Barton *et al.*, 1980; Sasser *et al.*, 1984) and chelating agents (Coleman *et al.*, 1982) has also been studied.

1.9 Possible mechanisms by which dietary components affect gastrointestinal lead absorption

1.9.1 Competition

Lead may compete with cations such as calcium and iron for sites on their respective intestinal transport proteins (Barton *et al.*, 1978; Flanagan *et al.*, 1979). It has also been suggested that calcium may indirectly affect lead absorption by affecting the synthesis of mucosal transport proteins (Aungst and Fung, 1985). However, the absence of saturable transport mechanisms indicates that lead transport is carrier independent (Blair *et al.*, 1979). It seems more likely that any competition between cations will occur at the tight junction between epithelial cells, either in the form of competition for anionic sites within the tight junctions, or competition for entry into the tight junctions from the bathing medium, or possibly a combination of both (Coogan, 1982). The influence of certain cations such as calcium on the permeability of the tight junction may also affect lead absorption (Hilburn *et al.*, 1980).

1.9.2 Speciation

Both the diet and intestinal secretions provide many anions and ligands with which lead can complex. The formation of various lead species may enhance or reduce absorption by forming more or less soluble lead complexes, by increasing or decreasing the suitability of soluble lead for transport by passive mechanisms, or possibly by increasing the amounts of lead able to be transported by active mechanisms.

To date, research studying the effect of various anions on lead transport has been limited. Partridge (1986) found that administering different lead salts to fasted rats caused differences in the amount of lead subsequently absorbed. Studies by Barton and Conrad (1981), and Aungst and Fung (1983), indicated that the presence of phosphate in

the diet caused a reduction in lead absorption leading both groups to conclude that the formation of insoluble lead phosphate rendered the lead unavailable for transport. However, the reduction in lead absorption may not have been solely a result of precipitation. Lead also readily forms stable, soluble lead phosphate ion pair complexes (maximum solubility of lead orthophosphate being 1.7×10^{-7}M (Coogan, 1982)), and will similarly form stable, soluble ion pair complexes with carbonate ions (maximum solubility of lead carbonate being 4.1×10^{-6}M (Coogan, 1982)). Both these soluble complexes could readily form in the gut *in vivo*, but to date the effect of their formation on lead transport has not been studied.

The effect of other dietary ligands on lead absorption has been widely studied. Chelating agents, such as citrate, folate and ascorbate are known to form stable complexes with lead. The formation of such complexes may account for the increased lead transport observed in the presence of these ligands, perhaps by altering the lipid solubility of the lead or the route of transport (Coleman *et al.*, 1982). Lead also forms complexes with components of bile, both proteins (Cikrt and Tichý, 1975) and salts (Partridge, 1986). The greater lead absorption seen when bile flow is normal compared to when it is reduced (Cikrt and Tichý, 1975; Partridge, 1986) may be due to the formation of such complexes and their subsequent incorporation into the enterohepatic circulation.

1.10 Aims of the present research

Initial studies will extend present data on the transport of lead in the intestine by determining whether indeed lead can be transported actively against a concentration gradient in the duodenum as suggested by Barton (1984). The effect of the presence of food in the intestine on the absorption of lead and its subsequent distribution round the body will be studied, taking into account the amount of lead in the blood and the proportion of lead present in various organs by virtue of their blood content. The possible lead species present in the soluble phase of the gut washings from fed and fasted rats will be elucidated by a combination of gel filtration chromatography and a number of simple chemical tests. The effect of the various soluble lead species elucidated on the transport of lead across the gut will be studied in the living animal by a combination of whole body studies and a modified *in vivo* perfusion technique.

Chapter 2

STUDY OF LEAD TRANSPORT IN EVERTED DUODENAL SACS FROM THE RAT

2.1 Introduction

Clinically evident lead poisoning usually results from the absorption of lead across the gastrointestinal tract (Hilburn, 1979), but evidence as to the site and mechanism of absorption is conflicting. Grunden and Stantić (1975) and Blair *et al.* (1979) found no difference in the amount of lead absorbed by the various regions of the small intestine, and Cikrt (1970) found no significant difference between the duodenal and ileal absorption of lead. However, Gerber and Deroo (1975) found that more lead was absorbed in the jejunum than duodenum or colon, and the duodenum has been quoted as the principal region of lead absorption in suckling rats (Henning and Leeper, 1984) and adult rats (Barton, 1984).

A number of mechanisms for lead uptake have been suggested by various workers. Blair *et al.* (1979) and Hilburn *et al.* (1980) suggest that in the rat, lead is transported into the serosal compartment by a process of passive diffusion linked to water transport, occurring through the tight junctions between epithelial cells. Others have suggested that there may be two mechanisms for lead absorption in the small intestine. Observations from work with mice led Flanagan *et al.* (1979) to conclude that although lead was primarily absorbed by a diffusion-type mechanism, a second carrier-mediated process, enhanced by a low dietary iron level, contributed to lead absorption. Later work by Aungst and Fung (1981b), suggested a model of lead transport which included both a carrier-mediated component and passive diffusion, although they suggested that the contribution of passive diffusion to the total lead flux was relatively minor.

Barton (1984) produced evidence for active lead transport. Everted sacs of rat duodenum were incubated in lead-containing buffers for up to two hours, and accumulation of lead on the serosal side of the intestinal membrane against a concentration gradient was demonstrated. Transport of lead in the jejunum and ileum, however, was shown to be passive (Barton, 1984).

The results obtained by Barton were corroborated to a certain extent by Coleman (1979), Coogan (1982) and Heaven (1985), who also demonstrated the passive transport of lead across the jejunum and ileum. However, work by Coleman (1979) did not support Barton's evidence for active transport in the duodenum. The aim of this investigation was to determine if indeed lead can be actively transported against a concentration gradient in the duodenum. The method described by Barton (1984) was

followed as closely as possible.

2.2 Materials and methods

2.2.1 Chemicals

All chemicals used throughout these studies were AnalaR grade (or equivalent) and were supplied by Aldrich Chemical Company Ltd., Gillingham, U.K., Fisons Ltd., Loughborough, U.K., Sigma Chemical Company Ltd., Poole, U.K. and B.D.H. Ltd., Poole, U.K.. Gases were of medical grade and were supplied by the British Oxygen Company, Wolverhampton, U.K.. "Sagatal" (sodium pentobarbitone) was supplied by May and Baker, Dagenham, U.K..

2.2.2 Radiochemicals

Lead-203 (half life, 52.1 hours) prepared by firing accelerated electrons at a thallium target, followed by purification by chromatography (Horlock *et al.*, 1975), was supplied as lead-203 chloride in isotonic saline by the Medical Research Council Cyclotron Unit, Hammersmith Hospital, London, U.K.. The sample was assayed for stable thallium and iron by the Cyclotron Unit and in all cases the concentrations of these metals was found to be negligible.

2.2.3 Animals

All rats used throughout these studies were male Wistars weighing between 180-200g, bred by Bantin and Kingman Ltd., Hull, U.K.. They were maintained on Heygates Rat and Mouse Breeding Diet (Pilsbury's Ltd., Birmingham, U.K.) and tap water *ad libitum*, and kept in an animal house at 20°C with a twelve hour light cycle. Animals to be fasted were placed in cages with wire grid bottoms (to prevent coprophagy) for 16 hours overnight, and had access to only tap water until sacrificed.

2.2.4 Detection and measurement of radioactivity

Lead-203 (used as the lead tracer) is a gamma emitter. All samples were estimated for lead by counting the gamma emissions over the range 60-425 KeV for 60 seconds on an L.K.B. Wallac 1282 CompuGamma (L.K.B. Wallac, Turku, Finland). The CompuGamma automatically corrected the counts for background, radioactive decay,

and also rejected any samples that did not reach twice the background counts.

The amount of stable lead in an unknown sample was calculated by determining the relationship between the activity of the lead tracer and the number of moles of stable lead in a standard sample. The standard sample consisted of a known volume of the original radioactive solution which was taken at the start of each experiment and counted together with the unknown samples.

2.2.5 Buffer solutions

See appendix A.1.1.

2.2.6 Preparation and incubation of everted sacs

Viable sacs (appendix 2), were prepared and incubated as described by Barton (1984). Rats were anaesthetised by an intraperitoneal injection of "Sagatal" (sodium pentobarbitone) at a dose of 40mg/kg body weight and the duodenum removed, rinsed with ice-cold washing solution to remove residual food debris, and everted as detailed in appendix A.2.2.4. The animal was then killed by cutting into the thoracic cavity through the diaphragm and rib cage. Each sac was filled with 0.5mls of incubation buffer and suspended in 7mls of the same buffer. A mixture of 95% O_2/5% CO_2 was bubbled through the mucosal buffer medium at approximately 20-30mls/minute throughout the incubation period. The incubation was performed in a water bath at 37°C with shaking (unless stated otherwise) at 80 oscillations/minute. Incubation times of 30, 60 and 120 minutes were routinely used.

After incubation, the intestine was blotted on filter paper moistened with washing solution and the serosal fluid drained into a sample vial. The volume of the serosal buffer recovered was assessed by weighing and its radioactivity measured (section 2.2.4). The radioactivity of 1ml of mucosal buffer and the drained and blotted intestine was similarly measured. The ratio of the concentrations of lead in the serosal compartment to that in the mucosal compartment was calculated and is expressed as [S]:[M].

2.2.7 The effect of shaking the incubation sacs on [S]:[M] ratios

Nowhere in the work published by Barton (1984) was there any mention of the duodenal sacs being shaken during incubation. The effect of shaking was therefore studied. Sacs were prepared as in section 2.2.6 and incubated in a water bath at 37°C for 60 minutes. Six sacs were shaken at 80 oscillations/minute and six sacs were incubated without shaking.

Results showed that although [S]:[M] ratios for both groups were significantly lower than unity (0.68 ± 0.15 for the shaken sac, and 0.34 ± 0.02 for the unshaken sac), the ratio was significantly higher for the shaken sacs than for the unshaken sacs ($p<0.05$). It was therefore decided that all incubations would be performed in a water bath at 37°C shaking at 80 oscillations/minute.

2.2.8 Recovery of lead

The amount of lead in the incubation system was calculated before and after each experiment. The average total lead recovery for each incubation was 85% ± 2% (n=32). To determine the location of the "missing" 15% lead, incubations were done as described in section 2.2.6 but without intestinal tissue. Following incubation, all glassware was thoroughly washed in concentrated "Lipsol" (L.I.P. Ltd., Shipley, Yorkshire, U.K.), a chelating detergent, and the washings counted. Approximately 10% of the lead was found to have adhered to the sides of the incubation vessel, 2% was lost in pipettes during transfer of labelled buffers and 1% adhered to the tubes supplying the incubation system with 95% O_2/5% CO_2.

2.2.9 Statistics

All results presented are the means of not less than six observations, and are expressed as means ± standard error of means. One-way analysis of variance was used to determine any significant changes in [S], [M], [S]:[M], mucosal uptake or tissue binding of lead with time .

All [S]:[M] ratios were tested to see if they were significantly greater than unity using a one-tailed t-test (Snedecor and Cochran, 1980).

Table 2.1 The movement of lead in the everted duodenal preparation of the rat.

Time (mins)	n	[S] pmol/ml	[M] pmol/ml	[S]:[M] ratio	Net mucosal uptake (pmol)	Lead bound to intestine pmol/g wet wt.
0	-	1000	1000	1	0	0
30	6	227±35	492±34	0.48±0.09	3550±238	5180±459
60	7	255±65	367±26	0.68±0.15	4430±181	6650±830
120	13	211±32	236±22	0.98±0.18	5350±155	10100±616
A.N.O.V.A		N.S.	$p<0.001$	N.S.	$p<0.001$	$p<0.001$

Everted sacs prepared from rat duodenum, injected with 0.5mls of buffer containing $1x10^{-6}$M lead chloride labelled with lead-203 were incubated in 7mls of the same buffer at 37°C in a water bath shaking at 80 oscillations/minute. Results are expressed as means ± standard error of means.

N.S. refers to no significant difference in means over 30-120 minutes.

2.3 Results

In this study, the [S]:[M] ratio did not rise to a value significantly greater than unity over the whole incubation period. After 30 minutes, the [S]:[M] ratio was significantly lower than the initial value of unity and although the values at 60 and 120 minutes appear to be higher than that at 30 minutes, the difference is not statistically significant (table 2.1).

The changes in the [S]:[M] ratio were a result of changes in the lead concentration on either side of the intestinal membrane. After the initial fall in the concentration of lead on the serosal side of the membrane after 30 minutes incubation, no further changes in concentration were observed (table 2.1). However, mucosal lead concentrations fell significantly over the whole experimental period, and this was seen as a significant increase in net mucosal uptake over this time period. Lead bound to the intestinal tissue also increased significantly over the 120 minute incubation. After 120 minutes, 63% ± 3% of the total lead in the incubation system had bound to the sac.

2.4 Discussion

2.4.1 Comparison of the results of this study with those presented by Barton (1984)

The method used by Barton (1984) to demonstrate active transport of lead in the everted sac prepared from the rat was followed as closely as possible. The criterion for active transport was a rise in the [S]:[M] ratio away from the value of unity, which existed at the start of the incubation period, to a value significantly higher than unity. In Barton's investigation, despite an initial fall in the [S]:[M] ratio after 30 minutes to a value of 0.73 ± 0.05, by 120 minutes, a ratio of 6.06 ± 0.79 had been achieved, corresponding to a fall in mucosal lead concentrations and a rise in serosal concentrations. However, in this study, the [S]:[M] ratio did not rise to a value significantly greater than unity over the incubation period, suggesting that active transport was not occurring. By 30 minutes, the ratio had fallen to a value of 0.48 ± 0.09 and although apparently increasing to 0.98 ± 0.18 after 2 hours, this increase was not significant. As in Barton's study, there was a decrease in lead concentration in the mucosal compartment over the 2 hour period, corresponding to an increase in net mocosal uptake, but there was no consequent increase in serosal lead concentrations. The essential difference between the results of the two

studies seems, therefore, to be due to an apparent inability by the sacs in this study to transfer lead against a concentration gradient into the serosal compartment. However it is possible that in this study, lead may have been transported across the sac and bound to the serosal surface, in which case it would be undetectable in the serosal buffer.

The results from this study compare favourably with those quoted by Grunden and Stantic (1975). They used a modified Krebs-Ringer buffer containing 0.7×10^{-6}M lead chloride labelled with lead-203 on either side of the rat duodenum. Incubation at 37°C in a shaking incubator for 90 minutes produced a mean [S]:[M] ratio 1.10 ± 0.16 (n=8), a value not significantly greater than unity.

The percentage of total available lead bound to the tissue after incubation for 90 minutes was found to be 55% (Grunden and Stantic, 1975) and 80% after 120 minutes (Barton, 1984). These values correspond favourably with those obtained in this study for 60 and 120 minute incubations (i.e. 42% and 63% respectively).

2.4.2 Possible reasons for the differences between this study and that of Barton (1984)

Although the method quoted by Barton and that used in this investigation are fundamentally the same, two important differences do exist.

1. The volume of buffer injected into the sac

In his investigation, Barton injected 0.8mls of labelled buffer into the 6cm duodenal sac. In this study, such a volume was found to be too large for a sac this size and consequently a volume of 0.5mls was preferred. If indeed 0.8mls of buffer was used by Barton, the resulting distention of the duodenal tissue would probably have caused premature tissue damage. Indeed, Barton reported that, when examined by light microscopy 60 minutes after incubation, the sac tissue showed submucosal oedema and early loss of absorptive cells in the crypts, and at 2 hours, there was definite clubbing of villi, shedding of villus tips and considerable crypt cell loss. Coogan (1982) showed that jejunal sacs, filled with 0.5mls of Krebs-Henseleit buffer and incubated in 10mls of the same for 50 minutes, exhibited no such tissue damage. It may be concluded that the movement of lead into the serosal compartment observed by Barton may have been a consequence of premature tissue damage.

Inaccuracies in Barton's work may also have resulted from the effects of

massive binding of lead to the serosal surface of the tissue in conjunction with the large volume of buffer injected into the sac. Binding of lead to the serosal surface reduces the serosal lead concentration, but this reduction is less apparent the larger the volume of buffer in the serosal compartment. This problem may be highlighted when considering two hypothetical everted duodenal sacs of identical length, one with 0.8mls of a buffer in the serosal compartment, the other with 0.5mls of the buffer, which are incubated for the same duration under identical conditions in the same buffer. Equimolar concentrations of lead are placed in both mucosal and serosal compartments. Assuming no transport of lead from mucosal to serosal compartments in either sac, only binding of lead to the mucosal and serosal surfaces (a factor identical in either case since the sacs are the same length), after incubation, the sac filled with 0.8mls of buffer will have a higher serosal lead concentration than the sac filled with 0.5mls of buffer because the amount of lead removed from the serosal buffer and bound to the serosal surface, as a proportion of the total amount of lead present, is smaller. Using the results presented here, but assuming an incubation volume of 0.8mls (as used by Barton, 1984) and not 0.5mls, and identical tissue binding irrespective of the volume of buffer in the serosal compartment, the calculated [S]:[M] ratios would be 1.04, 1.45 and 2.15 for 30, 60 and 120 minutes respectivley. These values, although greater than unity, do not indicate active transport, they simply reflect the fall in mucosal lead concentration due to binding, and a constant serosal lead concentration. The [S]:[M] ratios calculated by Barton (1984) will reflect similar inaccuracies. Clearly, the use of [S]:[M] ratios as a criterion for active transport in circumstances where tissue binding is occurring, is unsatisfactory.

2. <u>The use of lead-203 versus lead-210</u>

Although use of a different lead isotope should not affect transport itself, lead-210 decays to bismuth-210 and polonium-210. It is possible that the presence of bismuth or polonium may interfere with lead transport across the duodenum. Also, lead-210 is essentially a beta emitter, only 4% of disintegrations resulting in gamma emission. Hence, for maximum counting efficiency, lead-210 should be counted by liquid scintillation, but Barton used a Packard Auto-Gamma Spectrometer. Coleman (1979) has shown that the efficiency of counting lead-210 by gamma emission to be only 2-3%. To overcome this, he suggests the sample should be of very high activity, or should be counted for long periods of time. Barton uses buffers containing only 9,000

c.p.m./ml prior to incubation, and uses the average of two one minute counts in his calculations. Therefore, inaccuracies in his counting may have led to large errors in his results.

2.4.3 The effect of shaking on the [S]:[M] ratio

In his experimental procedure Barton fails to state if sacs were shaken during incubation. The results in section 2.2.7 show that although shaking the incubation sacs at 80 oscillations/minute will increase the 60 minute [S]:[M] ratio significantly, the value is still not high enough to be comparable with that quoted by Barton. It may be suggested, however, that shaking the incubating sacs at speeds above 80 oscillations/minute will increase the [S]:[M] ratios to values comparable with those of Barton by reducing the unstirred water layer in the mucosal compartment (Lherminier and Alvarado, 1981), and consequently increasing serosal transport of the lead cation. However, very rapid shaking of everted sacs is likely to lead to tissue damage.

2.4.4 Suitability of the everted sac for studies of lead transport

The everted sac is regarded as being a suitable technique for the study of lead transport for a number of reasons. Firstly, lead concentrations ranging from $1x10^{-5}M$ to $1x10^{-6}M$ (usually used in transport studies) have been shown to have no adverse effect on sac viability (Coleman, 1979). Secondly, everted sacs prepared from various regions of the gastrointestinal tract have been shown repeatedly to be capable of transporting lead from mucosal to serosal compartments, transport being linear with respect to time down a concentration gradient (Blair *et al.*, 1979; Coleman, 1979; Coogan, 1982; Heaven, 1985). It can therefore be concluded that despite the problems associated with the everted sac technique outlined in section 6.1, and its unsuitability for studies involving [S]:[M] ratios where massive tissue binding occurs (section 2.4.2), viable everted sac preparations incubated for up to an hour can be used to study lead transport.

2.5 Conclusion

Despite the very close similarity in methodology between the work published by Barton (1984) and that performed in this study, there was no agreement between the results. Unlike Barton's investigation, [S]:[M] ratios significantly greater than unity were

not demonstrated, and there was no apparent transport of lead to the serosal compartment. Possible reasons for the inability to substantiate Barton's findings have been discussed.

Chapter 3

TISSUE DISTRIBUTION OF LEAD IN FED AND FASTED RATS

3.1 Introduction

Experiments to determine tissue distribution of lead in rats under various experimental conditions have been performed by a number of workers. Differences in lead accumulated in a number of tissues of animals under various dietary conditions have been studied by Bratton *et al.* (1981); Louis-Ferdinand *et al.* (1982); Sasser *et al.* (1984); Spickett *et al.* (1984); Ashraf and Fosmire, (1985); Heaven, (1985) and Partridge, (1986). Tissue distribution has also been used to study the effect of both time and increased dietary zinc on the excretion of lead previously deposited in the rat (Castellino and Aloj, 1964; Cerklewski, 1984). In all these cases, the concentration of lead in only a number of the major organs was determined for comparison purposes and to give some idea as to the scale of absorption.

Preliminary studies of tissue distributions (unpublished data) involved a limited number of organs and failed to establish the full extent of lead distribution round the body after an oral dose of lead acetate labelled with lead-203. Recoveries rarely exceeded 80%. It was also noticed that the recovery of lead was consistently higher in fed rats than fasted rats. Three major areas of possible lead accumulation had not been accounted for in previous investigations namely muscle, bone and blood. It was realised that any of these could be acting as a lead pool.

1. Muscle

Previous experiments by Sasser *et al.* (1984) indicated that although muscle constitutes the largest fraction of body mass, the lead concentrations in muscle were less than 0.01% of the administered dose per gram of tissue. Hence, muscle was not considered to be a major lead pool. It was also realised that to determine the amount of lead in muscle would involve removal and counting of all muscle tissue, a very difficult and very inaccurate procedure.

2. Bone

The skeleton contains the largest quantity of body lead (DeMichele, 1984) and has been described as the principal retention area for lead. Analysis of post-mortem tissue for lead indicated that more than 95% of the total body burden is stored in the bone as relativly insoluble triphosphate (Schroeder and Tipton, 1968; Barry, 1975).

The majority of the tissue distribution experiments in which bone was studied, involved sacrifice of the animal a number of weeks after dosing with lead, giving

ample time for any deposition in bone to occur (Six and Goyer, 1970; Cerklewski, 1984). Work by Sasser *et al.* (1984) showed that six hours after dosing fed control rats with lead, its concentration per gram of femur represented less than 1% of the dose administered. Preliminary experiments showed femur lead concentrations to be less than 0.03% of the administered dose after four hours in both fed and fasted rats (unpublished data). Such observations suggest that four hours was too short a time for large amounts of lead to accumulate in the bone. This, together with the realisation that accurate skeletal lead measurement would involve complete hydrolysis of soft tissue round the skeleton, a lengthy process when working with a tracer of short half-life (such as lead-203), prompted the study of blood as the major pool of lead after four hours.

3. Blood

Lead absorbed through the gastrointestinal tract is distributed throughout the body via the blood stream but some of the lead, approximately 2% of the total body burden (Baloh, 1974), remains within the blood. Again, tissue distribution experiments allowing a long absorptive time for lead, show decreasing blood lead levels (Sasser *et al.*, 1984) as the metal moves rapidly from this pool into soft tissue and ultimately into bone (Hilburn, 1979; DeMichele, 1984). Work by Castellino and Aloj (1964) showed that one hour after intra-venous injection of a dose of lead, almost 21% of the dose was still concentrated in the blood. Studies by Sasser *et al.* (1984) demonstrated that six hours after an oral dose of lead was given to fed control rats, approximately 0.06% of the dose was present per gram of blood. The possibility of a substantial percentage of the dose administered being found in blood, together with the relative ease with which the size of this possible lead pool could be determined (by blood volume measurement) prompted the measurement of blood lead concentrations.

The aim of the present investigation, therefore, was to examine the tissue distribution of lead four hours after administration of a dose of $1x10^{-6}M$ lead acetate solution labelled with lead-203. A period of four hours was chosen to allow the amount of lead transported into the body with time, to reach a plateau (Conrad and Barton, 1978). The method for measuring blood volumes would allow, not only the determination of the total amount of lead in the blood, but would also allow the lead found in the organs to be subdivided into lead within the organ tissue, and lead in the blood contained within the organ tissue.

3.2 Materials and methods

3.2.1 Chemicals

All chemicals required for this investigation were supplied as detailed in section 2.2.1.

3.2.2 Radiochemicals

Lead-203 was supplied as described in section 2.2.2. Iodine-125 (half life, 60 days), which, attached to human serum albumin, was used to determine blood volume, was obtained from the Radiochemical Centre, Amersham, U.K.. The amount of free iodine in the sample was determined by 10% trichloroacetic acid precipitation. In the preparation used in this investigation, 98% of the iodine-125 was attached to the albumin.

3.2.3 Animals

Rats were allowed access to food and water *ad libitum*. Animals to be fasted were placed in cages with wire grid bottoms (to prevent coprophagy) for 18 hours overnight and had access to only tap water until sacrificed (see section 2.2.3).

3.2.4 Detection and measurement of radioactivity

Like lead-203, iodine-125 is a gamma emitter. Samples were estimated for lead-203 as detailed in section 2.2.4. Iodine-125 was estimated by counting the gamma emissions over the range 18-100 KeV over a 60 second period. In cases where both the activity of lead-203 and iodine-125 in the various samples had to be determined, the CompuGamma was programmed to count both labels simultaneously. The use of such a dual label programme was possible as lead-203 and iodine-125 emit gamma radiation of different energies, the main energy value for lead-203 being 0.279MeV and that for iodine-125, 0.035 MeV. The programme accounted for overlap by counting standards and estimating cross-over.

3.2.5 Determination of blood volume

The method used for the determination of blood volume was based on that of Aust *et al.* (1951), Wang (1959) and Wiseman and Irving (1963). In all cases, the blood volume was measured by the dilution principle, involving the injection of a suitable test

substance into the blood, allowing it to disperse evenly throughout the blood, and then measuring the extent to which the substance had been diluted. The greater the volume of blood, the less concentrated the test substance after dispersal. The only factors that needed to be known were the total quantity of test substance put into the blood, and the concentration in blood after dispersal. In this case, human serum albumin labelled with iodine-125 (I-125 H.S.A.) was used to measure plasma volume, the total blood volume being calculated from the values for plasma volume and haematocrit (ratio of red cells to plasma).

3.2.5.1 Cannulation of the jugular vein and carotid artery

Animals which were either fed or had been fasted as described in section 3.2.3, were anaesthetised by an intra-peritoneal injection of "Sagatal" (sodium pentobarbitone) at a dose of 40mg/kg body weight and laid so their heads lay proximally and their tails, distally. An incision was made on the right side of the neck and the surrounding skin was removed to expose the right external jugular vein. Membranous tissue surrounding the vein was dissected away and the vein isolated. A ligature of silk suture (Sutures Ltd., Newtown, Powys, U.K.) was used to tie off the proximal end of the vein, and a loop of suture was loosely tied round the distal end of the vein. A small incision was made in the vessel wall just in front of the proximal ligature, using a pair of fine butterfly scissors. Into the incision was inserted 8-12mm of a length of polythene tubing (inner diameter 1mm, outer diameter 1.6mm, Portex Ltd., Kent, U.K.) attached to a 25G syringe needle, previously heparinised with heparinised saline (50 units of heparin/ml 0.9% saline). Once in place, the cannula was secured by the distal ligature. To the syringe needle was attached a heparinised, 1ml polypropylene syringe containing 200µl of heparin-saline solution.

The left carotid artery of the rat was exposed by a mid-line incision along the neck, followed by careful dissection through the muscles on the left of the trachea. Once isolated, the proximal part of the artery was ligated and a loose ligature placed round the distal end. A length of suture was threaded under the far distal end of the artery and held tightly to leave the vessel free of surrounding tissue and to constrict it, thus preventing the backflow of blood when the artery was cut. A small incision, just in front of the proximal ligation allowed the polythene tubing of a second cannula to be inserted and pushed gently

towards the aorta. Once in position, the distal ligature was tightened, fastening the cannula in place.

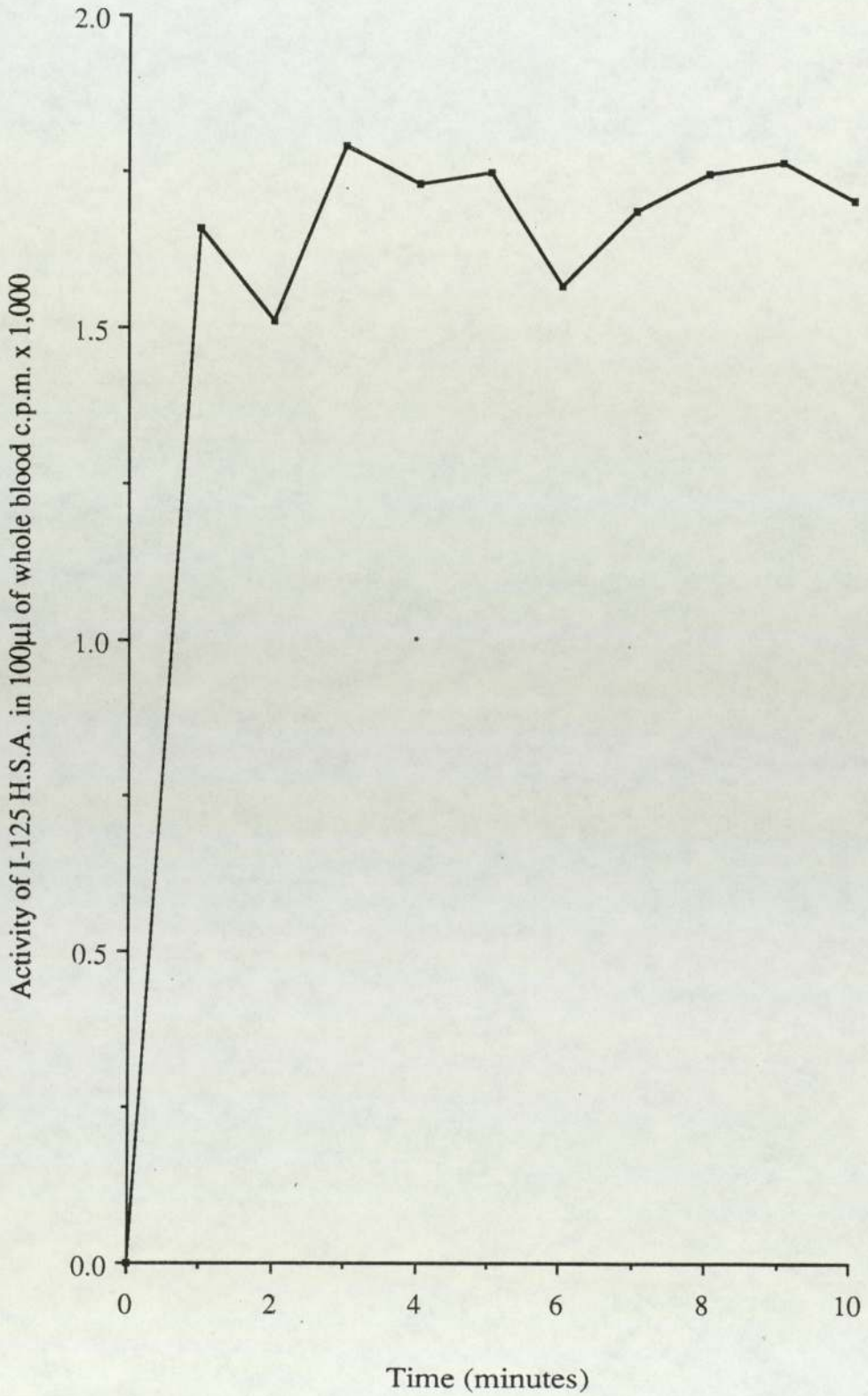
3.2.5.2 Determination of a suitable mixing time for I-125 H.S.A.

When using I-125 H.S.A. to determine plasma volume, it is necessary to allow enough time for it to mix with and be fully diluted by the plasma (the mixing time). If too long a time is allowed, unknown amounts of label may be lost from the circulation, so the shortest mixing time has to be determined.

A volume of I-125 H.S.A. (10μCi/kg body weight) was injected into the jugular vein of a fasted rat from a Hamilton syringe attached to the inserted cannula (section 3.2.5.1). The syringe was then washed out with 100μl of heparinised saline. To determine the time necessary for the I-125 H.S.A. to equilibrate within the blood system, approximately 125μl of blood was withdrawn from the canula in the left carotid artery, and exactly 100μl was pipetted into a counting vial. The pipette tip was also placed in the vial which was then half filled with 3mls of distilled water and shaken. This procedure was carried out every minute for ten minutes. Bleeding through the arterial cannula between syringe changes was prevented by clamping the polythene tube with a pair of artery clamps.

The activity of the I-125 H.S.A. in the blood was measured by counting the gamma emissions as described in section 3.2.4. The counts per minute were then plotted against time. The resulting graph (figure 3.1) showed a rise in the activity of I-125 H.S.A. in the blood, reaching a maximum after three minutes. This was followed by a levelling off in activity, suggesting the I-125 H.S.A. had been distributed evenly round the blood system after three minutes and, consequently, this was the time interval routinely used between injection of I-125 H.S.A. and withdrawal of blood in later experiments. This agrees with the mixing time used by Rieke and Everett (1957). In fed rats, a time of three minutes was found to give unrealistically high blood volumes, indicating that the iodinated albumin had not equilibrated and a time of five minutes was found to be more suitable. It was assumed that the label lost from the blood was negligible during the three or five minute mixing periods, as the activity of I-125 H.S.A. in the blood remained constant for up to ten minutes following injection (figure 3.1).

Figure 3.1 Graph to show the presence of I-125 H.S.A. in rat blood with time



3.2.6 Distribution of lead in the blood and organs of a rat after oral administration of a lead dose

A dose of 0.3mls of $1x10^{-6}$M lead acetate containing 4μCi of lead-203 was administered orally by gastric intubation to a rat which had either been fasted as described in section 3.2.3 or which had been allowed food *ad libitum* during the period prior to sacrifice. After 3.5 hours the jugular vein was cannulated as detailed in section 3.2.5.1, and 10μCi of I-125 H.S.A./kg body weight was injected into the jugular vein to allow the determination of blood volume as described in section 3.2.5. In this instance, 1ml of blood was removed by cardiac puncture, placed in a heparinised counting vial with approximately 3mls of distilled water and counted on an L.K.B. CompuGamma (section 3.2.4). Haematocrits of undiluted blood were obtained using heparinised capillary tubes 75mmx1.2mm (L.I.P. (Equipment and Services Ltd., Shipley, Yorkshire, U.K.). The tubes were centrifuged at 2,000 r.p.m. for 30 minutes and the haematocrit read directly from the tube.

After four hours (approximately 5-10 minutes after the injection of I-125 H.S.A.), the animal was killed by cutting into the thoracic cavity through the diaphragm and rib-cage, and various major organs were removed and washed in isotonic saline. A known proportion of the liver was removed; the activity of lead-203 and iodine-125 in this sample was used to calculate the activities in the whole liver. The small intestine was removed and divided into its three main regions. The duodenum extended from the pyloric sphincter to the ligature of Trietz, and the jejunum and ileum, from the ligature of Trietz to the ileocaecal valve. The jejunum and ileum were separated by cutting the second section in half, the half nearest the pyloric sphincter being the jejunum and the half nearest the ileocaecal valve being the ileum. The luminal contents of each section were washed out with isotonic saline, collected and counted separately. The stomach, caecum and colon were counted together with their luminal contents. The activity of lead-203 and iodine-125 in the various organs was determined on an L.K.B. CompuGamma programmed to count both labels simultaneously (section 3.2.4).

3.2.7 Calculation of results

3.2.7.1 Blood volume

The plasma volume of the rat was measured by observation of the dilution

effect the plasma had on a known activity of I-125 H.S.A. after injection into the jugular vein. However, this value had to be corrected for free iodine-125 in the I-125 H.S.A. (section 3.2.2), and for any counts associated with albumin remaining on the jugular cannula after injection. It was assumed that a negligible amount of I-125 H.S.A. remained in the Hamilton syringe after it had been washed through with heparinised saline.

A haematocrit value, corrected for plasma trapped in the red cell fraction after centrifugation (43%), allowed calculation of the plasma volume of 1ml of blood removed from the rat. It was known that the activity of the sample was due solely to I-125 H.S.A. in the plasma and hence a value for the total plasma volume of the rat could be calculated from the equation quoted by Guyton (1982):

$$\text{Total plasma volume} = \frac{\text{counts per minute injected}}{\text{counts per minute removed/ml plasma}}$$

The total plasma volume was converted to a value for total blood volume using the equation quoted by Wang (1959), which takes into account haematocrit, the plasma trapping factor (P.T.F.) of the haematocrit quoted as 0.96 by Huang and Bondurant (1956) and the unequal distribution of erythrocytes round the body (the F_{cell} value) quoted as 0.986 by Huang and Bondurant (1956)

$$\text{Total blood volume} = \frac{\text{plasma volume} \times 100}{100\text{-haematocrit} \times \text{P.T.F.} \times F_{cell}\ \text{number}}$$

The plasma volumes of each organ was calculated by comparing the counts per minute due to the I-125 H.S.A. in the organ with the counts per minute obtained from 1ml of plasma. The organ blood volumes were calculated from the organ plasma volumes using the equation quoted by Wang (1959). It was assumed that all the I-125 H.S.A. in the organs was in the blood, and none had entered the organ tissue itself.

3.2.7.2 Lead concentrations

Once the lead concentration in 1ml of blood had been determined (section 3.2.6), it was possible to calculate the concentration of lead in the total blood volume. The amount of lead in the organs was also determined, the value obtained representing the

lead in the blood within the organ, and the lead in the organ tissue itself. Using previously calculated organ blood volumes, it was possible to calculate the amount of lead in the organ present by virtue of the blood, and subsequently the amount of lead in the organ tissue alone.

Knowing the concentration of lead in the 0.3mls of lead acetate given orally, and the concentration of lead in either the organs, blood or intestinal washings, it was possible to calculate the percentage of the administered dose present in each.

3.2.7.3 Statistics

The results were analysed statistically. The non-normal distribution of the data made use of the standard t-test impossible, and hence a non-parametric test had to be used, namely the Wilcoxon Rank Sum Test (Remington and Schork, 1970). This allowed analysis of the data by removing the effect of variability within one parameter (fed or fasted), hence allowing comparison of the variability between the two parameters. The fact that four animals had been used for each variable meant that the analysis could be performed only to the 5% level of significance.

3.3 Results

To enable comparisons to be made between fed and fasted animals, it is necessary to establish that, apart from dietary status, the two experimental groups of animals are the same. The blood volume per 100g body weight was shown to be similar in fed and fasted rats (table 3.1) and therefore comparisons of the two groups is possible.

The percentage of dosed lead recovered from the fed rats appeared to be higher than that recovered from the fasted rats, although the difference between the values was not significant (table 3.1).

3.3.1 Lead in the gastrointestinal tract

The amount of lead in the intestine varied greatly depending on the nutritional status of the animal. In the fed animal, approximately 25% of the total dose of lead remained in the stomach, whereas in the fasted animal, only approximately 0.5% of the dose was retained over the four hours (table 3.2). Consequently, over the four hour post-dosing period, 74.5% of lead entered the upper small intestine in the fed rat

Table 3.1 Blood volume, percentage of total dose administered (%T.D.A.) entering the body over the four hour period and percentage recovery for fed and fasted rats.

	Fed rat	Fasted rat
Blood volume/100g body weight (mls)	5.97 ± 0.49	5.09 ± 0.62
% T.D.A. entering the body	9.25 ± 2.94	30.10 ± 12.59
% recovery	91.70 ± 2.86	79.20 ± 8.78

Data expressed as means ± standard error of means for four animals.

No significant difference was found between the values for fed and fasted rats.

compared to 99.5% in the fasted.

A significant proportion of the administered lead remained associated with the duodenal, jejunal and ileal tissue, the proportion always being significantly higher in fasted rats compared with fed rats (table 3.2). A higher quantity of lead was found in the caecum and colon of fasted rats compared with fed rats, although the means were not significantly different. Faecal pellets collected over the four hour period after dosing did not contain any lead, presumably because they were formed before the lead had reached the colon.

In all regions of the intestine, the amount of lead in the blood associated with the segments was negligible when compared to the amount of lead in the segment itself (table 3.2). More lead was found in the blood associated with all the intestinal regions of fasted animals than fed animals, (with the exception of the caecum); this can not be accounted for by an increase in the blood volume of the intestinal regions of fasted rats compared with fed rats (table 3.3).

3.3.2 Lead in the internal organs

The percentage of the total dose administered entering the body (i.e. the percentage of lead that can not be accounted for by the intestine or intestinal washings) appeared to be higher in fasted rats compared to fed, but the values were not significantly different (table 3.1). Similar values for lead uptake in fed and fasted rats of 7-8% and 30% respectively, were obtained by Partridge (1986). This apparent difference was reflected in the summed total of lead to be found in the internal organs of these animals, the value being considerably higher in fasted compared with fed rats (table 3.4).

With the exceptions of heart and lungs, there was an increase in the amount of lead within the organ tissue of fasted rats compared to fed rats, which was significant in all organs except the brain (table 3.4). The total blood volume contains more lead than any of the organs. In the case of the heart and lung, there was a greater amount of lead in the tissues from fed rats compared to those from fasted rats, this value being significantly different in the heart (table 3.4).

The amount of lead in blood associated with the organs was always greater in fasted animals than fed animals (table 3.4). This, however, was not caused by an increased organ blood volume in fasted rats (table 3.5). The increase can be explained by

Table 3.2 Distribution of lead in the gastrointestinal tract of fed and fasted rats.

Organ	Dietary status	Lead in blood associated with organ (f.moles)	Lead in organ tissue and contents# or organ tissue only (f.moles)	% T.D.A. in organ and associated blood
#Stomach	fed	0.458±0.458*	76400±29400*	25.5±9.78*
	fasted	29.8±9.49	1410±348	0.48±0.12
Duodenum	fed	1.87±0.92	143±37.0*	0.05±0.01*
	fasted	37.8±24.3	4960±2790	1.67±0.94
Jejunum	fed	11.0±4.65	1710±275*	0.57±0.09*
	fasted	37.8±16.3	18100±10400	6.04±3.46
Ileum	fed	8.96±3.77*	2110±556*	0.71±0.18*
	fasted	74.0±29.7	11000±2670	3.70±0.9
#Caecum	fed	2.91±1.87	15900±8200	5.29±2.73
	fasted	0.0±0.0	110000±39200	36.5±13.1
#Colon	fed	2.43±1.24	1080±553	0.36±0.18
	fasted	15.9±12.4	12800±9330	4.28±3.11

Rats dosed with 0.3mls of $1x10^{-6}$M lead acetate labelled with 4μCi of lead-203 and left for four hours before sacrifice. All organs were washed with isotonic saline before counting. Results are expressed as means ± standard error of means for four animals.

The stomach, caecum and colon were counted together with their luminal contents.

* Denotes a difference between the two experimental groups ($p<0.05$).

Table 3.3 Blood volumes of the various intestinal regions in fed and fasted rats.

Organ	Blood volume (mls)	
	Fed rat	Fasted rat
Stomach	0.02 ± 0.02	0.04 ± <0.01
Duodenum	0.02 ± <0.01	0.04 ± 0.02
Jejunum	0.14 ± 0.03	0.09 ± 0.04
Ileum	0.12 ± 0.02	0.10 ± 0.01
Caecum	0.04 ± 0.01	0.00 ± 0.00
Colon	0.03 ± 0.01	0.02 ± 0.01

Blood volumes are means ± standard error of means for four animals. No significant difference was found between the blood volumes of the organs of fed and fasted rats.

Table 3.4 Distribution of lead in the internal organs of fed and fasted rats

Organ	Dietary status	Lead in blood associated with organ (f.moles)	Lead within organ tissue (f. moles)	% T.D.A. in organ and associated blood
Brain	fed	2.37±0.815*	9.81±3.07	<0.001*
	fasted	31.2±13.8	83.7±73.0	0.04±0.02
Liver	fed	115.0±45.6*	212±74.1*	0.11±0.01*
	fasted	1910±1020	5560±3390	2.49±1.47
Spleen	fed	5.95±2.59	30.4±5.89*	0.01±<0.001*
	fasted	63.1±24.1	192±98.2	0.09±0.04
Kidneys	fed	25.7±10.7*	310±11.1*	0.11±0.006*
	fasted	307±114	8140±4890	2.82±1.67
Heart	fed	10.1±3.51*	15.7±3.54*	0.006±0.001*
	fasted	85.9±30.8	1.45±1.45	0.03±0.01
Lungs	fed	18.5±5.49*	1080±634	0.36±0.21
	fasted	261±107	411±207	0.22±0.10
Blood	fed	1160±461*	-	0.39±0.15*
	fasted	11100±4650	-	3.72±1.55
Total	fed	-	-	0.93±0.12*
	fasted	-	-	8.51±4.36

Rats dosed with 0.3mls of $1x10^{-6}$M lead acetate labelled with 4μCi of lead-203 and left for four hours before sacrifice. All organs were washed with isotonic saline before counting. Results are expressed as means ± standard error of means for four animals. The value for blood represents the total blood volume of the rat.

Total % T.D.A. in internal organs = % T.D.A. in blood-free organs + % T.D.A. in total blood

* Denotes a difference between the two experimental groups ($p<0.05$).

Table 3.5 Blood volumes of various internal organs in fed and fasted rats.

Organ	Blood volume (mls)	
	Fed rat	Fasted rat
Brain	0.04 ± <0.01	0.04 ± <0.01
Liver	1.59 ± 0.06	2.11 ± 0.40
Spleen	0.08 ± 0.01	0.09 ± 0.02
Kidney	0.35 ± 0.02	0.41 ± 0.07
Heart	0.14 ± 0.02	0.14 ± 0.03
Lungs	0.31 ± 0.07	0.33 ± 0.06

Blood volumes are means ± standard error of means for four animals. No significant difference was found between the blood volumes of the organs of fed and fasted rat.

the greater amount of lead in the blood of fasted rats compared with fed rats (table 3.4) which, since the total blood volumes are comparable, indicates a higher concentration of lead in the blood of fasted compared to fed animals.

The amount of lead in the blood of internal organs contributed considerably to the final percentage of the administered dose calculated for organ and associated blood. In the liver, over a quarter of the lead present was found in the blood associated with the organ. Such figures show the importance of accounting for blood lead when performing such distribution experiments.

3.4 Discussion

3.4.1 Lead in blood

The values obtained for both total blood volume (table 3.1) and organ blood volumes (tables 3.3 and 3.5) are in close agreement with previously recorded values (Huang and Bondurant, 1956; Hamilton, 1962, and Conrad and Barton, 1978; Triplett *et al.*, 1985, respectively), indicating that the method used for their determination was satisfactory. It has been suggested that anaesthetic (Rieke and Everett, 1957) and the use of iodinated albumin, which is not homologous with that of the experimental animal (Gregersen and Rawson, 1959), will lead to false values for total blood volume and hence organ blood volumes, but this does not seem to have happened here.

The concentration of lead in the blood of fasted animals was significantly greater than that in fed rats (table 3.4). This reflects the increased absorption of lead in fasted animals (table 3.1).

3.4.2 Recovery of orally administered lead

One of the primary aims of the work detailed in this chapter was to improve the recovery of orally administered lead, four hours after dosing, by taking into account lead in blood as well as lead in the major organs. However, no such improvement was achieved. This suggests that an important area for lead accumulation has still not been accounted for. Two major tissues have not been examined; muscle and bone. It has been calculated that the clearance rates for lead from an exchangeable pool, including blood, to soft tissue and bone, are similar (Chamberlain, 1985). This would seem to indicate that either of these sites could contain the lead which has not been accounted for. Accurate

determination of the amount of lead in either site is practically very difficult, as outlined in section 3.1. It is interesting to note that despite the greater absorption of lead from the intestine in fasted compared to fed rats, total recoveries appear lower (table 3.1). This is probably due to the fact that in fasted rats, more lead is available to the "exchangeable pool" and therefore more is transferred to tissues in which lead is not determined.

3.4.3 Lead in the gastrointestinal tract

The amount of lead entering the upper duodenum after four hours in fasted rats was 99.5%, agreeing with the value of 99.6% quoted by Heaven,1985, and 97.8% quoted by Partridge (1986). In the fed rat, the values were more variable being 74.5% in this study, compared to 85.3% quoted by Heaven (1985) and 82.2% quoted by Partridge (1986) possibly reflecting variations in the rate of gastric emptying. Again, both in this study and that by Heaven (1985) and Partridge (1986), the amount of lead bound to intestinal tissue was found to be greater in fasted rats than fed rats four hours after dosing. Such observations may be explained by the fact that lead entering the intestinal lumen in the fed rat is surrounded by food, reducing its chances of reaching the gut wall and being bound or absorbed. Some lead may form non-absorbable complexes with constituents of the diet, preventing its uptake or binding. Alternatively, there may be competition between lead and various dietary constituents, such as other metals, for uptake sites. Hence, in the fed rat, the percentage of the dose administered which is bound to the gut wall is small, most of the lead remaining in the food bulk and being accounted for in the intestinal washings. In the fasted rat, lead moves quickly through the stomach and rapidly enters the intestine, where an absence of food allows its easy access to the mucosal surface, causing increased tissue binding (table 3.2) and absorption (table 3.4).

It is unlikely that differences in tissue binding between the different intestinal segments of the animals in each experimental group reflect differences in sites of lead absorption. It is more likely to be due to the amount of food actually available for binding as the food bulk passes through that part of the intestine, or differences in luminal surface areas of various intestinal segments. Coleman (1979), Miller *et al.* (1983) and Partridge (1986) all suggest that the high binding capacity of the intestinal tissue may act as a protective mechanism by reducing the luminal concentration of free lead, thereby reducing

the rate of lead transport. In contrast, Heaven (1985) suggests tissue binding is part of the transport process. It is possible that the intestine acts as a store for lead, a reduction in the luminal concentration of the cation causing slow release of lead from the tissue into the lumen, thereby providing a constant supply of lead cation for transport.

Lead present in the caecum is probably found mainly in the food residues, tissue binding being low due to the inability of the ion to reach the caecum wall because of the large food mass present. The caecum of both fed and fasted animals acts as a point for lead accumulation. The presence of lead in the caecum gives some idea as to the rapidity by which it is moved along the intestine - the transit time. During the four hours after dosing, 7% of the lead entering the intestinal lumen reached the caecum in the fed rat, whilst in the fasted animal this value was 37%. The lower percentage in the fed animal can not be accounted for by an increased lead uptake (table 3.1), or by increased tissue binding (table 3.2) and must therefore have been due to a slowing of lead movement through the intestine during the four hours after dosing, due to its association with food. A reduced transit time in fed compared to fasted animals was also reported by Heaven (1985). These experimental observations disagree with the suggestion made by Coogan (1982) that feeding may reduce lead absorption in part by stimulating peristalsis, thereby increasing the transit time of a lead dose and shortening the length of time the lead is in contact with the gut wall. Decreased peristalsis in the fasted rat would, he suggested, reduce transit time and consequently increase lead absorption. The experimental evidence in this chapter and that obtained by Heaven (1985) demonstrates that transit time is not increased on feeding and the decrease in the absorption of lead in fed compared to fasted rats must therefore be caused by the interaction between lead and food.

3.4.4 Lead in the internal organs

Lead concentrations within the tissue of internal organs, with the exception of the heart and lungs, are greater in fasted compared to fed rats (table 3.4). In the heart and lung there is an apparent decrease in lead concentration in fasted rats compared to fed, this being significant in the case of the heart. This suggests that the lungs and heart have a limited capacity to accumulate lead, an observation also made by Miller *et al.* (1983).

The amount of lead found within an organ seems to be related to its size and

degree of vascularization, the larger the organ, the more lead it is able to accumulate. However, the kidneys accumulate as much lead during the four hours after dosing as the liver, even though they are smaller. Even when this size difference is taken into account, the kidneys have been found to contain five times more lead than the liver, per gram of tissue (Heaven, 1985). Hejtmancik *et al.* (1982) also found that the highest lead concentration in any soft tissue was observed in the kidneys. This may be due to their degree of vascularisation. The kidneys receive 25% of the cardiac output (Rose, 1983), and together with their ability to selectively filter and concentrate, they are a prime target for lead accumulation.

The brain, one of the principal sites for the toxic effects of lead, shows a lower lead concentration than any other tissue, agreeing with the observation of Partridge (1986). A similar observation was made by Mykkänen *et al.* (1979) and Sasser *et al.* (1984) who took the size of the brain into account when comparing it with the other organs. Mykkänen *et al.* (1979) suggested that the brain may be protected by regulatory factors limiting the transport of lead from the blood into the brain. It is possible that the blood-brain barrier has a role in this regulation.

The amount of lead in an organ is not, therefore, a direct indication of the affinity of the cells within the organ for lead. Organ size, vascularization and the presence of possible "regulatory factors" and protective mechanisms must be taken into account.

3.5 Summary

The distribution of lead round the body of a male Wistar rat, four hours after an oral dose of $1x10^{-6}$M lead acetate solution was studied and the main sites of lead accumulation determined. Blood volumes of rats were determined by the dilution principle, using I-125 H.S.A. as the test substance. The amount of lead in the blood was determined, and it was shown that there was a greater percentage of lead in the blood compared to any of the internal organs in both experimental groups of animals, but that a significant proportion of lead was still unaccounted for. Further investigations to try and obtain total lead recovery after its absorption through the gut, will have to take into account bone and muscle.

In the rat, nutritional status was shown not only to greatly influence lead absorption from the intestine (fasted rats taking up approximately three times the amount

of lead taken up by fed rats, table 3.1), but also its distribution throughout the body.

Chapter 4

DETERMINATION OF THE SOLUBLE LEAD SPECIES FORMED IN THE LUMEN OF THE GASTROINTESTINAL TRACT OF THE RAT

4.1 Introduction

The overall daily dietary intake of lead from food and water is estimated at 100μg for adults and 50μg for children (Southwood, 1983). However on average, adults absorb 10% of the dose, and children, 53% (Southwood, 1983). The discrepancy between the amount of lead ingested and that absorbed is due to the formation of various soluble and insoluble lead species in the gut lumen which alter its bioavailability (Karhausen, 1973; Kehoe, 1976; Chamberlain *et al.*, 1978). To assess accurately the bioavailability of a lead dose, the speciation (i.e. the individual physico-chemical forms of lead which make up its total concentration) must be determined. To date there has been little research in this area.

Lead is a soft Lewis acid that readily forms covalent bonds with soft bases. It forms stable soluble ion pair complexes with phosphate, carbonate and hydroxide anions, it binds with sulphur donors, (the thiolate ligand being the most avid monodentate bioligand), and complexes with phosphate, carboxyl and amino groups on organic biomolecules such as amino acids, organic acids, peptides, proteins and polysaccharides (Rickard and Nriagu, 1978). The complexing of lead with any of these ligands, either before ingestion or once in the gut lumen, could affect the amount absorbed into the body because of differences in the solubility and chemical behaviour of the various complexes. Exactly which lead-ligand complexes are formed depends on the relative concentration of various ligands and the formation constants for the complexes.

Partridge (1986) used gel filtration chromatography to separate the lead species in the supernatant phase of the gut contents of rats following an oral lead dose. The resulting elution profile for the fed rat gave a single peak; that for the fasted rat produced a similar peak and a second minor peak. He suggested that the large peak found in both fed and fasted animals was a mixture of the lead cation and lead-ligand complexes possibly lead-histidine and lead-bile salts. However it seems unlikely that lead-bile salt complexes constitute a major part of the species in this peak, as it is shown later in his study that the peak is not noticeably reduced on ligation of the bile duct. The minor peak formed in the fasted animal (named "Pb-X"), remained unidentified, although its failure to be absorbed after isolation and readministration into the rat suggested its possible role as a protective complex. It was also suggested that "Pb-X" was similar to the low molecular weight zinc binding ligand shown by Song *et al.* (1984) to be involved in zinc

absorption.

The aim of the present investigation was to determine an identity for "Pb-X". However, rather than producing the complex *in vivo*, (Partridge, 1986) an attempt was made to produce it by incubating lead with gut washing supernatants *in vitro*. This would, it was thought, lead to an increase in the formation of the various soluble lead species by removing other potential binding sites such as the gut wall or particulate matter in the gut lumen, with which lead may preferentially complex *in vivo*.

4.2 Comparison of the production of lead species *in vivo* and *in vitro*

In initial studies, the chromatographs of the lead species produced *in vivo* were compared with those produced during an *in vitro* incubation to ensure that the latter technique was valid.

4.2.1 Materials and Methods

4.2.1.1 Chemicals

All chemicals required throughout this investigation were supplied as described previously (see sections 2.2.1 and 2.2.2). Sephadex G-15 was supplied by Sigma Chemical Company Ltd., Poole, U.K..

4.2.1.2 Animals

As section 3.2.3.

4.2.1.3 The gel chromatography system used in the speciation of lead from the soluble phase of rat gut washings

The gel chromatography system was identical to that described by Partridge (1986). Sephadex G-15 was allowed to swell in excess buffer ($1x10^{-2}$M lead acetate solution, acidified to pH 3.8 with 1M acetic acid) for 24 hours at room temperature. The suspension was then washed with approximately 1000mls of the same buffer and degassed under vacuum. The slurry was resuspended and packed under pressure using a peristaltic pump (Gilson Minipuls 2, Anachem Ltd., Luton, U.K.) running at 3.5mls/minute, in a glass chromatography column measuring 60cmx1.6cm (Amicon

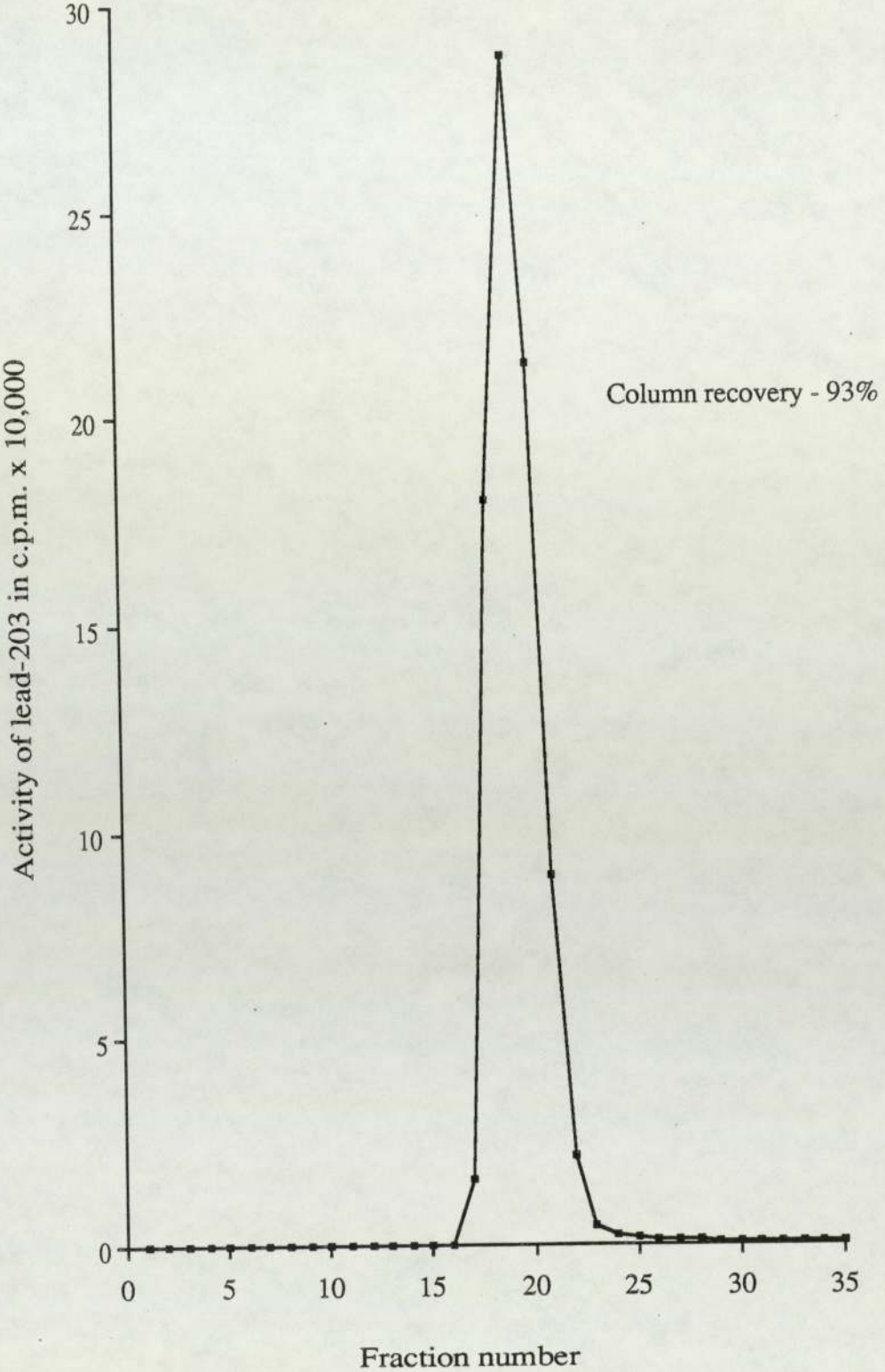
Wright, Wright Scientific Ltd., Stonehouse, U.K.). The column was sealed and the gel bed washed ascendingly with approximately 800mls of degassed buffer. All samples were 5ml in volume and were run on the gel column ascendingly at 3mls/minute, using $1x10^{-2}$M lead acetate buffer (pH 3.8) as eluent. For all elution profiles, thirty five 6ml samples were collected using an L.K.B. Redirac 2112 fraction collector, (L.K.B. Instruments Ltd., Croydon, U.K.). Each column was used only once.

The total exclusion volume was determined by running 5mls of distilled water labelled with 1μCi tritiated water (New England Nuclear, Du Pont (U.K.) Ltd., Stevenage, U.K.) through the column. Exactly 1ml from each of the thirty five eluted samples was mixed with 10mls of scintillation cocktail (Optiphase Safe, Fisons Ltd., Loughborough, U.K.) in a glass scintillation vial and counted for 10 minutes each on a Beckman L.S.7500 liquid scintillation counter (Beckman Instruments Inc., Irvine, California, U.S.A.). Quenching was corrected by comparison with a curve of differentially quenched standards of known activity. The total exclusion volume was found at fraction 19. The void volume was determined using Blue Dextran 2000 (approximate molecular weight - 2,000,000 Pharmacia (Great Britain) Ltd., Milton Keynes, U.K.) and was found at fraction 8. Lead acetate ($1x10^{-6}$M) labelled with 1μCi lead-203 was eluted over fractions 17-22, with the peak at fraction 19 (figure 4.1.1).

4.2.1.4 Preparation of lead species by incubating the soluble phase of rat gut washings with lead *in vitro*

Rats which had been fasted as outlined in section 3.2.3 or allowed food *ad libitum*, were killed by cervical dislocation. The small intestine was excised from pyloric sphincter to ileocaecal junction, and washed through with 11mls of ice-cold isotonic saline. The washings were centrifuged at 100,000 x g for 1 hour at 4°C in an M.S.E. Superspeed 50 ultracentrifuge (Measuring and Scientific Instruments Ltd., Crawley, U.K.) and the supernatant decanted. Exactly 9.9mls of supernatant were incubated with 0.1mls of $1x10^{-4}$M lead acetate labelled with 1μCi lead-203 chloride giving a final lead concentration of $1x10^{-6}$M. Incubations were performed in a Tecam shaking water bath (Jennings Lab. Suppliers, Nottingham, U.K.) at 37°C, shaking at 80 oscillations/minute. After two hours, the washings were recentrifuged at 100,000 x g for 1 hour after which a 5ml sample was analysed by the gel filtration chromatography system detailed in section

Figure 4.1.1 Elution profile of a lead standard ($1x10^{-6}$M lead acetate labelled with 1μCi lead-203).



Gel - Sephadex G-15

Eluent - $1x10^{-2}$M lead acetate (pH 3.8)

Flow rate - 3mls/minute

Fraction volume - 6mls

See section 4.2.1.3 for detailed column specifications.

4.2.1.3. Any cloudiness in the eluted fractions was removed by centrifugation (100,000xg for one hour). The lead in the column fractions was determined by counting the gamma emissions of lead-203 on an L.K.B. CompuGamma as described in section 2.2.4. The various lead species separated were expressed as a percentage of the amount of lead in the original incubation.

4.2.2 Results

4.2.2.1 Comparison of the lead species prepared by the *in vitro* incubation of gut washing supernatants from fed and fasted rats

Incubation of the supernatant phase of gut washings with lead *in vitro* produced an insoluble lead species in both fed and fasted rats, a substantially greater amount being formed in the washings from fed rats than in the gut washings from fasted animals (table 4.2.1). After centrifugation and separation of the remaining lead species on the gel column, the amount of lead associated with each peak was calculated, the initial peak ("Pb-X") comprising fractions 8-10, and the second peak ("Pb-Y") fractions 17-22. The amount of "Pb-X" and "Pb-Y" produced in fasted animals was greater than that produced in fed animals (table 4.2.1). Lead species eluted between "Pb-X" and "Pb-Y" and those eluted after "Pb-Y" were termed "undefined species" and were produced to a greater extent in fasted rats compared to fed rats. The fact that these preliminary experiments were not repeated meant that it was not possible to determine whether any of these differences were statistically significant.

4.2.2.2 A comparison of the *in vivo* and *in vitro* preparation of lead species

The elution profiles obtained following chromatographic separation of the lead species prepared *in vitro* (this study) were compared to those obtained by Partridge (1986) after *in vivo* preparation (figures 4.2.1 and 4.2.2). Comparison of the chromatographs obtained for fed rats (figure 4.2.1) revealed one important difference; although the large second peak ("Pb-Y") was observed in the chromatographs from both studies, the *in vitro* incubation also produced the initial "Pb-X" peak which was not found after *in vivo* preparation i.e. after oral dosing of lead. Oral dosing of lead also failed to produce the undefined lead species which were eluted prior to "Pb-Y" during the *in vitro* incubation, although the elution of lead in fractions subsequent to "Pb-Y" was

Table 4.2.1 Species formed when the supernatant phase of gut washings from either fed or fasted rats is incubated *in vitro* with $1x10^{-6}$M lead acetate.

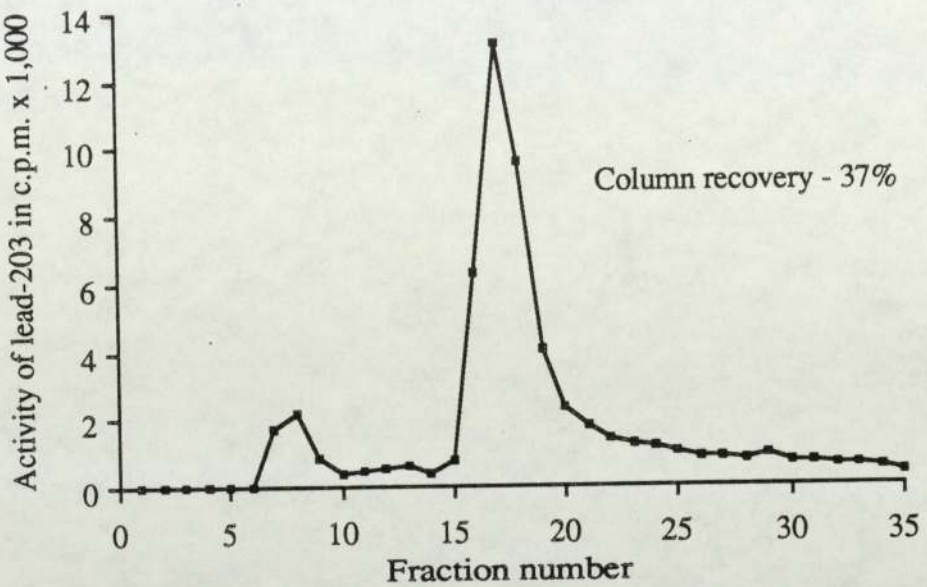
	Species formed	% lead as individual species	
		Fed rat	Fasted rat
	"incubation precipitate"	55.0	1.3
#	"Pb-X"	1.0	1.6
#	"Pb-Y"	8.0	34.8
#	"undefined species"	3.2	11.7
	% column recovery	37	50

Species separated by the gel filtration chromatography system described in section 4.2.1.3 (see figures 4.2.1 and 4.2.2).

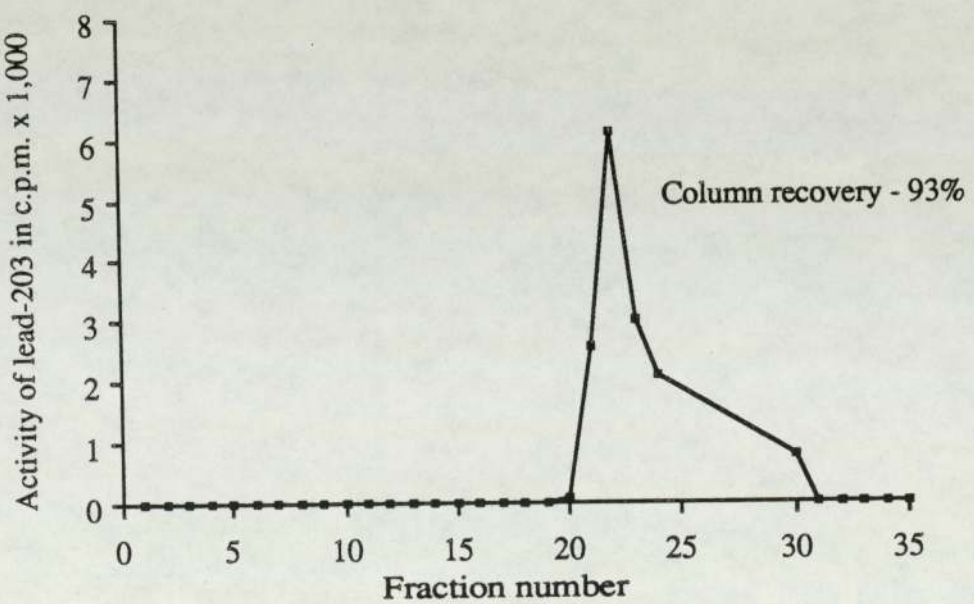
It is assumed that "Pb-X" is eluted over fractions 8-10, and "Pb-Y" is eluted over fractions 17-22.

Figure 4.2.1 The elution profiles obtained following chromatographic separation of the lead species formed in the gut washing supernatants of the fed rat *in vitro* (this study) and *in vivo* (Partridge, 1986).

In vitro (this study)



In vivo (Partridge, 1986).

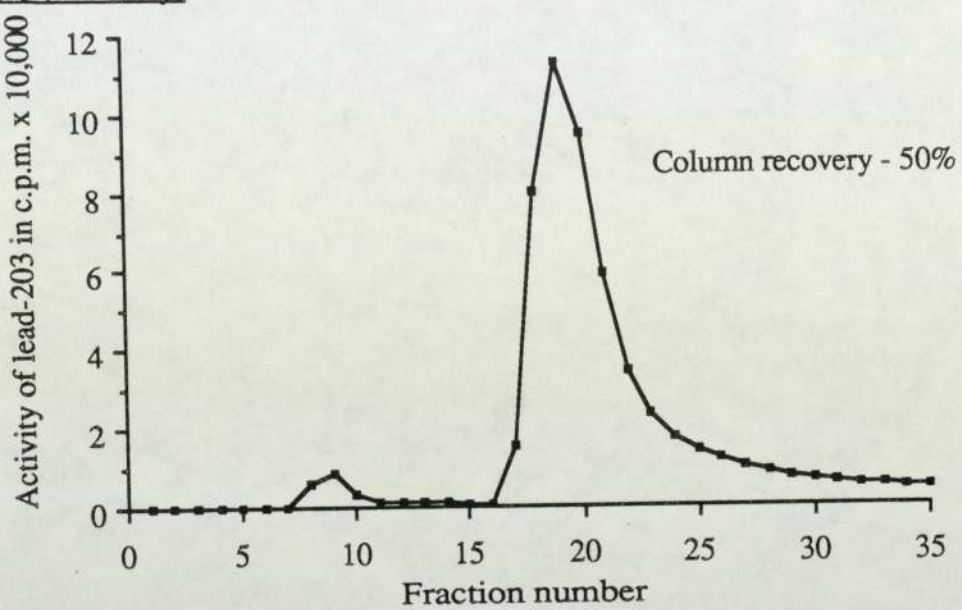


Gel	-	Sephadex G-15
Eluent	-	$1x10^{-2}$M lead acetate (pH 3.8)
Flow rate	-	3mls/minute
Fraction volume	-	6mls

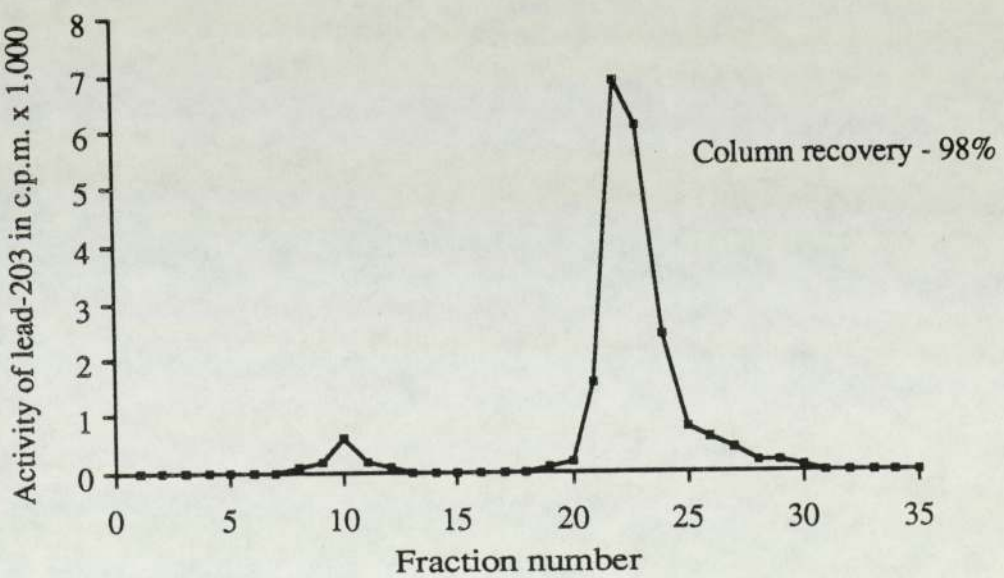
See section 4.2.1.3 for detailed column specifications.

Figure 4.2.2 The elution profiles obtained following chromatographic separation of the lead species formed in the gut washing supernatants of the fasted rat *in vitro* (this study) and *in vivo* (Partridge, 1986).

In vitro (this study)



In vivo (Partridge, 1986).



Gel	-	Sephadex G-15
Eluent	-	$1x10^{-2}$M lead acetate (pH 3.8)
Flow rate	-	3mls/minute
Fraction volume	-	6mls

See section 4.2.1.3 for detailed column specifications.

common to both *in vitro* and *in vivo* preparations. The chromatographs obtained from the fasted animal (figure 4.2.2) was similar after *in vivo* and *in vitro* preparation. Both showed large peaks at the "Pb-Y" position, with a smaller peak at the "Pb-X" position. However, both peaks "Pb-X" and "Pb-Y" produced *in vitro* were larger than those produced *in vivo*. No "undefined species" were eluted in the fractions between the "Pb-X" and "Pb-Y" peaks *in vivo* but were present *in vitro*. Undefined lead species were eluted after the "Pb-Y" peak in both preparations.

4.2.3 Discussion

4.2.3.1 Insoluble lead species

The insoluble lead species formed during the *in vitro* incubation (table 4.2.1) may be insoluble lead salts such as lead carbonate, lead phosphates or perhaps insoluble lead-protein complexes, the ligands being derived from the gut wall in fasted animals, and the gut wall and diet in fed animals. It is possible that these species are formed in the gut lumen after the oral administration of lead outlined by Partridge (1986), and removed from the gut washings with other particulate matter during centrifugation prior to loading the supernatant on the gel column. As a result of the formation of this incubation precipitate, there is less soluble lead available for complexing with soluble ligands in the fed compared to fasted rat.

4.2.3.2 Comparison of the elution profiles obtained on separation of gut washing supernatants from fed and fasted rats following incubation with lead *in vitro*

The elution profiles obtained following incubation of gut washing supernatants from fed and fasted rats with lead *in vitro* contained peaks "Pb-X" and "Pb-Y" and "undefined species" (figures 4.2.1 and 4.2.2), suggesting that the same species may be formed regardless of dietary status. The area of the gastrointestinal tract in which these species are formed (table 4.2.2) may give some idea as to where their ligands originate. It appears that in fasted rats, the ligands in "Pb-X" and the "undefined species" are derived from the gut wall as neither species is formed in the stomach. However, in fed rats "Pb-X" and "undefined species" are formed in both the stomach and intestine, suggesting that the ligands may be derived from gut wall and diet. These

Table 4.2.2 The formation of "Pb-X", "Pb-Y" and undefined lead species in the soluble phase of gut washings from the stomach and small intestine following *in vitro* incubation with lead.

	Fed rat		Fasted rat	
	Stomach*	Intestine	Stomach*	Intestine
"Pb-X"	+	+	-	+
"Pb-Y"	+	+	+	+
"undefined species"	+	+	-	+

\+ indicates species are formed

\- indicates species are not formed

* indicates unpublished observation

preliminary studies give no further insight into the origin or identity of the "Pb-Y" species.

4.2.3.3 Comparison of the elution profiles obtained on separation of gut washing supernatants from fed and fasted rats after either an oral lead dose i.e. *in vivo* (Partridge,1986) or incubation with lead *in vitro*

A comparison of the chromatographs produced from supernatants of gut washings obtained following lead dosing i.e. *in vivo* (Partridge, 1986) and those incubated *in vitro* show a number of similarities. In fed rats, although *in vitro* incubation of gut washing supernatants with lead showed two lead peaks at positions "Pb-X" and "Pb-Y" (figure 4.2.1), oral dosing of lead did not produce the initial "Pb-X" peak. This may be due to a dilution effect caused by the difference in the amount of saline used to wash out the intestine (20mls in the study by Partridge (1986), compared to 11mls in this study) which would either cause "Pb-X" to dissociate if the lead-ligand complex was weak, or may dilute "Pb-X" to such a degree that it is not present in sufficient quantity to be detected. Alternatively, lead in the lumen of the fed rat may bind preferentially to particulate matter from the diet, resulting in a lack of free ionic lead with which the "X" ligand can complex. In such situations the "Pb-Y" peak would be comprised solely of lead complexes and contain no free ionic lead. Similar explanations may account for the reduction in the "undefined species" in the elution profiles after separation of gut washing supernatants from fed and fasted rats following dosing. In fasted rats, both "Pb-X" and "Pb-Y" species are formed *in vivo* and *in vitro* (figure 4.2.2) although larger amounts are formed *in vitro* possibly reflecting the increased amount of lead available to complex with the soluble ligands rather than bind with the gut wall.

The percentage of lead recovered when lead standards were eluted from the columns, both in this study (figure 4.1.1) and in that by Partridge (1986), was high (93% and 92% respectivly) suggesting that the amount of lead bound to the gel is negligible. However, the recovery of lead from the columns on which gut washing supernatants were run in this study was low when compared to the elution of the lead standard (figure 4.1.1) and the gut washing preparations *in vivo* (Partridge, 1986) (figures 4.2.1 and 4.2.2). This may be due to the formation of more lead-ligand complexes *in vitro* which

either bind to the gel or are insoluble in the acetic acid used to lower the buffer pH (such as lead hydroxide and lead orthophosphate (Greninger *et al.*, 1975)), and hence precipitate out on the column rather than being eluted. Indeed, the slight cloudiness in the initial fractions of the column may be the result of some of the insoluble species, formed on loading the sample, passing through the gel beads and being eluted.

This initial study demonstrates that the incubation of lead and gut washing supernatants *in vitro* is a viable alternative to dosing lead orally, when studying the formation of soluble lead species, a greater quantity of "Pb-X" (the complex under investigation) and "Pb-Y" being produced. Consequently, the *in vitro* incubation technique will be used in subsequent investigations of lead speciation.

4.3 Investigation into the nature of the lead species separated by gel chromatography, with particular reference to the "Pb-X" peak

4.3.1 Introduction

As demonstrated by the chromatographs produced by Partridge (1986) and those in this study (figures 4.2.1 and 4.2.2), the species "Pb-X" is always eluted in the void volume. Elution at this point indicates that the complex has a molecular weight in excess of 1,500 and it is therefore likely that the initial peak is either a single lead species, or a number of unresolved species formed from the complexing of lead with ligands from the gut wall in the fasted rat and both the gut wall and diet in the fed rat. This, together with the fact that large amounts of protein are found in the gut lumen, derived from both the diet and the gut wall whose thiol groups would strongly complex with lead, suggest that "Pb-X" may be a lead-protein complex(es). The *in vitro* incubation of gut washings and lead, described in section 4.2.1.4, was used to study the effect of heat on "Pb-X" to determine how strongly the lead and ligand are complexed. Any effect of heat on both the "Pb-Y" peak and the undefined lead species was also noted. The presence of protein, peptide chains and amino acids in the samples eluted from the column was determined by the method of Lowry *et al.* 1951 (appendix A.3.1). The effect of the removal of protein from the gut washings by precipitation with trichloroacetic acid (T.C.A.) following incubation with lead, on "Pb-X" and "Pb-Y" was also studied. Where appropriate, differences between the mean values for test and control samples were tested for significance using Student's t-test.

4.3.2 Materials and methods

4.3.2.1 Chemicals

All chemicals required for this investigation were supplied as described in sections 2.2.1 and 2.2.2.

4.3.2.2 Animals

As section 3.2.3

4.3.2.3 The effect of heat on the chromatographic profile of the gut washing supernatants from fed and fasted rats

Centrifuged gut washings from fed and fasted rats were incubated with $1x10^{-6}$M lead acetate labelled with lead-203 as detailed in section 4.2.1.4. Following incubation and centrifugation, the gut washings were divided into two 5ml samples. One sample was analysed by gel filtration chromatography (section 4.2.1.3). The second sample was heated to 100°C for two minutes in a boiling water bath, centrifuged as previously and analysed on a second identical gel chromatography system. Cloudiness in any of the eluted samples was removed by centrifugation (see section 4.2.1.4). The lead in the column fractions was determined by counting the gamma emissions of lead-203 (section 2.2.4). The effect of heating the gut washings from fasted rats for ten minutes following incubation with lead was also studied. The fractions eluted from the gel columns were centrifuged if cloudy and assayed for both lead (section 2.2.4) and peptides, amino acids and protein (appendix A.3.1).

4.3.2.4 The effect of T.C.A. on the chromatographic profile of the gut washing supernatant from a fasted rat

Centrifuged gut washings from a fasted rat were incubated with $1x10^{-6}$M lead acetate labelled with lead-203 as described in section 4.2.1.4. Following incubation and centrifugation, the gut washings were divided into two 5ml samples. To one sample was added 1ml of distilled water, prior to analysis by gel filtration chromatography (section 4.2.1.3). To the second sample was added 1ml of 20% T.C.A., the precipitate formed removed by centrifugation (100,000 x g for 1 hour) and the supernatant analysed

on a second identical gel chromatography system. Cloudy fractions were cleared by centrifugation (section 4.2.1.4), and assayed for lead (section 2.2.4) and peptides, amino acids and protein (appendix A.3.1).

4.3.2.5 Determination of the protein profile obtained when gut washing supernatants from fed and fasted rats are eluted on the gel chromatography column before and after heating for two minutes

Gut washing supernatants from fed and fasted rats were incubated with $1x10^{-6}$M lead acetate, but without the addition of the lead-203 tracer. The incubation, heating and assay procedures were identical to those described in section 4.3.2.3.

4.3.3 Results

4.3.3.1 Lead profiles obtained when gut washing supernatants from fed and fasted rats are eluted on Sephadex G-15 before and after heating

The criterion for determining the amount of the various lead species was the same as previously (sections 4.2.1.4 and 4.2.2.1). The results for the control samples in this study (table 4.3.1) correspond closely to the values obtained in the preliminary study (table 4.2.1).

A significantly greater amount of incubation precipitate was formed during incubation of fed compared to fasted gut washing supernatants (table 4.3.1). The amount of "Pb-X" formed by fasted rats was not significantly different from that formed by fed rats, although significantly more "Pb-Y" was found in fasted compared to fed rats. Significantly more "undefined species" were also found in chromatographs from fasted rats compared to those from fed rats. Samples from fed and fasted animals produced similar column recoveries.

Heating produced a precipitate in gut washings from both fed and fasted rats, the amount of precipitate formed being independent of the dietary status of the animal prior to sacrifice (tables 4.3.2 and 4.3.3). Heating also altered the lead chromatographs from both fed and fasted animals (figures 4.3.1 and 4.3.3). In fed rats, heating caused complete removal of "Pb-X" and a significant fall in the amount of both "Pb-Y" and the

Table 4.3.1 Species formed when the supernatant phase of gut washings from either fed or fasted rats is incubated *in vitro* with $1x10^{-6}$M lead acetate.

Species formed	% lead as individual species		
	Fed rat	Fasted rat	
"incubation precipitate"	64.7 ± 8.8	7.0 ± 1.6	**
# "Pb-X"	0.4 ± 0.2	0.8 ± 0.5	
# "Pb-Y"	9.7 ± 2.0	39.2 ± 2.1	**
# "undefined species"	3.3 ± 1.0	10.7 ± 0.5	**
% column recovery	49 ± 3	57 ± 2	

Species separated by the gel filtration chromatography system described in section 4.2.1.3 (see figures 4.3.1 and 4.3.3).

Results are expressed as means ± standard error of means for four animals. It is assumed that "Pb-X" is eluted over fractions 8-10 and "Pb-Y" is eluted over fractions 17-22.

** Denotes a difference between the two experimental groups ($p<0.001$).

Table 4.3.2 The effect of heating for two minutes on the species formed in the gut washing supernatant of the fed rat after *in vitro* incubation with $1x10^{-6}$M lead acetate.

Species formed	% lead as individual species	
	Before heating	After heating†
"incubation precipitate"	64.7 ± 8.8	65.2 ± 9.6
"heating precipitate"	-	23.7 ± 7.3
# "Pb-X"	0.4 ± 0.2	0.0 ± 0.0
# "Pb-Y"	9.7 ± 2.0	1.6 ± 0.3 **
# "undefined species"	3.3 ± 1.0	0.4 ± 0.1 *
% column recovery	49 ± 3	61 ± 3 *

Species separated by the gel filtration chromatography system described in section 4.2.1.3 (see figure 4.3.1).

† The incubation precipitate, formed during the two hour incubation period, was removed from the sample prior to heating.

Results are expressed as means ± standard error of means for four animals. It is assumed that "Pb-X" is eluted over fractions 8-10 and "Pb-Y" is eluted over fractions 17-22.

* Denotes a difference between the two experimental groups ($p<0.05$).

** Denotes a difference between the two experimental groups ($p<0.01$).

Table 4.3.3 The effect of heating for two minutes on the species formed in the gut washing supernatant of the fasted rat after *in vitro* incubation with $1x10^{-6}$M lead acetate.

Species formed	% lead as individual species	
	Before heating	After heating†
"incubation precipitate"	7.0 ± 1.6	6.8 ± 1.6
"heating precipitate"	-	43.5 ± 6.6
# "Pb-X"	0.8 ± 0.5	1.2 ± 0.4
# "Pb-Y"	39.2 ± 2.1	16.2 ± 2.1 **
# "undefined species"	10.7 ± 0.5	4.1 ± 0.9 **
% column recovery	57 ± 2	64 ± 6

Species separated by the gel filtration chromatography system described in section 4.2.1.3 (see figure 4.3.3).

† The incubation precipitate, formed during the two hour incubation period, was removed from the sample prior to heating.

Results are expressed as means ± standard error of means for four animals. It is assumed that "Pb-X" is eluted over fractions 8-10 and "Pb-Y" is eluted over fractions 17-22.

** Denotes a difference between the two experimental groups ($p<0.001$).

Table 4.3.4 The effect of heating for ten minutes on the species formed in the gut washing supernatant of the fasted rat after *in vitro* incubation with 1×10^{-6}M lead acetate.

	Species formed	% lead as individual species	
		Before heating	After heating†
	"incubation precipitate"	10.00	16.7
	"heating precipitate"	-	54.7
#	"Pb-X"	0.6	0.3
#	"Pb-Y"	40.4	6.7
#	"undefined species"	10.8	1.8
	% column recovery	60	54

Species separated by the gel filtration chromatography system described in section 4.2.1.3.

† The incubation precipitate, formed during the two hour incubation period, was removed from the sample prior to heating.

It is assumed that "Pb-X" is eluted over fractions 8-10 and "Pb-Y" is eluted over fractions 17-22.

unidentified lead species (table 4.3.2). Column recoveries were significantly improved on heating (table 4.3.2; figure 4.3.1). In fasted rats, there was no significant change in "Pb-X", however, there was a significant fall in the amount of "Pb-Y" and a similar significant fall in the amount of "undefined species" (table 4.3.3). Column recoveries were not significantly altered with heating (table 4.3.3; figure 4.3.3).

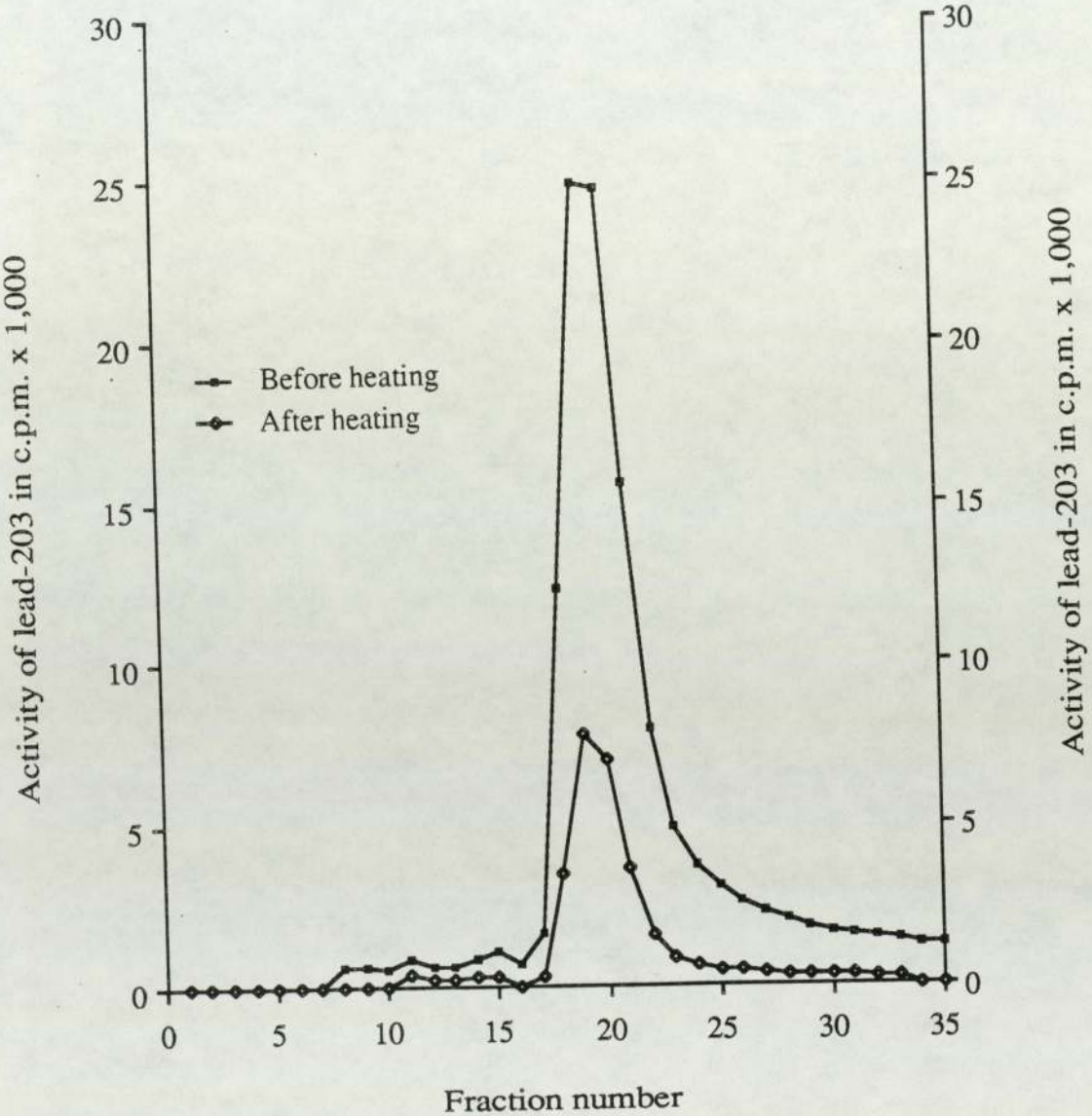
Gut washing supernatants from fasted rats were heated for ten minutes following incubation to determine whether a longer heating period would remove the "Pb-X" peak. Despite such heating, there was no change in "Pb-X" although the amount of "Pb-Y" and the "undefined species" was reduced as it had been after two minutes (table 4.3.4). The recovery of lead from the test column was similar to that from the control column (table 4.3.4).

4.3.3.2 Protein profiles obtained when gut washing supernatants from fed and fasted rats are eluted on a gel chromatography column before and after heating

The chromatographs revealed that the gut washings from both fed and fasted rats contained protein, which was eluted in the void volume, and also smaller peptide chains or amino acids with a molecular weight less than 1,500, which were eluted in later fractions (figures 4.3.2 and 4.3.4). More protein and peptides or amino acids were detected in the column fractions following elution of gut washing supernatants from the fed rat (figure 4.3.2) compared to the corresponding fractions obtained from the fasted rat (figure 4.3.4), both before and after heating. Nevertheless, the position of the peaks from fed and fasted animals, both before and after heating, corresponded well, being eluted at fractions 9, 12, 14 and 20 in all but one case. Chromatographs from both fed and fasted rats showed the largest amount of protein to be eluted in fraction 9.

Heating the gut washings from the fed rat for two minutes did not appear to affect the protein at fraction 9/10 although the amount of peptides or amino acids in the latter peaks was reduced by half (figure 4.3.2). In contrast, heating the gut washing supernatant from the fasted animal for two minutes appeared to reduce the amount of protein eluted in fractions 9/10 to an appreciable extent, but have little effect on the later protein profile (figure 4.3.4). Heating the gut washing supernatant from the fasted rat for ten minutes appeared not to affect either the position or the height of the protein, the

Figure 4.3.1 The effect of heating for two minutes on the elution profile obtained following chromatographic speciation of the lead species formed in the gut supernatants of the fed rat *in vitro*.



Column recovery (before heating) - 46%

Column recovery (after heating) - 63%

Gel - Sephadex G-15

Eluent - 1×10^{-2}M lead acetate (pH 3.8)

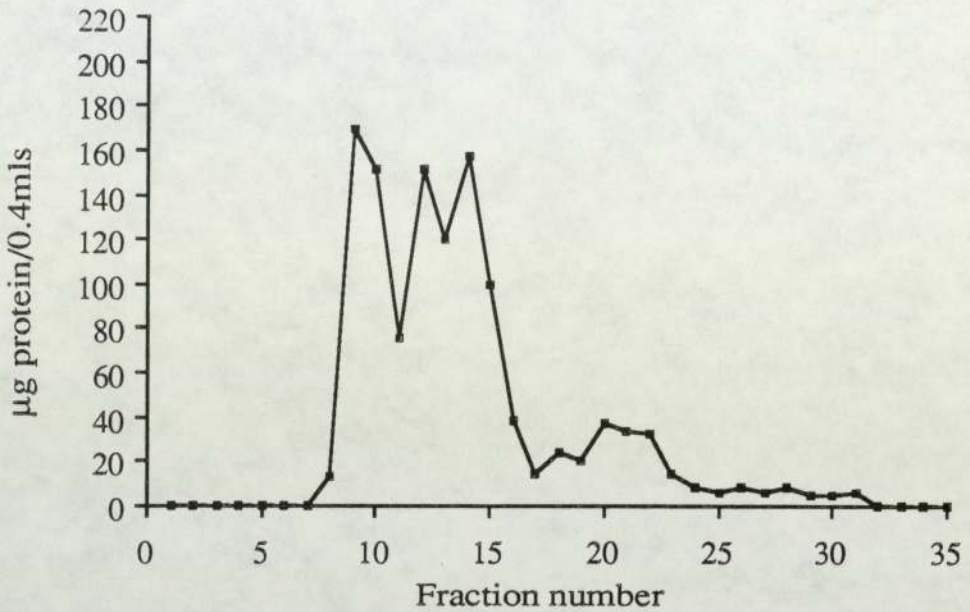
Flow rate - 3mls/minute

Fraction volume - 6mls

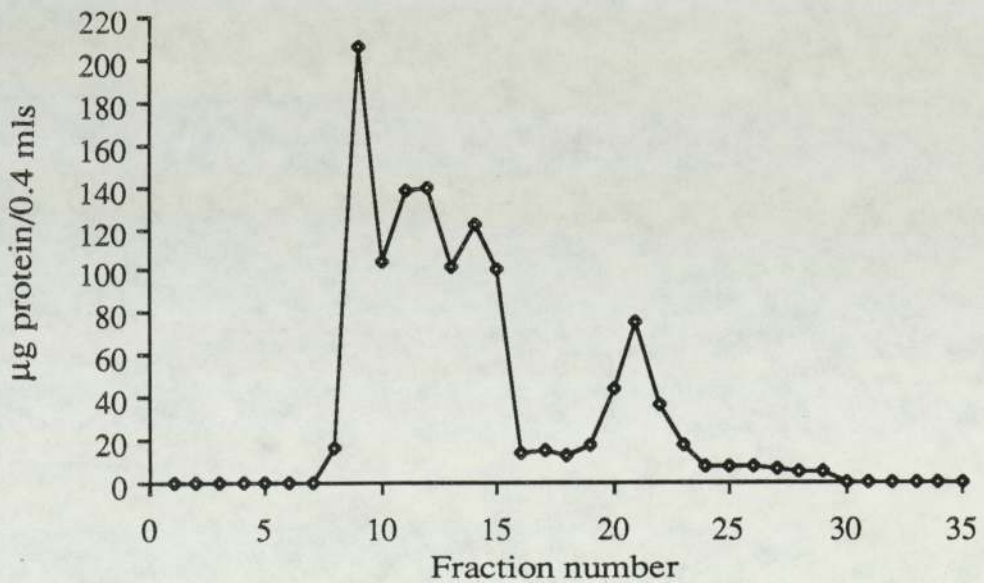
See section 4.2.1.3 for detailed column specifications.

Figure 4.3.2 The effect of heating for two minutes on the elution of protein, peptides and amino acids from the gut washing supernatant of the fed rat.

Before heating



After heating



Gel - Sephadex G-15

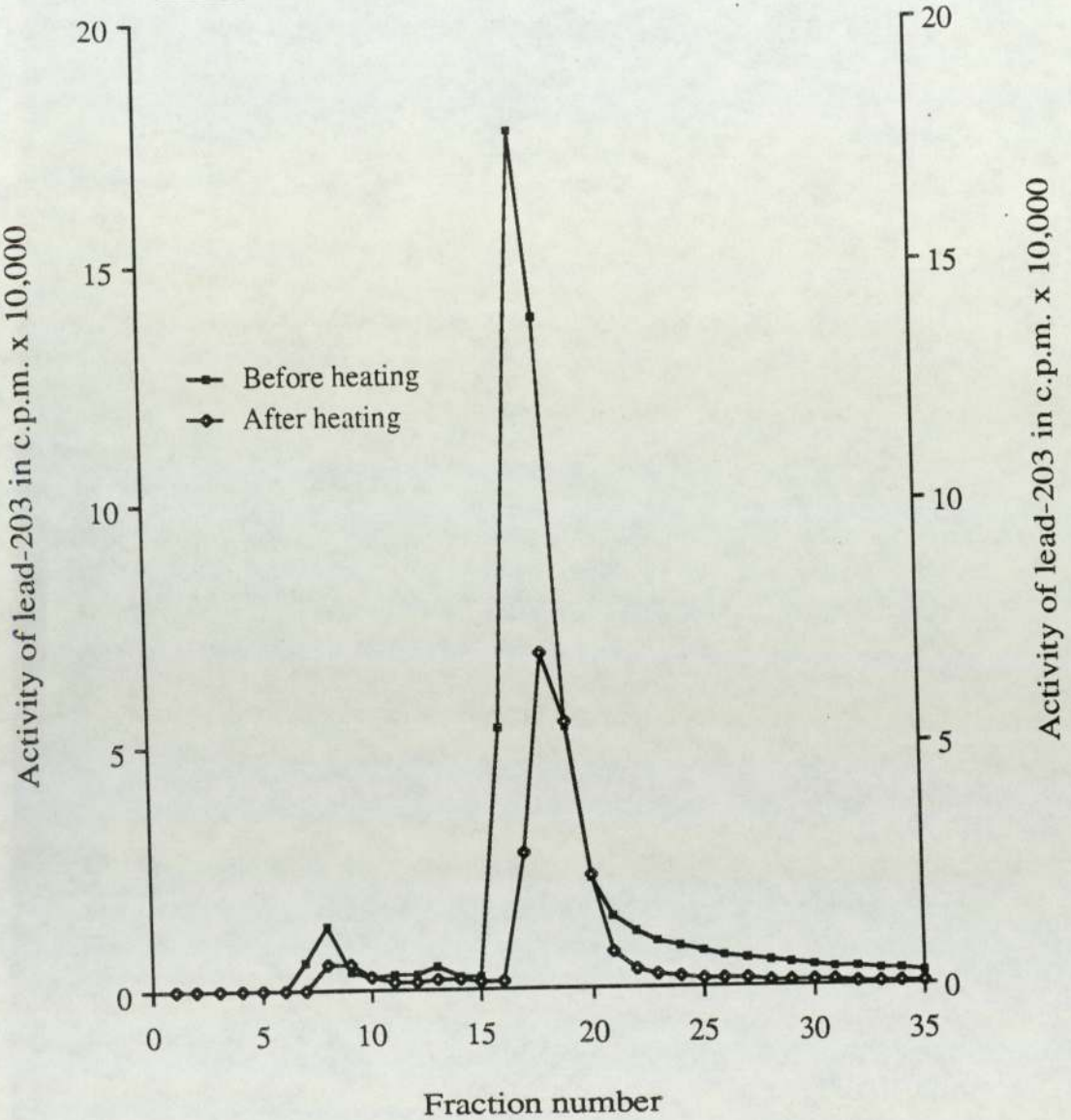
Eluent - $1x10^{-2}$M lead acetate (pH 3.8)

Flow rate - 3mls/minute

Fraction volume - 6mls

See section 4.2.1.3 for detailed column specifications.

Figure 4.3.3 The effect of heating for two minutes on the elution profile obtained following chromatographic speciation of the lead species formed in the gut supernatants of the fasted rat *in vitro*.



Column recovery (before heating) - 62%

Column recovery (after heating) - 65%

Gel - Sephadex G-15

Eluent - $1x10^{-2}$M lead acetate (pH 3.8)

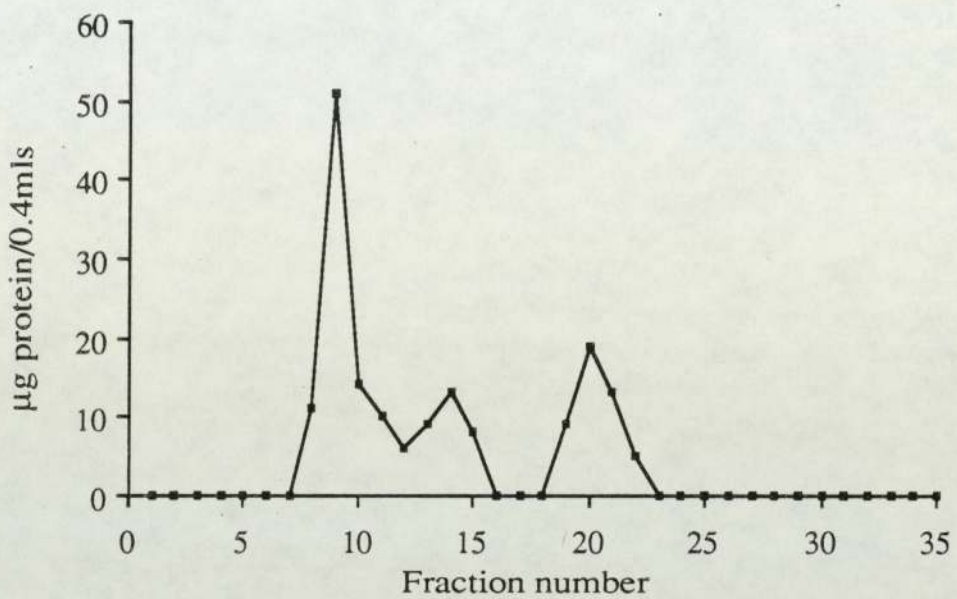
Flow rate - 3mls/minute

Fraction volume - 6mls

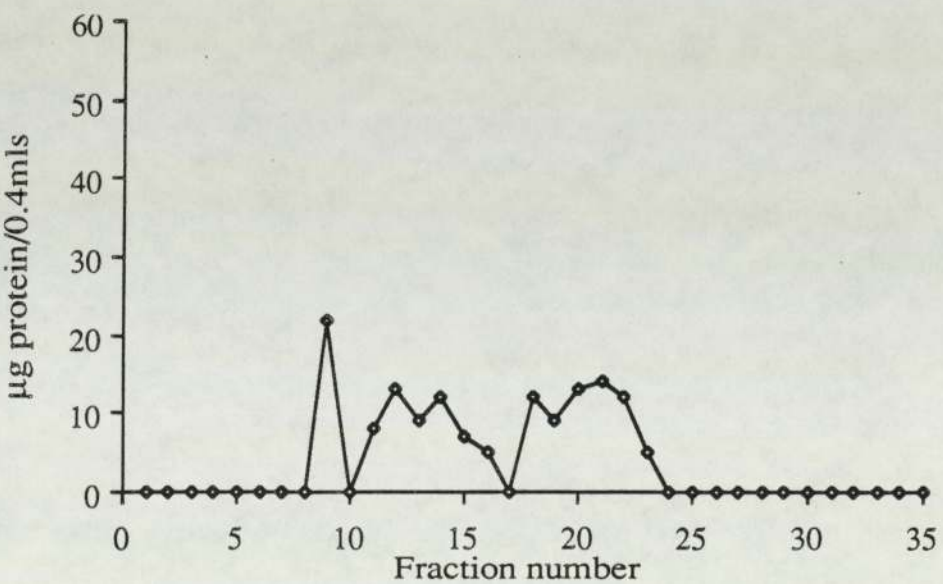
See section 4.2.1.3 for detailed column specifications.

Figure 4.3.4 The effect of heating for two minutes on the elution of protein, peptides and amino acids from the gut washing supernatant of the fasted rat.

Before heating



After heating



Gel - Sephadex G-15

Eluent - $1x10^{-2}$M lead acetate (pH 3.8)

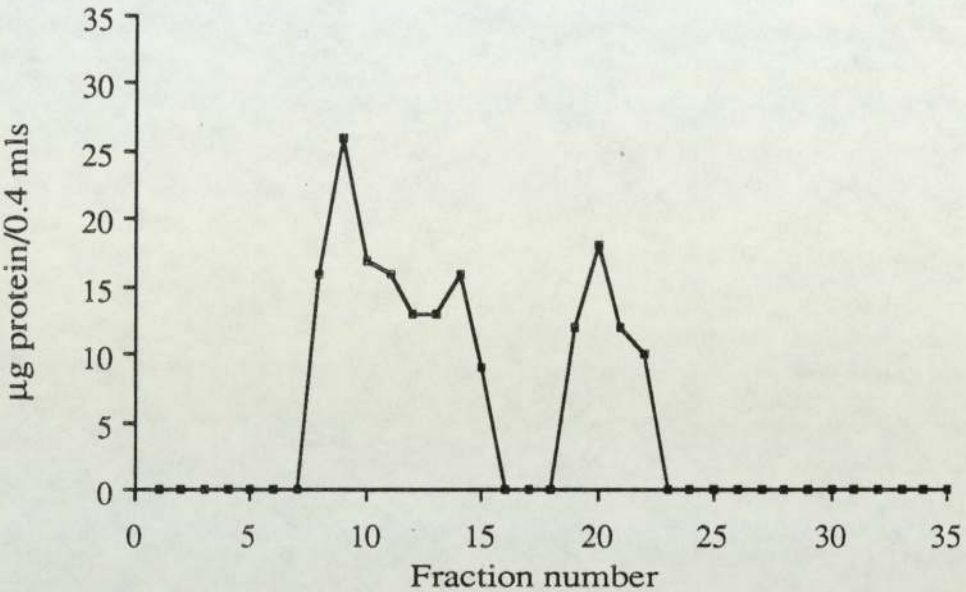
Flow rate - 3mls/minute

Fraction volume - 6mls

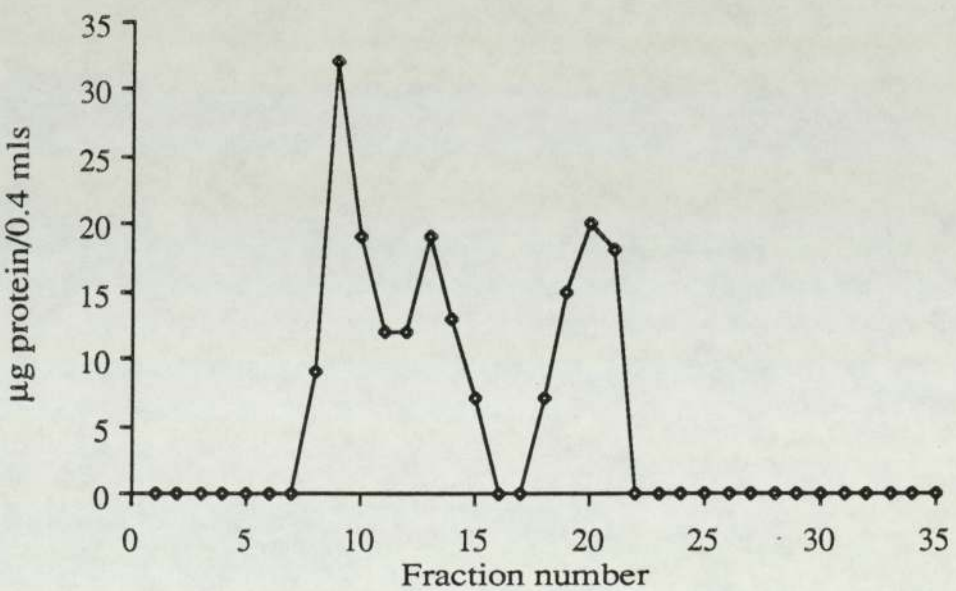
See section 4.2.1.3 for detailed column specifications.

Figure 4.3.5 The effect of heating for ten minutes on the elution of protein, peptides and amino acids from the gut washing supernatant of the fed rat.

Before heating



After heating



Gel - Sephadex G-15

Eluent - $1x10^{-2}$M lead acetate (pH 3.8)

Flow rate - 3mls/minute

Fraction volume - 6mls

See section 4.2.1.3 for detailed column specifications.

peptide or the amino acid peaks to any great extent (figure 4.3.5).

4.3.3.3 The lead and protein profile obtained before and after addition of T.C.A. to the gut washing supernatant from a fasted rat

The addition of 20% T.C.A. caused the formation of a lead-containing precipitate. Subsequent analysis of the test supernatant by gel chromatography showed a six fold reduction in the amount of "Pb-X" compared to the control sample, and an almost three fold increase in the amount of "Pb-Y" (table 4.3.5). The amount of undefined lead species eluted after T.C.A. treatment was substantially lower than the control. The recovery of lead from the test column was over twice that of the control column (98% compared to 47% (table 4.3.5)).

Addition of T.C.A. to the gut washings reduced the amount of protein in the void volume by a third, but appeared to increase the amount of peptides or amino acids eluted in the latter peak (figure 4.3.6).

4.3.4 Discussion

4.3.4.1 Statistical comparison of the lead species formed following *in vitro* incubation of lead with gut washing supernatants from fed and fasted rats

As in the preliminary study, more insoluble lead species were formed during the incubation of gut washing supernatants from fed animals compared to fasted, this difference being statistically significant (table 4.3.1). It is likely that this is due to the presence of more ligands in the gut lumen of the fed rat compared to the fasted rat, derived from food and increased gut wall secretions with which lead can complex to form insoluble species.

There was no significant difference between the amounts of "Pb-X" formed in the fed and fasted rat (table 4.3.1) and it seems likely, therefore, that if the ligands in "Pb-X" in fasted rats are derived from the gut wall and those in fed rats are derived from both diet and gut wall (as suggested in section 4.2.3.2) and yet the amount of "Pb-X" is produced to the same extent in both, feeding must either reduce the amount of ligands derived from the gut wall, possibly by reducing the amount secreted, or cause the ligands to form soluble complexes with other ions from the diet in preference to lead. The larger

Table 4.3.5 The effect of the addition of 20% trichloroacetic acid (T.C.A.) on the species formed in the gut washing supernatant of the fasted rat following *in vitro* incubation with $1x10^{-6}$M lead acetate.

	Species formed	% lead as individual species	
		Before T.C.A.	After T.C.A.†
	"incubation precipitate"	27.28	27.15
	"T.C.A. precipitate"	-	0.22
#	"Pb-X"	0.18	0.03
#	"Pb-Y"	22.18	61.72
#	"undefined species"	5.41	0.03
	% column recovery	47	98

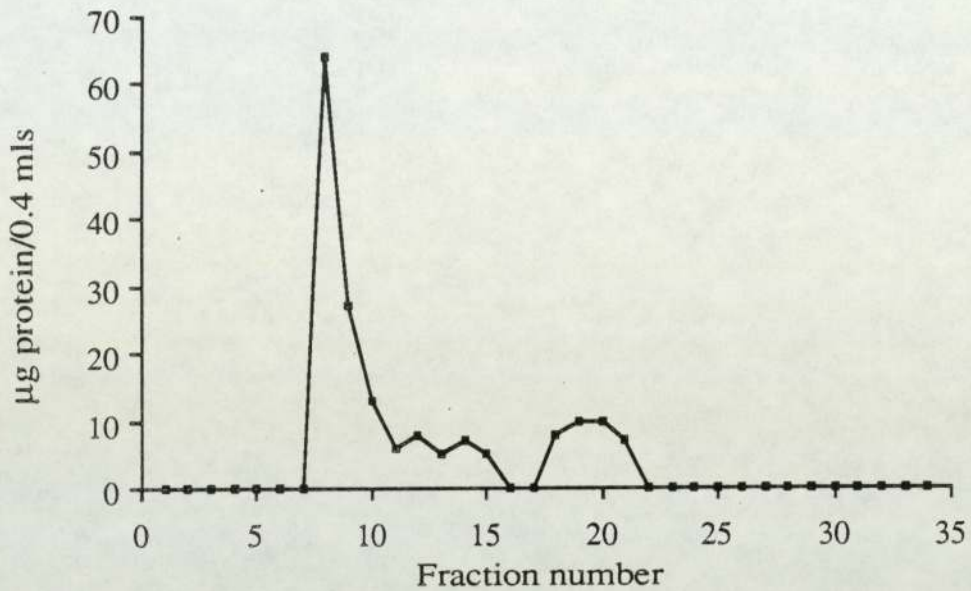
Species separated by the gel filtration chromatography system described in section 4.2.1.3.

† The incubation precipitate, formed during the two hour incubation period, was removed from the sample prior to the addition of T.C.A..

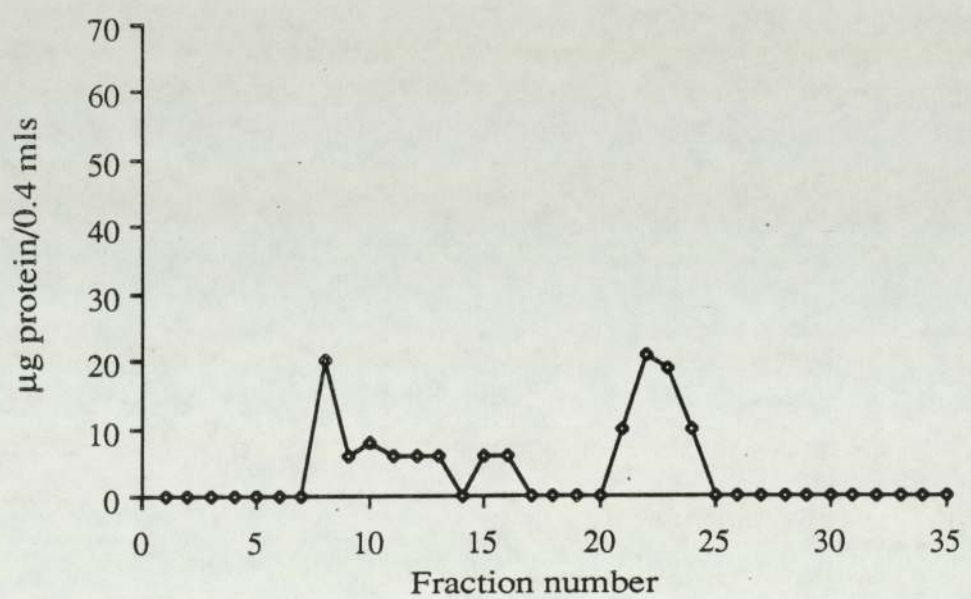
It is assumed that "Pb-X" is eluted over fractions 8-10 and "Pb-Y" is eluted over fractions 17-22.

Figure 4.3.6 The effect of the addition of 20% trichloroacetic acid on the elution of protein, peptides and amino acids from the gut washing supernatant of the fed rat.

Before addition of T.C.A.



After addition of T.C.A.



Gel - Sephadex G-15

Eluent - $1x10^{-2}$M lead acetate (pH 3.8)

Flow rate - 3mls/minute

Fraction volume - 6mls

See section 4.2.1.3 for detailed column specifications.

amount of "Pb-Y" produced in fasted compared to fed rats in the preliminary study (table 4.2.1), a difference shown to be significant by this study (table 4.3.1), may reflect an increase in the amount of lead as either inorganic ion pair species or as the free hydrated lead cation. The effect of ligand competition or availability may also account for the significant reduction in the number of undefined lead species found in the fed rat compared to the fasted rat (table 4.3.1).

4.3.4.2 The elution of proteins from the Sephadex G-15 column following separation of gut washing supernatants from fed and fasted rats

Large amounts of protein were eluted in fraction 9 as was expected, their molecular weight being in excess of 1,500 and it is therefore suggested that, as the lead and protein profiles co-chromatograph, peak "Pb-X" from both fed and fasted rats is a lead-protein complex. However, the protein assay also gave a positive result in later fractions including fractions 18/19 (the exclusion volume) (figures 4.3.2 and 4.3.4) suggesting that the undefined lead species eluted between peaks "Pb-X" and "Pb-Y" may be lead-peptide complexes and "Pb-Y" may be comprised not only of lead as inorganic ion pairs and the free hydrated lead cation, but also lead-amino acid complexes, such as lead-histidine as suggested by Partridge (1986). A positive result for the protein assay was also obtained subsequent to "Pb-Y" in fed rats (figure 4.3.2), possibly indicating the presence of lead-protein complexes whose movement through the column is slowed by their interaction with the gel. There is no positive reaction with the protein assay in the fractions subsequent to "Pb-Y" in fasted rats (figure 4.3.4). The lead in these fractions may therefore be either non-protein/amino acid lead species, or lead species which initially bind to the Sephadex gel but which are subsequently eluted as a result of the washing effect of the eluent on the gel, or the exchange of radioactive lead bound to the gel with non-radioactive lead from the eluent.

4.3.4.3 The effect of heating on the lead and protein profiles obtained from Sephadex G-15

The lack of any conclusive effect of heat on the protein profiles from fed and fasted rats (section 4.3.3.2) suggests that the large statistically significant changes in the lead profiles observed, are a result of changes in inorganic lead chemistry. Heating for

two and ten minutes did not cause dissociation of the "Pb-X" peak in fasted animals (figure 4.3.3; tables 4.3.3 and 4.3.4) suggesting the complexes are strongly associated, unlike those in the fed rat, which dissociate after two minutes heating (figure 4.3.1; table 4.3.2), the protein being eluted in the void volume, and the dissociated lead possibly in the exclusion volume with other free hydrated lead cations. The amount of "Pb-Y" in the gut washing supernatants of fed and fasted rats is significantly reduced after two minutes heating (tables 4.3.2 and 4.3.3). This reduction may be due to the removal of the soluble lead carbonate ion pair thought to exist in large quantities in the gut. Carbonate ions present in large quantities in the gut lumen, derived mainly from intestinal secretions (Keele and Neil, 1975), bind readily with lead, forming stable complexes as demonstrated by the large formation constant for the lead carbonate ion pair (Jackson and Sheiham, 1980). On heating to 100°C soluble lead carbonate is converted to basic lead carbonate which is insoluble in both hot and cold water (Greninger *et al.*, 1975) and would account for the formation of the heating precipitate (tables 4.3.2, 4.3.3 and 4.3.4). However, the percentage of precipitate formed can not be accounted for simply by the fall in the percentage of "Pb-Y" and it appears that other species not eluted in "Pb-Y" are precipitated on heating. One such species may be lead orthophosphate which is thought to be precipitated on the column due to its insolubility in acetic acid. Lead orthophosphate is also insoluble in hot water and, on heating, its removal as heating precipitate and the subsequent reduction in the amount of phosphate species loaded on to the colunmn, may result in the increase in recovery seen on heating, an effect significant in the fed state. Similarly, lead hydroxide, another lead species thought to be precipitated out on the gel column, is known to react with carbonates to form insoluble basic lead carbonate (Greninger *et al.*, 1975). It is possible, therefore, that heating gut washing supernatants catalyses this reaction, removing lead hydroxide from solution resulting in increased column recoveries.

4.3.4.4 The effect of the addition of T.C.A. to gut washing supernatants from the fasted rat on the lead and protein profiles

Addition of 20% T.C.A. to the gut washing supernatant from the fasted rat caused approximately 67% of the protein originally present in fractions 9/10 to be precipitated out of solution (figure 4.3.6) and a reduction in the amount of "Pb-X" of

83% (table 4.3.5). It is possible that the addition of more T.C.A. would have removed both the lead and protein peaks completely, although alternatively, the lead may be bound to a protein which is neither precipitated nor loses its metal ions when treated with T.C.A.. It appears that reduction in "Pb-X" by the T.C.A. is accounted for by the resulting precipitate (table 4.3.5), suggesting that little if any dissociation of lead and protein took place. The addition of T.C.A. appeared not to affect the amount of protein eluted between fractions 10 and 17, (figure 4.3.6) although the amount of lead eluted over these fractions is reduced (table 4.3.5). It is possible that in the presence of T.C.A., the peptides remain soluble but release the lead bound to them, perhaps as a result in the fall in pH, the dissociated lead being eluted in the "Pb-Y" peak. Dissociation of the lead-peptide complexes can not, however, account for the three fold increase in the amount of "Pb-Y" after T.C.A. treatment. It seems that the increase in "Pb-Y" is primarily a consequence of the high column recovery resulting from the addition of the T.C.A. to the supernatant (98% compared to 47%). It is likely that the reduction in pH causes dissociation of lead from other ligands (in addition to the peptides) which may otherwise have bound to the gel or precipitated out in the eluent.

4.3.5 Summary

The results discussed in section 4.3.4 demonstrate that the same lead species are not formed in both fed and fasted animals as was originally suggested in section 4.2.3.2. It appears that the lead in the "Pb-X" peak is associated with protein derived from the gut wall in fasted animals, whereas, in fed animals, the protein is derived from both the diet and the gut wall. The complexes from fed rats are dissociated if heated to 100°C for two minutes, whereas some or all of the complexes from fasted rats remain stable for up to ten minutes at 100°C and in the presence of T.C.A.. "Pb-Y" from both fed and fasted rats appears to be a mixture of hydrated lead cation, inorganic lead species (the lead carbonate ion pair being especially prevalent) and lead-amino acid complexes such as lead-histidine as previously suggested by Partridge (1986). The species eluted between the "Pb-X" and "Pb-Y" peaks in both fed and fasted animals are possibly lead-peptide complexes (the peptide being derived from diet and gut wall in fed animals and the gut wall only in fasted animals) which dissociate to a certain extent when heated to 100°C for two minutes but dissociate completely in the presence of T.C.A.. Lead eluted

after "Pb-Y" following separation of gut washing supernatants from fed rats appears to be bound to proteins whose movement through the column is slowed by interaction with the gel. In fasted rats however, this lead is not protein-bound and may be lead species which initially bind to the column and are later removed as either a result of the washing effect of the eluent, or the exchange of radioactive lead bound to the gel with non-radioactive lead in the eluent. It appears that the low column recoveries are due to the formation of lead complexes which bind to the gel or are precipitated on the column being insoluble in acetic acid (e.g. lead hydroxide and lead orthophosphate (Greninger *et al.*, 1975)).

4.4 Lead-metallothionein: a heat stable lead-protein complex possibly present in "Pb-X"

4.4.1 Introduction

The majority of lead in the soluble phase of gut washings from a fasted rat appears to be bound to a protein which neither releases its metal ion on heating to 100°C for up to ten minutes nor following treatment with T.C.A.. A protein with similar properties known to be found in the intestinal lumen of rats is metallothionein.

The metallothioneins are a group of cytosolic metalloproteins with a molecular weight of between 6,000-7,000, depending on their metal composition (Dunn *et al.*, 1987). All mammalian metallothioneins are structurally similar. They are single chain polypeptides of sixty-one residues containing no aromatic amino acids, histidines or disulphide bonds, but having a high cysteine content (33%) (Kotsonis and Klaassen, 1981; Kägi *et al.*, 1984; Bremner, 1987a; Dunn *et al.*, 1987) and thus a high affinity for binding heavy metals (Foulkes and McMullen, 1986). The metal ions bind exclusively through thiolate co-ordination complexes involving all twenty cysteine residues (Bremner, 1987b; Dunn *et al.*, 1987). Metallothionein proteins are also very heat stable. They are not destroyed by heating at 80°C for ten minutes (Cherian and Goyer, 1978), heating being used to remove extraneous proteins in a number of assays for metallothionein (Eaton, 1985; Scheuhammer and Cherian, 1986). Metallothionein is also resistant to precipitation by T.C.A., a property exploited in the assay for metallothionein described by Piotrowski *et al.* 1973.

Trace amounts of metallothionein are normally present in most organs of eukaryotic species. Basal levels of metallothionein-like proteins in six animal species was

found to be highest in the liver (54-496 μg/g tissue wet wt.), kidney (101-305 μg/g tissue wet wt.) and intestine (127-257 μg/g tissue wet wt.) (Zelazowski and Piotrowski, 1977). However, exposure to a number of factors (reviewed in detail by Cousins, 1985; Hamer, 1986; Dunn *et al.*, 1987; Bremner, 1987a) and the parenteral or dietary administration of a number of metals especially cadmium, zinc, or copper can increase the concentration of metallothionein in the liver, kidney and intestine (Dunn *et al.*, 1987). Lead administered either intravenously or parenterally to experimental rats can also induce hepatic zinc-metallothionein (Maitani *et al.*, 1986), or possibly lead-metallothionein (Ikebuchi *et al.*, 1986).

Of particular interest in this study is the presence of intestinal metallothionein. In man, an intestinal metallothionein concentration of 7μg/g tissue wet wt. has been reported (Heilmaier *et al.*, 1987), although in the rat, values of 10μg/g tissue wet wt. (Heilmaier *et al.*, 1987), 40μg/g tissue wet wt. (Kello *et al.*, 1979; Onoska and Cherian, 1981) and 205μg/g tissue wet wt. (Zelazowski and Piotrowski, 1977) are quoted. The variation in these basal levels possibly reflects differences in analytical technique and variations in animal environment. Intestinal metallothionein is thought to play a key role in regulating the absorption of the potentially toxic elements cadmium (Squibb *et al.*, 1976) and mercury (Dunn *et al.*, 1987) as well as the nutritionally essential zinc (Cousins, 1979) and copper (Dunn *et al.*, 1987). Although the exact mechanism by which regulation is achieved is not fully understood, in the case of zinc (and possibly other ions) it appears that of the ion that enters the mucosal cell, a portion is rapidly transferred to the plasma and a portion is bound to metallothionein. An increase in zinc status causes the increased synthesis of thionein (metal-free metallothionein), an increase in the amount of cytosolic zinc-metallothionein, and therefore a reduction in the amount of zinc transported. A reduction in zinc status reduces metallothionein synthesis resulting in less sequestration of the ion and hence an increase in the amount of zinc transferred to the plasma (Cousins, 1979).

Besides being found in intestinal mucosa, metallothionein may also be found in the gut lumen as a result of desquamation of epithelial cells (Squibb *et al.*, 1976; Valberg *et al.*, 1977; Webb and Cain, 1982) and its excretion in bile (Sato and Bremner, 1984; Bremner *et al.*, 1986; Bremner, 1987b; Dunn *et al.*, 1987). Initially, the protein may be bound to cadmium or zinc, but such complexes may not survive in the luminal

environment. It is known that in tissue, the ratio of metals in the protein is subject to the concentration of the metals and their relative affinity to the protein (Heilmaier *et al.*, 1987). Indeed, lead is known to have a very high capacity to displace zinc from metallothionein *in vitro* (Waalkes *et al.*, 1984). Metallothionein proteins are also known to readily exchange metals with either free metal ions in the cytosol or ions bound to other ligands at physiological pH without protein degradation (Albergoni and Piccinni, 1983; Dunn *et al.*, 1987). It is suggested that during the *in vitro* incubation of gut washing supernatants from the fasted rat, two important conditions exist that may promote formation of the lead-metallothionein complex:

1. In fed rats, exogenous soluble dietary components (including protein) may compete with endogenous metallothionein for lead. However, in fasted rats, a reduced amount of dietary components removes the competition, in effect increasing the amount of lead available for binding with metallothionein.

2. In the fed state, lead may have to compete with high concentrations of other dietary ions for binding sites on the metallothionein. A relatively higher concentration of lead in the gut washing supernatant of fasted rats during the incubation ($1x10^{-6}M$) compared to other ions, coupled with the high capacity of lead to displace zinc from metallothionein compared to other ions (as shown *in vitro* by Waalkes *et al.*, 1984) may promote the formation of the lead-metallothionein complex.

Although it appears likely that lead-metallothionein is formed during the *in vitro* incubation of the gut washing supernatant from the fasted rat, there is some doubt as to whether the complex would be stable once loaded onto the Sephadex column. It is known that on exposure of metallothionein to low pH, the protein loses its metal ions, forming thionein (Kägi and Vallee, 1961; Vašák and Armitage, 1986) and chromatography involving metallothionein should therefore be performed at a pH above 7.0 (Dunn *et al.*, 1987). It could be argued that running intestinal supernatants on a column at a pH of 3.8 would therefore cause the dissociation of any lead-metallothionein complex. Synthesis of lead-metallothionein *in vitro* would enable the stability of the complex on a column at pH 3.8 to be assessed.

Initially, the ability of lead and metallothionein to form a complex *in vitro* was studied by incubating the two, and eluting the mixture on a Sephadex G-15 column with $7x10^{-6}M$ sodium hydroxide at pH 7.0, a pH at which little dissociation of the

complex would occur (Dunn *et al.*, 1987). The effect of heating on this complex was also tested. Further investigations compared the stability of the complex on columns eluted with $7x10^{-6}$M sodium hydroxide (pH 7.0) and $1x10^{-2}$M lead acetate (pH 3.8), the buffer previously used to elute gut washing supernatants. Finally, the effect of heating the lead-metallothionein complex before elution on a column with $1x10^{-2}$M lead acetate (pH 3.8) as eluent was tested to determine whether heating would render the complex more prone to dissociation at low pH.

4.4.2 Materials and methods

4.4.2.1 Chemicals

See sections 2.2.1 and 2.2.2. Metallothionein (forms 1 and 2) from rabbit liver, was obtained from Sigma Chemical Company Ltd., Poole, U.K..

4.4.2.2 The formation of lead-metallothionein *in vitro* and the effect of heating on the complex

The incubation comprised of $3x10^{-5}$M metallothionein (2mg of metallothionein in an incubation volume of 10mls assuming a molecular weight for metallothionein of 6,600) and $1x10^{-6}$M lead acetate labelled with 1μCi of lead-203. After two hours in a water bath at 37°C shaking at 80 oscillations/minute, the incubation mixture was divided into two 5ml samples. One sample was analysed by a chromatography system similar to that described in section 4.2.1.3, except that a solution of $7x10^{-6}$M sodium hydroxide (pH 7.0) was used in place of the $1x10^{-2}$M lead acetate solution both during column preparation and as eluent. The second sample was heated to 100°C for two minutes in a boiling water bath as previously (section 4.3.2.3), and analysed on a second identical chromatography system. The lead in the column fractions was determined by counting the gamma emissions of lead-203 (section 2.2.4).

4.4.2.3 Comparison of the stability of lead-metallothionein on a column eluted with $7x10^{-6}$M sodium hydroxide at neutral pH and $1x10^{-6}$M lead acetate at pH 3.8

Lead and metallothionein were incubated as described in section 4.4.2.2..

After incubation, the 10 ml sample was divided into two 5ml samples. One sample was analysed by the chromatography system eluted with $1x10^{-2}$M lead acetate (pH 3.8) described in section 4.2.1.3, the other by the chromatography system eluted with $7x10^{-6}$M sodium hydroxide (pH 7.0) described in section 4.4.2.2. Column fractions were analysed for lead as previously (section 2.2.4).

4.4.2.4 Comparison of the elution of lead-metallothionein on a column eluted with $1x10^{-2}$M lead acetate at pH 3.8, before and after heating

The incubation and heating procedure was identical to that described in section 4.4.2.2. Both test and control samples were analysed on a gel chromatography system using $1x10^{-2}$M lead acetate as eluent as described in section 4.2.1.3. The fractions were assayed for lead (section 2.2.4).

4.4.3 Results

The amount of lead-metallothionein complex formed, showed some variation between comparable control values, possibly as a result of differences in the basal amounts of ions bound to the metallothionein samples supplied. However, a lead-metallothionein complex was formed *in vitro*, being eluted at the "Pb-X" position (fractions 8/9) on the column run at neutral pH (table 4.4.1). The complex remained stable despite heating to 100°C for two minutes. Apart from the lead-metallothionein complex, no other lead was eluted from the column and consequently column recoveries for both the heated and unheated samples were very low.

Comparison of the chromatographic profiles from the column eluted with $7x10^{-6}$M sodium hydroxide solution (pH 7.0) and that eluted with $1x10^{-2}$M lead acetate (pH 3.8) (table 4.4.2) demonstrated that, despite a reduction in the amount of lead-metallothionein eluted from the latter column, the recovery of lead was substantially increased due to the elution of a peak at the "Pb-Y" position. A small amount of lead was also eluted as "undefined species" in the fractions on either side of this peak.

Heating had no effect on the amount of lead being recovered at the "Pb-X" and "Pb-Y" positions or in the fractions not associated with either peak when using $1x10^{-2}$M lead acetate (pH 3.8) as eluent (table 4.4.3).

Table 4.4.1 The elution profiles obtained from a Sephadex G-15 column (using $7x10^{-6}$M sodium hydroxide pH 7.0 as eluent) before and after heating an incubation mixture of lead and metallothionein for two minutes.

Species formed	% lead as individual species	
	Before heating	After heating
"Pb-X"	0.8	0.8
"Pb-Y"	-	-
"undefined species"	-	-
% column recovery	0.9	1.0

Species separated by the gel filtration chromatography system described in section 4.4.2.2.

It is assumed that "Pb-X" is eluted over fractions 8-10 and "Pb-Y" is eluted over fractions 17-22.

Table 4.4.2 The elution profile obtained on incubating lead and metallothionein *in vitro* followed by elution on a Sephadex G-15 column using either $1x10^{-2}$M lead acetate (pH 3.8) or $7x10^{-6}$M sodium hydroxide (pH 7.0) as eluent.

Species formed	% lead as individual species	
	Lead acetate	Sodium hydroxide
"Pb-X"	0.6	1.7
"Pb-Y"	91.5	-
"undefined species"	1.6	-
% column recovery	95	2

Species separated by the gel filtration chromatography system described in section 4.4.2.3.

It is assumed that "Pb-X" is eluted over fractions 8-10 and "Pb-Y" is eluted over fractions 17-22.

Table 4.4.3 The elution profiles obtained from a Sephadex G-15 column (using $1x10^{-2}$M lead acetate pH 3.8 as eluent) before and after heating an incubation mixture of lead and metallothionein for two minutes.

Species formed	% lead as individual species	
	Before heating	After heating
"Pb-X"	0.03	0.04
"Pb-Y"	89.39	87.74
"undefined species"	3.65	2.37
% column recovery	94	95

Species separated by the gel filtration chromatography system described in section 4.2.1.3.

It is assumed that "Pb-X" is eluted over fractions 8-10 and "Pb-Y" is eluted over fractions 17-22.

4.4.4 Discussion

All mammalian metallothioneins studied reveal similar structural features (Bremner, 1987a; Dunn *et al.*, 1987), so it was assumed that metallothionein from rabbit liver could be used in these studies in the absence of a suitable commercially available intestinal metallothionein.

Detection of lead in the "Pb-X" position of the column eluted with $7x10^{-6}$M sodium hydroxide (pH 7.0) (table 4.4.1) indicated that the lead-metallothionein complex can be synthesised *in vitro* and that it co-chromatographed with the "Pb-X" peak from the gut washing supernatants of fed and fasted rats (figures 4.3.1 and 4.3.3). However, unlike the complex from fed rats, the lead-metallothionein complex showed heat stability, agreeing with previous observations (Eaton, 1985; Scheuhammer and Cherian, 1986). The failure to elute lead after the "Pb-X" position, together with the low recoveries from the columns, suggested that lead not bound to metallothionein was bound to the gel, an observation substantiated when comparing the elution profiles obtained from the column eluted with $1x10^{-2}$M lead acetate (pH 3.8) and $7x10^{-6}$M sodium hydroxide (pH 7.0) (table 4.4.2). The elution of a peak at the "Pb-Y" position containing hydrated lead cation which had not bound to metallothionein, together with the substantially higher recovery of lead obtained on elution with lead acetate suggests that any free hydrated lead cation loaded onto the column will bind to the gel unless the gel is pre-treated with high concentrations of lead. It was for this reason that Partridge (1986) used a $1x10^{-2}$M lead acetate eluent with a pH of 3.8, in an attempt to reduce the interaction of lead from the samples with the ionised carboxyl groups within the gel. Use of low pH buffer in these studies appears to cause some dissociation of the lead-metallothionein complex. Such dissociation possibly accounts for the presence of the undefined lead species following elution with lead acetate, which in this case are not lead-peptide complexes as identified following the chromatographic separation of the lead species from gut washing supernatants (section 4.3.4.2). Heating the lead-metallothionein complex was shown not to render it more prone to dissociation at a pH of 3.8 (table 4.4.3).

In all cases where the incubation mixture was eluted using lead acetate as eluent, the recovery of lead from the column was consistently high (< 93%), being substantially higher than those obtained when eluting gut washings from the column using the same eluent (49-61%). This further suggests that the insolubility of certain lead

complexes formed during the incubation of gut washing supernatants with lead in the eluent, is the cause of the poor column recoveries (section 4.3.4.3).

4.4.5 Conclusions

The stability of both synthesised lead-metallothionein and the "Pb-X" complex from fasted rats on the gel column at pH 3.8, together with their heat stability and elution positions at fractions 8/9, indicate that "Pb-X" from fasted rats may include lead-metallothionein complexes.

4.5 Resolution of the "Pb-X" peak formed on incubation of lead with the gut washing supernatant of a fasted rat, by gel chromatography using Sephadex G-75

4.5.1 Introduction

Elution of "Pb-X" in the void volume of Sephadex G-15 indicates that the species present have a molecular weight in excess of 1,500 (section 4.3.1). Resolution of these species may be achieved by re-chromatography of the peak on Sephadex G-75, a gel which separates peptides and globular proteins over a molecular weight range of 3,000-80,000. Metallothionein is frequently isolated using this gel (Valburg *et al.*, 1976; Ikebuchi *et al.*, 1986; Scheuhammer and Cherian, 1986).

The aim of this investigation was therefore to resolve the lead-protein complexes formed during the *in vitro* incubation of lead with the gut washing supernatant from a fasted rat which are eluted in the void volume of Sephadex G-15, both before and after heating, by re-chromatography on a calibrated G-75 column. The molecular weights of the proteins eluted from the G-75 column would be determined and the possible presence of metallothionein verified by comparison of the position of the eluted proteins with that of metallothionein and lead-metallothionein.

4.5.2 Materials and methods

4.5.2.1 Animals and chemicals

As in sections 2.2.1, 2.2.2, 3.2.3 and 4.4.2.1. Sephadex G-75 was supplied by Sigma Chemical Company Ltd., Poole, U.K..

4.5.2.2 The gel chromatography system used to separate the lead-protein complexes formed during the *in vitro* incubation of gut washing supernatants from fasted rats

Initial separation of gut washing supernatants was achieved using the Sephadex G-15 chromatography system described in section 4.2.1.3. Resolution of the "Pb-X" peak was achieved on a Sephadex G-75 column. Sephadex G-75 was allowed to swell in excess buffer (0.1M Tris-acetate buffer, pH 7.0) for at least 36 hours at room temperature. The suspension was washed with approximately 1000mls of the Tris buffer and degassed under vacuum as previously (section 4.2.1.3). The slurry was resuspended and packed under pressure using a peristaltic pump running at 1.5mls/minute, into a glass chromatography column 60x1.6cm. The column was sealed and washed ascendingly with approximately 800mls of degassed buffer. Samples were 2mls in volume, and were run ascendingly at 1ml/minute using the 0.1M Tris-acetate buffer (pH 7.0) as eluent. Thirty five 6ml samples were collected as previously (section 4.2.1.3).

4.5.2.3 Calibration of the Sephadex G-75 column

Sephadex G-75 may be calibrated for molecular weight determination (Whitaker, 1963; Andrews, 1964; Freifelder, 1982). In this study, the method detailed by Whitaker (1963) and recommended by the suppliers of the chromatography gel (Sigma Chemical Company Ltd., 1986) was used, a calibration graph being obtained on plotting the log of the molecular weight of standard proteins against their respective ratios of elution volume (V_e) to void volume (V_o).

Identical concentrations (1mg protein/2mls distilled water) of ovalbumin (M.W. 43,000), carbonic anhydrase (M.W. 29,000) cytochrome C (M.W. 12,400) and insulin (M.W. 5,700) were eluted from the Sephadex G-75 column. The elution position of each protein (except cytochrome C whose presence in the eluted fractions was clearly visible, it being a red chromaphore), was determined by assaying the fractions eluted for protein by the method detailed by Lowry *et al.* (1951) (appendix A.3.1). The void volume and total exclusion volume of the column were determined as described previously (section 4.2.1.3) and were found at fractions 8 and 19 respectivly. The ratio of elution volume to void volume (V_e/V_o) for each protein was plotted against the log of

the corresponding molecular weight giving a linear calibration graph (figure 4.5.1). Metallothionein (1mg protein/2mls distilled water) and the lead-metallothionein complex synthesised in section 4.4.2.2 were found to co-chromatograph at fraction 16.

4.5.2.4 The formation of "Pb-X", and its subsequent resolution on Sephadex G-75, before and after heating for ten minutes

The "Pb-X" complex was formed by incubating the gut washing supernatant from a fasted rat with lead. The procedure for incubation, heating and separation on G-15 was identical to that described previously (section 4.3.2.3). The "Pb-X" peak was identified in fractions 8-10 by counting the gamma emissions from lead-203 (section 2.2.4), the fractions pooled, evaporated overnight in a Vertis freeze-drier (Techmation Ltd., Edgware, U.K.), rehydrated by addition of 2mls of distilled water and eluted on the G-75 column (section 4.5.2.2). Resulting fractions were assayed for lead (section 2.2.4) and protein (appendix A.3.1).

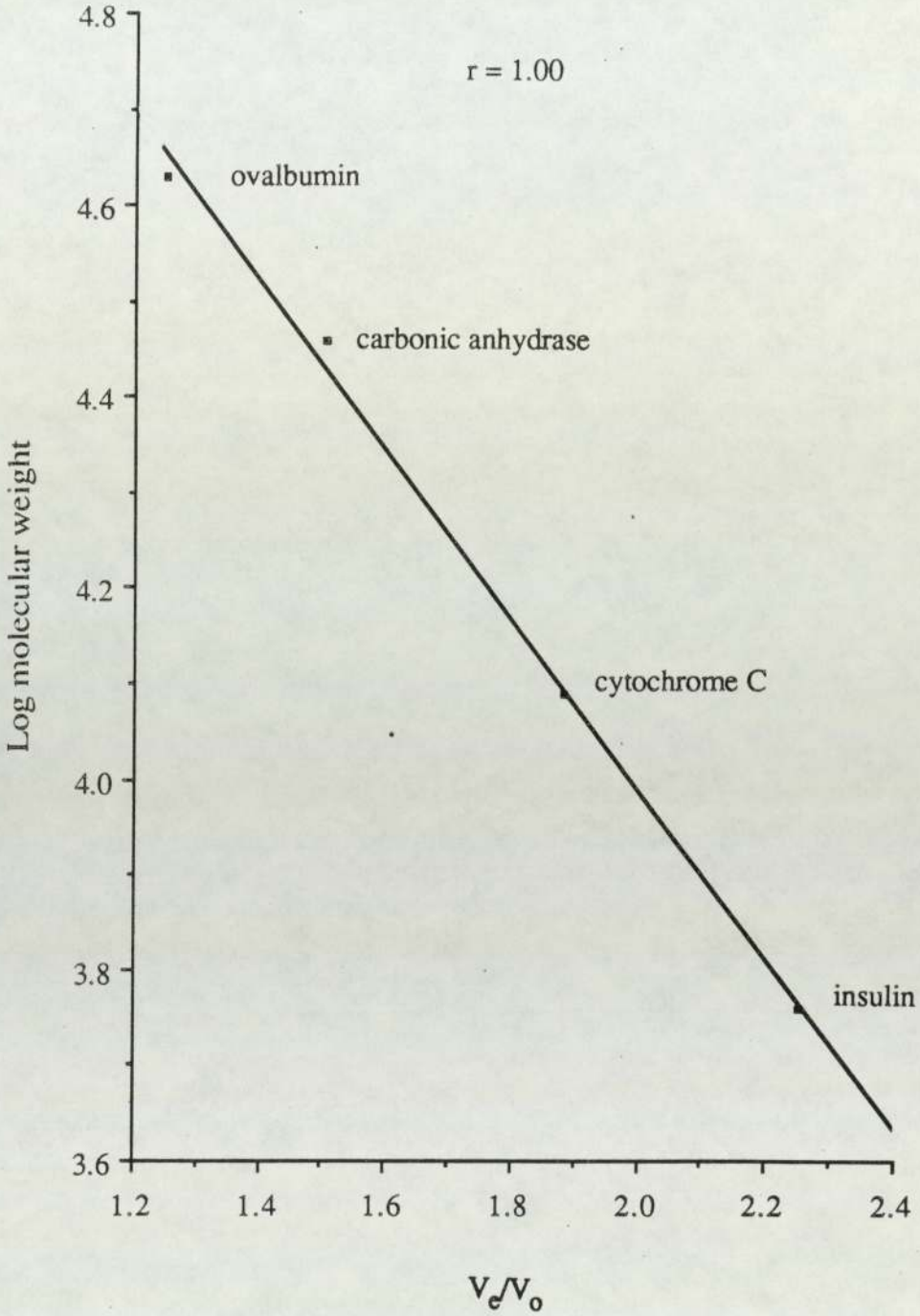
4.5.3 Results

No lead was detected in any of the samples eluted from the G-75 column. Proteins from the control and heat treated samples were eluted in two distinct bands (figure 4.5.2). Some protein was eluted in the void volume, indicating a molecular weight in excess of 80,000. The rest of the protein was eluted in fractions 15-22, being within a molecular weight range of 3,000-13,000. Protein was detected at fraction 16 of both heat treated and control profiles, a position corresponding to the elution of the metallothionein and lead-metallothionein species.

4.5.4 Discussion

Resolution of the "Pb-X" peak on G-75 indicated the presence of a number of proteins whose elution was not affected by heating. The metallothionein and lead-metallothionein species were eluted at fraction 16 giving them a V_e/V_o ratio of 2, a ratio at which few other metal binding proteins are eluted (Dunn *et al.*, 1987), and agreeing with published values of 1.6-2.0 (Cherian *et al.*, 1978) and 1.8 (Dunn *et al.*, 1987). Elution at this position indicated the species had a molecular weight of 10,000,

Figure 4.5.1 Calibration graph for Sephadex G-75.



Gel - Sephadex G-75

Eluent - 0.1M Tris-acetate buffer (pH 7.0)

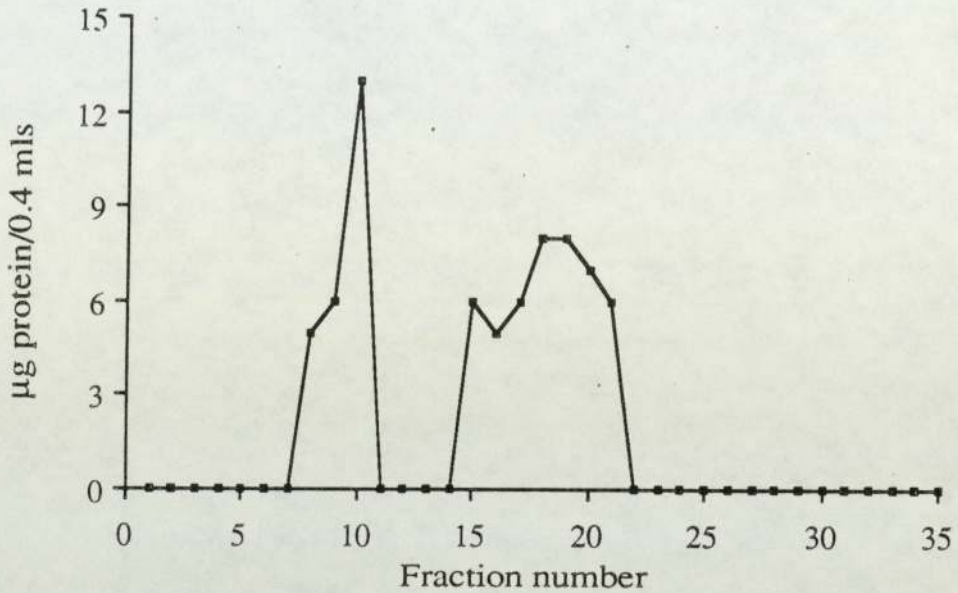
Flow rate - 1ml/minute

Fraction volume - 6mls

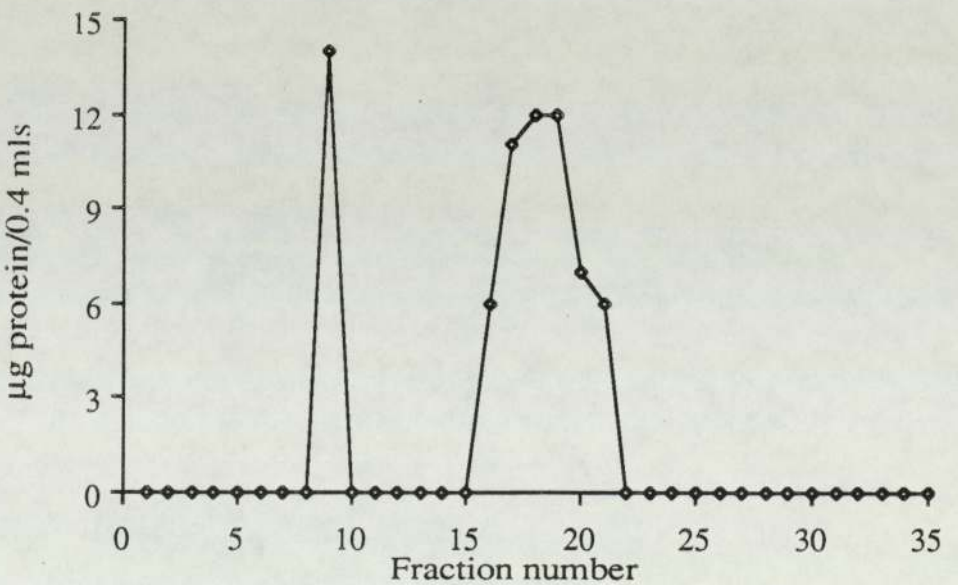
See section 4.5.2.2 for detailed column specifications.

Figure 4.5.2 Elution of the proteins from the gut washing supernatant of the fasted rat and identified as "Pb-X" on Sephadex G-15, following re-chromatography on Sephadex G-75.

Before heating



After heating



Gel	-	Sephadex G-75
Eluent	-	0.1M Tris-acetate buffer (pH 7.0)
Flow rate	-	1ml/minute
Fraction volume	-	6mls

See section 4.5.2.2 for detailed column specifications.

which agrees with that quoted by Kägi and Vallee (1961). However, determination of the molecular weight of metallothionein from amino acid sequencing data gives a value of 6,000-7,000 depending on metal composition (Dunn *et al.*, 1987). This value is thought to represent the real molecular weight, the apparent molecular weight obtained from gel chromatography being a result of the hindered movement of the protein through the gel due to its prolate-related ellipsoid shape (Kägi *et al.*, 1974; Dunn *et al.*, 1987).

The presence of protein in fraction 16 of the elution profile, both before and after heating the gut washing supernatant, suggests the presence of metallothionein. However, due to the complete decay of lead-203, it was not possible to demonstrate the exact location of the lead in relation to the protein.

Despite re-chromatography of the "Pb-X" peak on G-75, it appears that the proteins are still not completely resolved. Those proteins eluted in the void volume have molecular weights in excess of 80,000 and would need to be resolved further possibly on Sephadex G-100 or G-150 (fractionation range 4,000-150,000 and 5,000-300,000 respectivly). Proteins eluted later in the profile do not give distinct peaks and therefore should either be collected as smaller fractions than 6mls (in which case substantially more protein would have to be loaded on the column) or be further resolved on Sephadex G-25 or G-50 (fractionation range 1,000-5,000 and 1,500-30,000 respectivly).

The proteins eluted in the void volume of a G-15 column may alternatively be separated by gel electrophoresis. This technique has a lower detection limit for proteins than gel chromatography and has been used successfully by Farrar (1988), who detected a protein with a molecular weight similar to that of metallothionein in both fed and fasted animals. The possible presence of metallothionein in the fed rat is further evidence to suggest that it is competition between lead and dietary ions for binding sites on the protein, or competition with dietary proteins as potential ligands for lead, that prevents the formation of the lead-metallothionein complex by either *in vivo* and *in vitro* preparations (section 4.4.1).

4.6 General discussion

This investigation gives some insight into the possible types of lead species formed in the the gut washing supernatants of fed and fasted rats. It is reasonable to assume that as the chromatographs from the *in vitro* work compare favourably with those

obtained *in vivo* by Partridge (1986), the species formed under both experimental conditions are similar. However it must be remembered that *in vivo*, the presence of food in the lumen of the fed rats will result in strong competition for ionic lead, and hence the actual concentration of lead available to form soluble lead species will be reduced.

In the soluble phase of gut washings from fed and fasted rats there are a number of potential protein ligands derived from the gut wall in fasted animals and both the gut wall and the diet in fed animals. In fasted rats, proteins with a molecular weight in excess of 80,000, and others in the range 3,000-13,000 have been separated. However, despite being eluted at the same position on Sephadex G-15 (all share a molecular weight in excess of 1,500), the lead-protein complexes formed in both fed and fasted rat gut washing supernatants appear to be dissimilar. In fed animals the complexes are weak and dissociate after two minutes at 100°C. In contrast, a portion of the lead in the soluble phase of gut washings from a fasted rat appears to be bound to a protein which does not release its metal ion either following treatment with T.C.A., or on heating to 100°C for up to ten minutes.

The evidence presented in this study indicates that this protein may be metallothionein. Elution of lead-metallothionein on Sephadex G-75 results in an elution volume to void volume ratio (V_e/V_o) equal to 2.0, a position where few other metal binding proteins elute (Cherian *et al.*, 1978; Dunn *et al.*, 1987) and yet where a protein from the gut washing supernatant of a fasted rat is eluted. Other similarities between "Pb-X" and lead-metallothionein demonstrated by these studies, summarised in table 4.6.1, suggest that metallothionein is present in the intestinal lumen of fasted rats, and that lead-metallothionein may be one of the complexes eluted as "Pb-X" rather than the low molecular weight zinc binding ligand as previously suggested (Partridge, 1986), as this ligand is non-proteinatious, and, having a molecular weight of 1,000 (Song *et al.*, 1984), would not be eluted in the void volume of Sephadex G-15.

It has been suggested throughout these studies that the lack of lead-metallothionein complex in the fed rat does not necessarily suggest a lack of metallothionein in the gut lumen. As described previously, work by Farrar (1988) using gel electrophoresis has shown a protein band corresponding to metallothionein in fed as well as fasted rats. Indeed, the amount of metallothionein in the fed gut is probably higher than that in the fasted gut as a result of increased bile secretion and the presence of

Table 4.6.1 Similarities between an unidentified component of "Pb-X" and the lead-metallothionein complex, "Pb-Mt".

	"Pb-X"	"Pb-Mt"
Soluble lead-protein complex	√	√
Molecular weight > 1,500	√	√
May be formed in the gut lumen	√	√
Ligand possibly derived from the gut wall	√	√
Eluted at fraction 8/9 on Sephadex G-15 both before and after heating	√	√
Remains a soluble complex despite heating	√	√
Resistant to precipitation by T.C.A.	√	√
Has a V_e/V_o ratio of approximately 1.8 on Sephadex G-75	√	√

a food bolus increasing the rate of cellular desquamation (section 4.4.1). It appears likely that the absence of lead-metallothionein in the fed rat may largely be a result of either competition between the metallothionein and dietary ligands for lead, or between dietary ions and lead for binding sites on the metallothionein. Suggestions that in this study, fasting may have increased the concentration of intestinal metallothionein in response to the low levels of zinc in the gut lumen have been largely disproved by Richards and Cousins (1976). They demonstrated that amounts of intestinal metallothionein were not increased following a 24 hour fast, although, like Bremner and Davies (1975), they did observe an increase in levels of hepatic metallothionein.

In addition to the organic ligands, lead may form strong complexes with inorganic anions which are then eluted with the lead-amino acid complexes in "Pb-Y". A complex thought to be particularly prevalent is lead carbonate (section 4.3.4.3). Known to be converted to insoluble basic lead carbonate at 100°C, lead carbonate, together with lead orthophosphate (which is insoluble in hot water) and possibly any basic lead carbonate formed from the reaction of lead hydroxide with free carbonate ions, could account for the formation of the "heating precipitate". Other ion pairs present in "Pb-Y" may include lead sulphate and lead chloride. However, neither lead orthophosphate nor lead hydroxide are eluted in "Pb-Y" as they are insoluble in acetic acid, their precipitation on the gel column probably accounting for the low column recoveries.

Further identification of the potential lead ligands present in the lumen of fed and fasted rats is essential. Identification of the amino acid, peptides and proteins present in the gut by further resolution on different Sephadex gels, gel electrophoresis, ion exchange and high performance liquid chromatography (H.P.L.C.) would allow the ligands most likely to complex with lead, to be identified. Particular emphasis should be given to the identification of metallothionein as an important lead binding ligand, especially as it appears to plays a protective role in lead absorption (Partridge, 1986). Identification of the inorganic lead species is more difficult because of their low concentration in the gut. A number of techniques previously used for determination of lead species in natural and sea waters could be applied to the lead species in the gut, especially the ion selective electrode and anodic stripping voltametry.

4.7 Conclusions

The type of lead species formed in the rat gut depends on ligand bioavailability and competition. In both fed and fasted rats, lead-protein, lead-peptide, lead-amino acid and soluble inorganic lead ion pair complexes form, although some lead is still thought to be present as the free hydrated cation. In fed rats, less lead is complexed with peptides or is found as lead-amino acid complexes, as ion pairs or the free hydrated lead cation compared to the fasted rat probably as a result of the formation of significantly more insoluble lead species during incubation. A lead-protein complex thought to be formed in the gut washing supernatant of the fasted rat is lead-metallothionein. The effect of the formation of lead-metallothionein and the ion pair complexes on intestinal lead absorption will be assessed in chapters five and six.

Chapter 5

GASTROINTESTINAL ABSORPTION AND ORGAN DISTRIBUTION OF ORALLY DOSED LEAD ACETATE AND LEAD-METALLOTHIONEIN IN FASTED RATS

5.1 Introduction

*Metallothionein is a protein found naturally in the gut lumen of fasted and possibly fed rats as a result of desquamation of epithelial cells and secretion in bile (section 4.4.1), and appears to form a strong complex with the lead cation (as discussed in sections 4.4.1 and 4.4.4). Metallothionein is also found in the diet, in beef liver and kidney (Cherian, 1979; Klein *et al.*, 1986) and cereal (Cherian *et al.*, 1978) and is thought not to be destroyed by cooking (Cherian *et al.*, 1978; Cherian, 1979; Klein *et al.*, 1986; Massay *et al.*, 1988). It is therefore possible that as well as endogenous metallothionein, dietary metallothionein may also play an important role in the speciation of lead in the gut.

Originally it was not clear how much ingested metallothionein would reach the intestine, since it was not certain whether the protein was completely protected from degradation by proteolytic enzymes in the low pH environment of the stomach (Cherian, 1979) which in humans may be as low as 1.5, but which rises to between 2.5 and 4.0 during food intake. Work by Klein and associates (1986) demonstrated that although, below a pH of 3.5, metallothionein loses its associated ion and is subsequently digested by pepsin, above a pH of 3.5, nearly all the metal remains bound to the metallothionein which consequently remains resistant to proteolysis. Massay *et al.* (1988) also demonstrated that at low pH, metallothionein dissociates into metal ion and thionein, but unlike Klein *et al.* (1986), suggested that the apoprotein is resistant to digestion by proteases and reassociates with the metal ion in the more alkaline milieu of the small intestine. Despite uncertainty as to the fate of ingested metallothionein in the stomach, both pieces of evidence suggest that intact metallothionein from the diet may exist in the gut lumen. This view is further substantiated by Cherian (1979) who, following oral administration of cadmium-metallothionein to fasted mice, found that the major portion of the complex could be isolated intact from the intestinal mucosa.

Knowing that intact dietary metallothionein may reach the gut lumen, and bearing in mind the strong affinity of metallothionein for lead (Waalkes *et al.*, 1984), it is possible that the two will complex in the intestine in a similar manner to lead and endogenous metallothionein (section 4.4.1), especially if, as Massay *et al.* (1988)

* Throughout this chapter, metallothionein refers to thionein bound to an unspecified ion. Cadmium-, zinc-, or lead-metallothionein refers to thionein bound to cadmium, zinc or lead. Thionein refers to desalted metallothionein, lead-thionein, to lead bound to previously desalted metallothionein.

suggest, the protein entering the small intestine is desalted. Alternatively, large amounts of lead-metallothionein may itself be ingested and enter the intestine if, as demonstrated in rats by Ikebuchi *et al.* (1986), hepatic lead-metallothionein is induced following the exposure to lead of livestock used for food, possibly by their grazeing on contaminated pasture.

Despite the possibility that lead-metallothionein may be an important luminal species, little or no work has been done to study the effect of its formation on the intestinal absorption of lead. However, combining work by Partridge (1986) with that described in chapter 4, it appears that formation of lead-metallothionein may have a protective role, in that it reduces lead absorption (section 4.6). To date, most studies involving investigation of the role of metallothionein on the absorption of various ions have concentrated on determining the effect of the cadmium-metallothionein complex on cadmium absorption. Nevertheless, it is reasonable to assume that lead bound to metallothionein may behave in a similar manner.

The formation of cadmium-metallothionein complexes within the intestinal mucosa has been recognised as hindering the transfer of metal ion into the body during low level cadmium exposure (Squibb *et al.*, 1976; Kotsonis and Klassen, 1978) and evidence suggests that free cadmium-metallothionein present in the gastrointestinal lumen may have a similar protective effect. Substantially less cadmium was present in the internal organs of both mice and rats dosed with cadmium-metallothionein compared to those dosed with cadmium chloride (Cherian, 1979; Muller *et al.*, 1986) and intestinal perfusion of mouse duodenum and proximal jejunum with cadmium chloride and cadmium-metallothionein, showed similar amounts of the ion were removed from both perfusates, but significantly less cadmium from the cadmium-metallothionein perfusate passed into the body (Valberg *et al.*, 1977). A similar effect was observed by Foulkes and McMullen (1986) who perfused rat jejunum with either cadmium chloride or cadmium-metallothionein, and concluded that the transfer into the body of cadmium-metallothionein formed from endogenous metallothionein or taken up from a perfusate containing cadmium-metallothionein was very low.

Despite reducing cadmium transport, intracellular metallothionein will have limited effect on the transport of lead since little of the the lead cation enters the epithelial cell (Coogan, 1982). It appears however, that as free cadmium-metallothionein may play

a protective role by slowing the movement of cadmium across the intestinal mucosa, so the formation of free lead-metallothionein *in vivo* (as discussed in section 4.4.1) may cause a reduction in lead bioavailability either by the complex being sequestered in the intestinal mucosa or by being too large to move through the tight junctions between epithelial cells, which is the route thought to be taken by the hydrated lead cation (Blair *et al.*, 1979; Coleman, 1979; Coogan, 1982).

In this study a comparison will be made of the intestinal absorption and tissue distribution of lead orally dosed to rats as lead-metallothionein or as the hydrated lead cation.

5.2 Materials and methods

5.2.1 Chemicals and animals

As sections 2.2.1, 2.2.2, 3.2.3 and 4.4.2.1. See also section 4.4.4.

5.2.2 Methods

The formation of lead-metallothionein *in vitro* was demonstrated in section 4.4.2.2. In this study, 4.2×10^{-4}M metallothionein (5.5mg of metallothionein in an incubation volume of 2mls, assuming a molecular weight of metallothionein of 6,600) was incubated with 1×10^{-6}M lead acetate labelled with 1μCi of lead-203 per 0.3mls under identical conditions to those detailed in section 4.4.2.2.

Rats which had been fasted overnight (section 3.2.3) were orally dosed with 0.3mls of 1×10^{-6}M lead as lead acetate, or 1×10^{-6}M lead containing the lead-metallothionein complex. Both doses were labelled with 1μCi of lead-203. Following dosing the rats were returned to the cages with grid bottoms and only given access to water. After four hours the rats were sacrificed by cervical dislocation, and various organs removed, washed in isotonic saline and assayed for lead using the L.K.B. CompuGamma (section 2.2.4). The whole small intestine was removed from pyloric sphincter to ileocaecal valve and washed out with approximately 20mls of isotonic saline. Intestine and washings were assayed for lead separately. The amount of lead in each organ and in the gut washings was expressed as a percentage of the total dose of lead administered. The total percentage of lead in the internal organs (brain, liver, spleen, kidneys, heart and lungs) and the percentage of the total dose administered (% T.D.A.)

entering the body were used as indicators of the absorption of the administered species. The data was tested for significance using the Student's t-test.

5.3 Results

More lead appeared to enter the body of rats dosed with lead compared to those dosed with lead-metallothionein, twice as much appearing to accumulate in the major internal organs (tables 5.1 and 5.2). However, except for the kidney, the differences between the two groups were not statistically significant. Similarly, there was no significant difference between the amounts of lead associated with the gut washings from the two groups of animals, despite twice the amount of lead being associated with the washings from the lead-metallothionein dosed rats compared to those dosed solely with lead (table 5.3). The amount of lead remaining in the stomach was significantly greater in the rats dosed with lead compared to those dosed with lead-metallothionein. The amount of lead associated with the small intestine was independent of the type of lead species dosed (table 5.3).

5.4 Discussion

Oral dosing of the amounts of lead-metallothionein used in this study, had no significant effect on the absorption of lead from the intestine of fasted rats (table 5.1). Nevertheless, there was a reduction in the amount of lead in the major internal organs from the rats dosed with lead-metallothionein (table 5.2), and an increase in lead remaining in the intestine after four hours (table 5.3). It appears that the amount of lead-metallothionein complex present in the intestinal lumen was too small to have a significant effect on lead absorption, possibly as a result of some dissociation of the complex in the acid milieu of the stomach or because incubating lead and metallothionein did not result in the formation of large amounts of complex. From the data in tables 4.4.1 and 4.4.2, it is estimated that approximately 12-26% of the lead dosed was bound to metallothionein. The amount of lead present in the organs of the rats dosed with lead-metallothionein can therefore be accounted for by the amount expected to accumulate as a result of the transport of the 74-88% of lead not bound to metallothionein. It is possible that a more substantial effect of metallothionein on lead absorption could have been achieved by increasing the number of observations, thereby reducing experimental

Table 5.1 Percentage of the total dose administered (% T.D.A.) entering the body and the total % T.D.A. in the internal organs.

	lead dosed	lead-metallothionein dosed
% T.D.A. entering the body	43.7 ± 2.5	36.0 ± 6.0
Total % T.D.A. in the internal organs	10.0 ± 1.7	5.9 ± 0.9

Data expressed as means ± standard error of means for five animals. No significant difference was found between the values for rats dosed with lead and those dosed with lead and metallothionein.

Table 5.2 Distribution of lead in the major internal organs of fasted rats following an oral dose of either lead or lead-metallothionein.

Organ	% T.D.A. in organ and associated blood	
	Pb dosed	"Pb-Mt" dosed
Brain	0.03 ± 0.01	0.02 ± 0.01
Liver	4.63 ± 1.03	2.75 ± 0.62
Spleen	0.07 ± 0.01	0.05 ± 0.01
Kidneys	4.61 ± 0.61	2.77 ± 0.32 *
Heart	0.36 ± 0.11	0.19 ± 0.05
Lungs	0.34 ± 0.09	0.16 ± 0.03

Rats dosed with 0.3mls of $1x10^{-6}$M lead as lead acetate or lead-metallothionein labelled with 1μCi of lead-203 and left for four hours before sacrifice. All organs were washed with isotonic saline before counting. Results are expressed as means ± standard error of means for five animals.

* Denotes a difference between the two experimental groups ($p<0.05$).

Table 5.3 Distribution of lead in the gastrointestinal tract of fasted rats following an oral dose of either lead or lead-metallothionein.

Organ	% T.D.A. in organ and associated blood	
	Pb dosed	"Pb-Mt" dosed
#Stomach	0.35 ± 0.03	0.18 ± 0.03 *
Small intestine	11.09 ± 2.34	9.56 ± 2.07
#Caecum	35.40 ± 5.72	37.14 ±13.85
Gut washings	9.50 ± 2.59	17.09 ± 7.50

Rats dosed with 0.3mls of $1x10^{-6}$M lead as lead acetate or lead-metallothionein labelled with 1μCi of lead-203 and left for four hours before sacrifice. All organs were washed with isotonic saline before counting. Results are expressed as means ± standard error of means for five animals.

The stomach and caecum were counted together with their luminal contents.

* Denotes a difference between the two experimental groups ($p<0.01$)

variation, or by:

1. Reducing the acidity of the stomach.

Metallothionein complexes dissociate at acid pH, and it is therefore possible that metallothionein may exert a stronger influence over lead absorption in instances where stomach acidity is reduced. The reduction in acidity following feeding will increase the likelihood of intact complex reaching the small intestine, however its effect would probably be hidden due to the substantially greater effect of the presence of food which, in the main, reduces lead absorption by rendering it insoluble. It is also possible that dosing lead-metallothionein to younger animals may also lead to an increase in the amount of complex present in the intestine since pepsin, peptidase and gastric acid secretion is reduced in the young (Beach and Henning, 1988). A low stomach pH did not however, appear to cause dissociation of the complex in the study by Cherian (1979). The majority of cadmium-metallothionein orally dosed to fasted mice survived the low pH of the stomach and reached the intestinal mucosa intact. It is therefore likely that when all the metal ion administered is bound to metallothionein (as in the study by Cherian, 1979), a small amount of dissociation in the stomach causes little or no change in the overall effect of the complex in the gut lumen. However, a similar degree of dissociation following administration of a small amount of complex (as in this study) would reduce its concentration in the gut to a level at which the effect on absorption would be minimal.

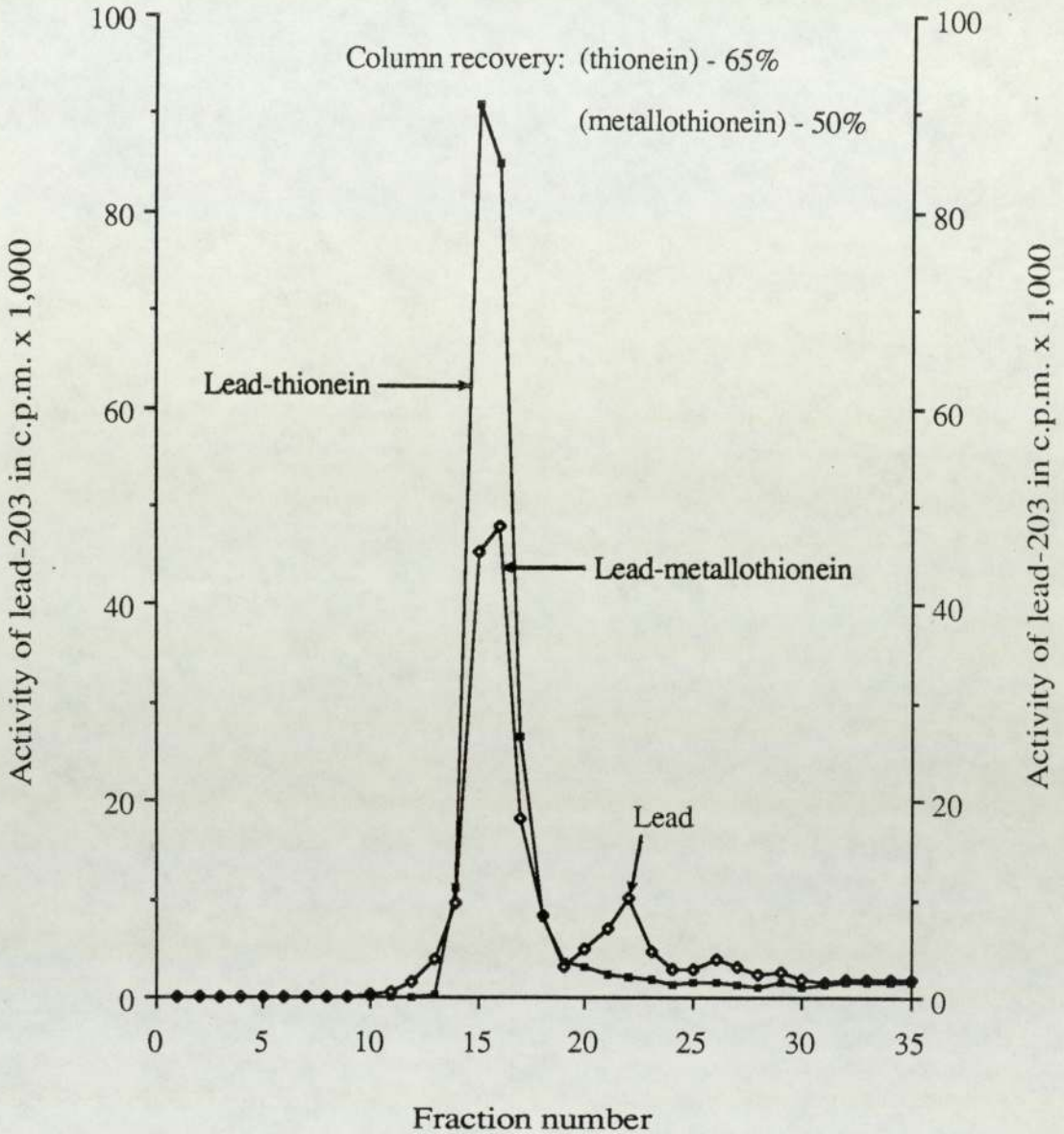
2. Increasing the amount of lead bound to metallothionein

The metallothionein preparation used in this study (section 4.4.2.1) contained approximately 7% metal as cadmium and zinc prior to its incubation with lead. Removal of these ions by desalting resulted in a doubling of the amount of lead bound to the protein (thionein) compared to the amount of lead which bound to untreated metallothionein (figure 5.1). It is likely that dosing lead-thionein formed by incubating thionein with lead would have resulted in a more profound reduction in the amount of lead absorbed.

3. Chronically administering lead-metallothionein

Since exposure to lead and metallothionein is chronic, long term dosing experiments may be of more use in determining differences in the absorption of lead and lead-metallothionein. Such studies are impossible using an isotope with a short half-life such as lead-203.

Figure 5.1 Profiles obtained following elution of identical quantities of lead-metallothionein and lead-thionein from a Sephadex G-75 column.



(1) Desalting: A solution of metallothionein (4mg/2mls distilled water) was acidified to pH2 with 0.1M hydrochloric acid and eluted on a Sephadex G-25 column. Column specifications are as detailed in section 4.2.1.3 except the gel was swollen and the column packed and eluted with 0.01M hydrochloric acid. Desalted metallothionein (thionein) was detected in eluted fractions by the Lowry assay (appendix A.3.1) and the sample neutralised with a 10mM sodium hydroxide solution.
(2) Incubation: Identical quantities of metallothionein and prepared thionein (0.6mg/2mls) were incubated with $1x10^{-6}$M lead acetate labelled with 0.2μCi lead-203 as in section 4.4.2.2.
(3) Chromatography: Samples were eluted on Sephadex G-75 (section 4.5.2.2). Lead-thionein and lead-metallothionein eluted at fraction 16, as did metallothionein (section 4.5.2.3). More lead bound to thionein than metallothionein, demonstrated by the larger amount of lead associated with the protein peak. A second peak at fraction 22 following elution of lead-metallothionein co-chromatographed with a lead standard and represented lead not bound to metallothionein.

It appears that the effect on lead absorption of the administration of exogenous lead-metallothionein, formed by the binding of lead to metallothionein already containing cadmium and zinc, is low. This, together with the dissociation of a portion of the complex in the low pH of the stomach of the fasted rat, results in only a small amount of lead-metallothionein reaching the small intestine and hence little or no effect on the transport of lead in the fasted animal is seen. A greater effect may be observed in cases where stomach acidity is reduced or larger amounts of lead-metallothionein are administered. The question as to whether endogenous lead-metallothionein formed in the gut lumen will reduce lead absorption remains, to a large extent, unanswered. Bearing in mind, however, the clear (although statistically non-significant) reduction in lead absorbed when a very small quantity of lead-metallothionein was administered (for reasons discussed earlier), lead-metallothionein formed *in vivo* (section 4.4.1), may have a more potent effect on lead absorption than the orally administered complex. Cerklewski and Forbes (1976) have implicated intestinal metallothionein in the protective effect of dietary zinc on lead toxicity. They observed that over a seven week period, as dietary zinc increased, the severity of experimentally induced lead toxicity in rats decreased, and concluded that the protection was largely a result of the inhibition of lead absorption at the intestinal level, possibly due to competition of lead and zinc for binding sites on intestinal metallothionein. It is possible that chronic administration of zinc resulted in induction of intestinal zinc-metallothionein which bound lead in the mucosa or in the gut lumen, thus reducing its transfer into the body. Brewer *et al.* (1985) suggested that dietary zinc supplementation adequate to induce significant intestinal metallothionein could protect or offer partial protection against lead toxicity. It is important to recognise that dietary components may not only affect lead absorption directly by competition or the formation of various species, but also indirectly, possibly as a result of their effect on the gut wall and its secretions.

Besides affecting bioavailability, the complexing of lead with metallothionein may also affect tissue distribution. Previous studies with cadmium-metallothionein demonstrated that when administered in the ionic form, cadmium will target the liver, whereas when complexed with metallothionein, it targets the kidney (Cherian *et al.*, 1978; Cherian, 1979). Although not observed following the acute dosing regime in this study, a similar pattern may be observed following chronic dosing of lead as

lead-metallothionein. Indeed if lead, like cadmium, was to be transported directly to the kidney, following its oral administration as a metallothionein complex, it may induce renal disease in a shorter period of time than inorganic lead salts. It is suggested that besides the total amount of lead in meat, the form of the lead, especially in kidney and liver, should be taken into account when estimating provisional limits of lead in food.

5.5 Conclusion

To date, the importance of metallothionein as a likely ligand for lead has largely been ignored. The reduction in the absorption of the "Pb-X" peak (containing lead-metallothionein) demonstrated by Partridge (1986), together with the work presented in this chapter implies that metallothionein may play a protective role in the gut lumen by complexing with lead, thereby reducing its absorption. However, it is clear that further investigation into the behaviour of the lead-metallothionein complex in the gut lumen is necessary, and its effect on absorption studied further by employing the techniques of everted sac or *in vivo* gut perfusion.

Chapter 6

THE IMPORTANCE OF THE FORMATION OF SOLUBLE LEAD ION PAIRS ON THE INTESTINAL ABSORPTION OF LEAD

6.1 Introduction

Besides forming stable soluble complexes in which the various ligands are linked to the central ion by covalent forces (section 4.1), lead also associates with anions purely as a result of electrostatic interaction, forming entities with no net charge, and which are stable enough to persist despite collisions with solvent molecules (Robinson and Stokes, 1968; Petrucci, 1971). Such associations are termed ion pairs. In solution, both complexes and ion pairs (which may be grouped under the term "species") are constantly being formed and broken down, a process which eventually reaches a state of equilibrium. These equilibria may be described in terms of formation constants (also called stability or association constants). Large values of formation constants indicate stable species which dissociate slowly into ion and ligand. Conversely, species with a low formation constant rapidly dissociate into individual ion and ligand (Snoeyink and Jenkins, 1980). A knowledge of formation constant data may also be used to indicate the species most likely to be formed when competition between either a number of ligands for a single metal ion or a number of metal ions for a single ligand exists, in that the species with the largest stability constant will be formed preferentially. Even when a ligand has formed a stable complex with a metal ion in solution, addition of a second ligand with the potential to form a complex with a higher formation constant (i.e. a more stable complex) than that already present will cause dissociation of the original species, the second ligand drawing the ion into a new species with itself (Snoeyink and Jenkins, 1980).

The lead species formed in the gut following oral administration of a lead dose will therefore depend on the relative concentration of various ligands and ions and the stability constants for the various species. Two potential ligands found in high concentrations in the gut lumen are phosphate ions, derived from the glycocalyx where they are formed from the enzymic hydrolysis of ATP (Koenig and Vial, 1970), and the carbonate ion, which may be derived either from bicarbonate ions secreted in the pancreatic juice and bile (Keele and Neil, 1975), or may be formed at the surface of the intestine by the enzymic activity of carbonic anhydrase, or may appear as a result of simple diffusion from plasma to lumen (Partridge, 1986). Both anions are also prevalent in the diet and are known to form stable ion pair complexes with lead as demonstrated by their large formation constants compared with other potential lead ligands:

	log K (formation constant for ion pair) (Jackson and Sheiham, 1980)
$Pb^{2+} + CO_3^{2-} \rightleftharpoons PbCO_3^0$	+ 7.09
$Pb^{2+} + H^+ + PO_4^{3-} \rightleftharpoons PbHPO_4^0$	+ 15.45

where formation constant, $K = \frac{[complex]}{[metal\ ion]\ [ligand]}$

It is thus conceivable that a proportion of lead in the gut will be associated with carbonate and phosphate ions derived from both the diet and gut wall, as ion pairs. The effect of the formation of such species on the transport of lead across the intestine is consequently of interest.

Previous studies have successfully used the *in vitro* everted sac technique of Wilson and Wiseman (1954) to demonstrate the passive movement of lead into the serosal space (Blair *et al.*, 1979; Coogan, 1982; Heaven, 1985). However, despite the relative ease with which everted sacs can be prepared, the easy sampling of mucosal and serosal fluids and the ability to rigidly control conditions on either side of the intestinal wall, the technique requires the tissue to be isolated from blood and lymphatic systems and nervous and hormonal control, reducing the means for an adequate supply of oxygen and nutrients and allowing the accumulation of absorbed solutes and toxins, both of which may influence the absorption under investigation. Such problems can largely be overcome by adequate oxygenation of the ionic medium in which the sac is being incubated and by keeping the incubation times as short as possible, allowing useful investigations to be conducted (section 2.4.4). Alternatively, ion transport may be studied by an *in vivo* perfusion technique in which the intestine remains *in situ*, with mesenteric blood supply intact, largely overcoming the inherent problems of the everted sac technique. In this investigation, the *in vivo* perfusion technique described by Morton (1982) was used to compare the transport of lead from a Tris buffer in which the cationic species was predominant, with that from a Krebs Henseleit buffer in which the predominant lead species were lead carbonate and lead phosphate ion pairs.

6.2 Materials and methods

6.2.1 Chemicals

All chemicals required throughout this investigation were supplied as in sections 2.2.1 and 2.2.2. Gases were supplied as in section 2.2.1. "Hypnorm" (0.315mg/ml fentanyl citrate and 10mg/ml fluanisone) and Diazepam were supplied by B.G. Spencer Ltd., Lichfield, U.K..

6.2.2. Animals

As section 3.2.3.

6.2.3 Buffer solutions

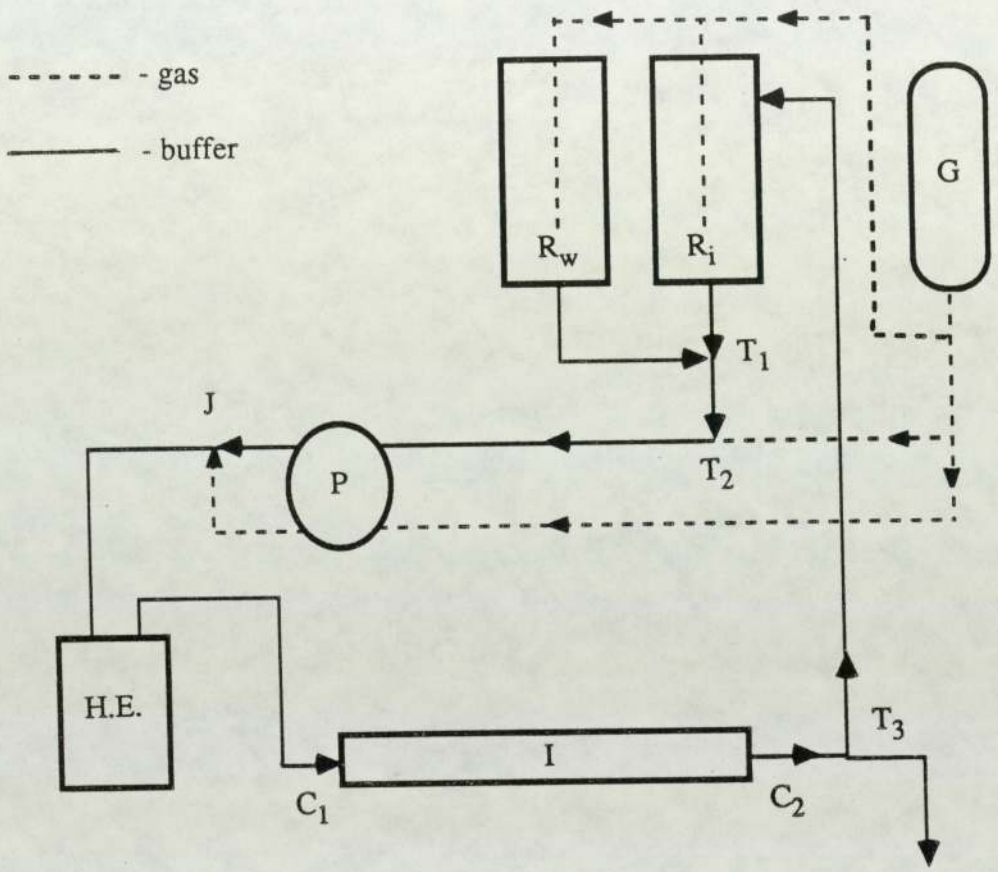
The composition of the Krebs-Henseleit bicarbonate buffer (K.H.B.) and the Tris buffer used in this investigation is detailed in appendices A.1.2 and A.1.3.

6.2.4 Development of the perfusion system

6.2.4.1 Apparatus

A diagrammatic representation of the apparatus is shown in figure 6.1. Two reservoirs with heating jackets, one containing the incubation buffer (R_i) and the other containing the washout buffer (R_w), were connected via two three way taps (T_1 and T_2), a peristaltic pump (P) and a heat exchanger (H.E.), to an inlet cannula C_1 inserted in the intestine at the ligature of Trietz (see section 6.2.4.2). The outlet cannula C_2 was placed approximately 20cm further along the jejunum, and was connected to the incubation reservoir R_i completing the perfusion circuit. Throughout the system, vinyl tubing (Portex Ltd., Kent, U.K.) with an inner diameter (i.d.) 3mm, and outer diameter (o.d.) of 4.25mm was used with the exception of the tubing used to gas the reservoirs, which was vinyl tubing with an i.d. of 1.5mm, and an o.d. of 2.1mm. The length of all connecting tubing was kept to a minimum to prevent excessive heat loss. The inlet and outlet cannulae were lengths of glass, 30mm long with an i.d. of 2mm and an o.d. of 3mm. One tip of each cannula was bevelled. Approximately 10mm from this tip was a slight constriction.

Figure 6.1 Diagram of the *in vivo* perfusion apparatus (Morton, 1982).



R_i - incubation reservoir

R_w - washout reservoir

$T_1 \rightarrow T_3$ - three-way taps

J - "T" junction

P - peristaltic pump

H.E. - heat exchanger

C_1 - inlet cannula

C_2 - outlet cannula

I - intestine (*in situ*)

G - gas supply

The incubation and washout buffers in reservoirs R_i and R_w were gassed directly, allowing adequate oxygenation. Gas also entered the system via a "T" junction placed approximately 2cms distal to the peristaltic pump at point J, causing segmented flow of the buffer and resulting in further oxygenation and mixing of buffers in the intestine. The three way tap T_2 allowed gas to enter the system enabling the gut lumen to be flushed out between consecutive perfusions.

The incubation and washout buffers were maintained at 37°C at the inlet cannula C_1 by circulating water at 45°C through the water jackets surrounding the reservoirs and by passing the buffer through a glass heat exchanger (H.E.), placed in a water bath at 45°C, prior to its entering the intestine. A lamp placed above the anaesthetised animal maintained the body temperature, which was monitored using a rectal thermometer. The intestine was protected from the heating effect of the lamp by positioning lint, saturated with isotonic saline, and backed with polythene, over the incision in the abdominal wall. The initial rectal temperature was 34°C and remained constant throughout the whole experimental period.

6.2.4.2 Cannulation of the proximal jejunum

Animals which had been fasted as described in section 3.2.3, were anaesthetised by an injection of "Hypnorm" administered intramuscularly into a hind-limb at a dose of 0.3-0.4mls/kg, followed by an intraperitoneal injection of Diazepam at a dose of 2.5mgs/kg. The abdomen was opened by a 3cm incision and the ligature of Trietz located. A small incision was made in the mesenteric membrane at the ligature of Trietz through which a length of silk suture (Sutures Ltd., Newtown, Powys, U.K.) was threaded and tied loosely. An incision in the intestine on the proximal side of the suture allowed the insertion of inlet cannula C_1 which was tied into place. A second incision was made on the distal side of a ligature placed round the jejunum approximately 20cm distal to the inlet cannula. The luminal contents of the intestine were washed out, onto a pad of cotton wool moistened with isotonic saline, with approximately 20mls of washout buffer at 37°C, introduced through the inlet cannula. The outlet cannula was inserted in the intestine through the second incision, and fastened into place. The cannulae were attached to the perfusion apparatus.

6.2.4.3 Determination of the viability of the perfusion system: the measurement of intestinal glucose uptake

Exactly 25mls of K.H.B. (pH 7.4) containing 20mM glucose was pipetted into the incubation reservoir R_i and at least 50mls of glucose free K.H.B. (pH 7.4) was pipetted into the washout reservoir R_w. Both buffers were gassed with a 95% oxygen 5% carbon dioxide mixture. The three way tap T_1 was positioned so as to draw perfusate from the incubation reservoir, T_2 was positioned to exclude entry of gas at this junction and T_3 was positioned so as to discard extraneous fluid in the intestine from the system. The peristaltic pump was set to run at 2mls/minute and switched on. The timing of the perfusion was started as the segmented buffer reached the inlet cannula, and the three way tap T_3 was switched to connect the outlet cannula with the incubation reservoir. Buffer returning to the incubation reservoir following perfusion was mixed by gasing. After 12 minutes 50 seconds, tap T_2 was turned to let the oxygen/carbon dioxide mixture into the system, and tap T_1 was positioned so K.H.B. minus glucose could be drawn from the washout reservoir. At time 13 minutes 20 seconds, T_2 was switched to stop the entry of gas into the system and allow washout buffer to be drawn from reservoir R_w. The plug of gas reached the inlet cannula at time 15 minutes and started to remove the incubation buffer. The washout buffer reached the inlet cannula at time 15 minutes 30 seconds and the lumen of the intestine was washed out for 10 minutes. Tap T_2 was then switched to introduce the oxygen/carbon dioxide mixture into the system and remove the washout medium from the lumen of the intestine. (The washout procedure and time was previously determined by perfusing a 20cm length of vinyl tubing (i.d. 3.0mm, o.d. 4.25mm) in place of the intestine. A glucose recovery of 95% ± 1% (mean ± standard error of means for three observations) was achieved with this washout procedure and time.)

The contents of the incubation reservoir were washed through tap T_1 into a 100ml volumetric flask which was then made up to volume. The perfusion was repeated following the introduction of a second 25mls of incubation buffer into the incubation

reservoir. A total of four consecutive 15 minute perfusions were performed with each rat. Following the last 15 minute perfusion, the rat was killed by cutting into the thoracic cavity. The length of intestine perfused was removed, washed out with 10mls of isotonic saline, cleaned of mesenteric tissue and placed in a drying oven at 80°C. The dry weight was measured until constant.

Perfusates were analysed for glucose by the assay detailed in appendix A.3.2. The luminal loss of glucose was calculated as the difference in the number of moles of glucose present in the incubation buffer before and after the 15 minute perfusion, and was expressed per gram dry weight of the intestine. A one way analysis of variance was used to determine whether there was a change in the luminal loss of glucose with time.

6.2.4.4 Results and discussion

A steady loss of glucose from the intestine over the four consecutive perfusion periods was the criterion used to determine whether the perfusion technique was valid (Steel *et al.*, 1981; Morton, 1982), and was demonstrated in this investigation (table 6.1). As the luminal loss of glucose remained at a steady state over the whole experimental period, the individual values for glucose loss over each 15 minute period for each animal were combined to give an overall mean (± standard error of mean) of 556 ± 33 μmoles glucose/g dry weight of intestine/15 minutes (n = 27).

6.2.4.5 Recovery of lead from the perfusion system

Exactly 25mls of K.H.B. containing 20mM glucose and $1x10^{-6}$M lead acetate labelled with 5μCi of lead-203, was circulated through the perfusion system as detailed in section 6.2.4.3 in which a 20cm length of vinyl tubing (i.d. 3.0mm, o.d. 4.25mm) was substituted for the intestine. After a 15 minute "incubation" followed by a 10 minute washout, the incubation reservoir was emptied and rinsed into a 100ml volumetric flask which was made up to volume. To determine the amount of lead adhering to the walls of the reservoir, the inner surface was washed with approximately 30mls of concentrated "Decon" (Fisons Ltd., Loughborough, U.K.), a chelating detergent, and carefully rinsed with distilled water. Washings were collected in a 100ml volumetric flask which was made up to volume. The "Decon" wash was found to have

Table 6.1 Luminal loss of glucose over four consecutive 15 minute periods.

Time periods (15 minute periods)	Luminal loss of glucose (μmoles glucose/g dry wt./15 mins.)
1	694 ± 61 (7)
2	520 ± 55 (7)
3	517 ± 70 (7)
4	483 ± 57 (6)

The rat proximal jejunum was perfused for four consecutive 15 minute periods with Krebs-Henseleit buffer (pH 7.4). Results are expressed as means ± standard error of means. The number of observations is given in brackets.

There was no change in luminal loss of glucose with time as demonstrated by one way analysis of variance.

removed all the lead bound to the inner surface of the incubation reservoir. The amount of lead removed from the reservoir by the two washes, and that adhering to the aspirator used to gas the incubation buffer, was determined by counting the emissions of lead-203 (section 2.2.4) and was expressed as a percentage of the lead in the original 25mls of incubation buffer.

Approximately 77% of lead was recovered from the system by simply washing the reservoir, 7% was removed from the inner surface of the incubation reservoir by the "Decon" wash, 6% was located on the aspirator and it therefore follows that approximately 10% of the lead adhered to the inner surface of the connective tubing. Determination of the luminal loss of radio-labelled lead from the buffer could not therefore be calculated from the difference in the amount of lead present in the buffer before and after perfusion, without taking into account the amount of lead bound to the apparatus. Consequently, after each 15 minute perfusion, following collection of the incubation buffer, the inner surface of the incubation reservoir was rinsed with "Decon" and washed thoroughly with distilled water and the washings counted, as was the aspirator. Subtracting the amount of lead recovered from the perfusion and from the apparatus, from that present at the start of the perfusion gave a value for the luminal loss of lead within approximately 10% of the actual value.

Viability studies identical to those described in section 6.2.4.3, except that the incubation reservoir was washed out with "Decon" followed by distilled water between consecutive perfusions, resulted in the steady loss of glucose from the intestine over the four consecutive perfusion periods. An overall mean (± standard error of mean) glucose loss, of 598 ± 22 μmoles glucose/g dry weight of intestine/15 minutes (n = 19) was obtained, a value not significantly different from that obtained in studies in which "Decon" was not used (section 6.2.4.4), when compared by Student's t-test. The use of detergent to remove lead bound to the reservoir did not, therefore, affect the viability of the preparation.

6.2.5 Comparison of the luminal loss and absorption of lead from the rat jejunum perfused with Krebs-Henseleit and Tris buffers

The perfusion procedure for both buffers was identical to that detailed in sections 6.2.4.3 and 6.2.4.5. Exactly 25mls of either Krebs-Henseleit or Tris buffer,

containing 20mM glucose (appendix A.1.2 and A.1.3) and $1x10^{-6}$M lead acetate labelled with 5μCi of lead-203, was perfused for 15 minutes followed by a 10 minute washout period with the appropriate glucose-free buffer (appendix A.1.2 and A.1.3). Perfusions with the Krebs-Henseleit bicarbonate buffer (K.H.B.) were gassed with a 95% oxygen/5% carbon dioxide mixture, whilst perfusions with the Tris buffer were gassed with 100% oxygen. Four consecutive perfusions were performed with each animal, the reservoir being washed out with "Decon" between each perfusion as described in section 6.2.4.5. The luminal loss of lead was calculated as described in section 6.2.4.5.

Following the final 15 minute perfusion, the rat was killed by cutting into the thoracic cavity. The length of perfused intestine was removed, washed out with 10mls of isotonic saline and assayed for lead using the L.K.B. CompuGamma (section 2.2.4), before its dry weight was determined (section 6.2.4.3). Various major organs were also removed, washed in isotonic saline and assayed for lead. The amount of lead in the organs was used to estimate the scale of lead absorption.

The effect of lead on the viability of the system was monitored by comparing the luminal loss of glucose from both K.H.B. and Tris buffer to that lost from lead-free perfusates (section 6.2.4.3). Similarly, possible changes in luminal loss of glucose or decreased intestine viability, as a result of perfusion with Tris buffer rather than K.H.B. could be monitored.

6.3 Results

6.3.1 Viability of the perfusion system following perfusion with lead

A steady luminal loss of glucose from the intestine over the four consecutive 15 minute periods was achieved irrespective of the buffer perfused. The overall mean glucose loss for the K.H.B. and Tris buffers was 532 ± 24 μmoles glucose/g dry wt/15 minutes (n=15) and 540 ± 24 μmoles glucose/g dry wt/15 minutes (n=15) respectively. Neither value was significantly different from that observed when lead-free K.H.B. was perfused and the reservoirs washed out with water only. It was concluded therefore that neither the presence of lead in the buffers, the washing of the reservoirs with "Decon" nor the use of a Tris buffer in place of K.H.B., caused a reduction in gut viability.

6.3.2 The loss of lead from the lumen and its transport into the body

The luminal loss of lead from the K.H.B. and Tris buffers remained in a steady state over the four consecutive perfusion periods (table 6.2) so the mean luminal loss of lead from each buffer was calculated (table 6.3). Comparison of the means by Student's t-test showed no significant difference in the luminal loss of lead. There was also no significant difference in the amount of lead bound to the intestines of the two experimental groups (10.0 ± 3.0ng/g dry weight, n=4 and 12.5 ± 1.0ng/g dry weight, n=4 for those perfused with K.H.B. and Tris respectively). However, significantly more lead entered the body, and subsequently accumulated in each of the organs examined, of the rats whose intestines had been perfused with the Tris buffer compared to those whose intestines had been perfused with K.H.B. (tables 6.4 and 6.5).

6.4 Discussion

Luminal loss of lead appeared to be independent of whether the major lead species present in the lumen was the hydrated cation (the species present in the Tris buffer) or the carbonate or phosphate ion pair (the species present in the K.H.B.). However, luminal loss does not only reflect loss by absorption but also loss due to the binding of lead to intestinal tissue (section 6.3.2), a process which may mask any differences in lead absorption resulting from the presence of the different lead species. It was also realised that differences in lead absorption may be too small to be detected when looking for changes in luminal loss at the nmole level. In the *in vitro* everted sac, lead transport is measured in pmoles (Coleman, 1979, Coogan, 1982, Heaven, 1985), and, assuming lead transport occurs to a similar degree *in vivo*, a 1000pmole difference in absorption between the two experimental groups would need to occur to produce a 1nmole difference in luminal loss. Therefore, in this study, absorption was also assessed by determining the amount of lead present in the internal organs. It was demonstrated that the intestinal absorption of lead was significantly reduced when the major lead species present in the lumen were the soluble carbonate and phosphate ion pairs rather than the hydrated cation (table 6.4). Before any conclusions can be drawn from this observation, it is necessary to determine the behaviour of the ion pair species in solution.

On formation of soluble ion pairs, an equilibrium is set up between the cation, anion and the ion pair species (see section 6.1). This equilibrium may be

Table 6.2 Comparison of lead lost from the lumen during four consecutive 15 minute perfusions with either Krebs-Henseleit buffer or Tris buffer containing $1x10^{-6}$M lead (pH 7.4).

Time periods (15 minute periods)	Mean lead lost from lumen nmoles/g dry wt/15 mins. Krebs buffer	Tris buffer
1	20.3 ± 2.5 (4)	17.5 ± 2.8 (3)
2	19.1 ± 3.3 (4)	18.5 ± 2.5 (3)
3	17.5 ± 0.5 (4)	18.2 ± 1.9 (4)
4	19.6 ± 3.1 (3)	19.5 ± 2.2 (4)

The rat proximal jejunum was perfused for four consecutive 15 minute periods with either Krebs-Henseleit buffer or Tris buffer (pH 7.4). Results are expressed as means ± standard error of means. The number of observations is given in brackets.

There was no change in luminal loss of lead with time as demonstrated by one way analysis of variance.

Table 6.3 Mean value for lead lost from the lumen during a 15 minute incubation period with either Krebs-Henseleit buffer or Tris buffer.

	Luminal loss of lead nmoles/g dry wt of intestine /15 mins	
Krebs buffer	19.1 ± 1.2	(n=15)
Tris buffer	18.5 ± 1.0	(n=14)

Results are expressed as means ± standard error of means. The number of observations is given in brackets.

No significant difference was found between the mean luminal lead loss for either experimental group.

Table 6.4 Total amount of lead present in the internal organs of rats following perfusion of the proximal jejunum with either Krebs-Henseleit buffer or Tris buffer containing $1x10^{-6}$M lead (pH 7.4).

	Total amount of lead in the internal organs (pmoles)	
Krebs buffer	41 ± 7	(n=4)
Tris buffer	600 ± 75	(n=4)

Results are expressed as means ± standard error of means. The number of observations is given in brackets.

The total amount of lead in the internal organs of rats perfused over four consecutive 15 minute periods with Tris buffer was significantly greater than in those of rats similarly perfused with Krebs-Henseleit buffer ($p<0.001$).

Table 6.5 Amount of lead present in various organs and associated blood following perfusion of the proximal jejunum with either Krebs-Henseleit buffer or Tris buffer containing $1x10^{-6}$M lead (pH 7.4).

	Lead within organ tissue (pmoles/organ)		
Organ	Krebs buffer	Tris buffer	
liver	28.5 ± 5.0 (4)	452.8 ± 44.8 (4)	**
kidney	9.5 ± 1.2 (4)	108.8 ± 27.6 (4)	*
spleen	0.9 ± 0.3 (4)	9.6 ± 1.2 (4)	**
heart	0.6 ± 0.2 (4)	8.7 ± 0.8 (4)	**
lung	1.4 ± 0.3 (4)	20.2 ± 3.0 (4)	**

Amount of lead present in various major organs after perfusion of the rat proximal jejunum after four consecutive 15 minute periods with either Krebs-Henseleit buffer or Tris buffer (pH 7.4). Results are expressed as means ± standard error of means. The number of observations is given in brackets.

* Denotes a difference between the two experimental groups ($p<0.02$)

** Denotes a difference between the two experimental groups ($p<0.001$)

described by the general equation:

$$\text{cation} + \text{anion} \rightleftharpoons \text{ion pair species} \qquad (1)$$

In both buffers, lead will form ion pairs with any anions present in solution. In K.H.B., the majority of lead will form stable, strongly associated, soluble ion pair complexes with carbonate and phosphate anions (as demonstrated by their large formation constants, see section 6.1). In contrast, the majority of lead in the Tris buffer is present as the hydrated cation. Initially it appears that the reduced transport of lead from the K.H.B. compared to the Tris buffer may therefore be due to the presence of lead as the ion pair species whose movement through the tight junctions between epithelial cells is slowed because of their large ionic radii. However, under normal physiological conditions the tight junctions are cation specific (Armstrong, 1987), and will therefore allow the transport of the free hydrated cation present in the lumen in preference to the chargeless ion pairs. Removal of the lead cation from the K.H.B., will disrupt the equilibrium described by equation 1, thereby causing it to move to the left, resulting in dissociation of the ion pair species. However, dissociation of the tightly associated lead carbonate and lead phosphate ion pairs will be slow, and it is therefore suggested that although the hydrated lead cation is the species that is transported, the rate of transport is limited by the rate of dissociation of the ion pairs present in solution. Both buffers will also contain a small proportion of lead as the soluble lead chloride and lead sulphate ion pair, but these species will have little or no effect in reducing lead absorption since they are weakly associated and rapidly dissociate on disruption of the equilibrium described previously. It is thought unlikely that the increased lead absorption from the Tris buffer is due to the formation of a rapidly transported Tris-lead complex. Tris is insoluble in lipids (Sigma Chemical Company Ltd., 1988), therefore if any Tris-lead complex had formed, it could not have been transported transcellularly. Paracellular transport of the complex would also have been unlikely for the same reasons outlined previously for the ion pairs. It may be concluded, therefore, that if any Tris-lead complex formed in the Tris buffer, it dissociated more rapidly than the lead carbonate or lead phosphate ion pair and therefore had no effect on the overall conclusions drawn from the experiment.

The solubility of lead salts in the gut is recognised as being the major determinant of lead absorption (Barton and Conrad, 1981; Aungst and Fung, 1983; Coleman *et al.*, 1983), dietary constituents enhancing the solubility of lead (such as

ascorbic acid and amino acids with sulphydryl groups) resulting in increased lead absorption (Conrad and Barton, 1978). However, the work presented in this chapter and in chapter 5 suggests that the type of soluble lead species present also influences lead transport. A reduction in the amount of lead in the gut as the free hydrated cation, or as weakly associated ion pairs, would seem to be advantageous in reducing lead absorption. The concentration of various lead ion pair complexes in the gut lumen and consequently the transport of lead will therefore depend on:

1. The type of lead species ingested

Holt *et al.* (1987) studied the effect of orally dosing different lead salts in solution on the intestinal absorption of lead. Salts with a high formation constant which form stable ion pairs in solution, such as lead phosphate and lead carbonate, resulted in reduced uptake of lead into the body, whereas salts with low formation constants, such as lead acetate and lead nitrate, which readily dissociate into anion and cation, resulted in increased lead uptake into the body. They concluded that the rate of transport of lead was determined by the rate of dissociation of the ion pair, the hydrated lead cation being the species which is transported. It appears therefore, that, contrary to a recent review (Nutrition Reviews, 1981) and the paper by Rabinowitz *et al.* (1980) in which it was suggested that gastric hydrochloric acid converts various lead compounds into a common species, namely lead chloride, different soluble lead salts may either survive the stomach, or may recombine following dissociation in the stomach, and exert different influences on the rate of lead absorption.

2. The luminal concentration of carbonate and phosphate anions

The type of soluble lead species in the gut does not depend entirely on the form in which they were administered. Once in the gut, weak ion pairs may dissociate, the lead cation forming stronger ion pairs with carbonate, phosphate and other anions depending on the relative concentration of each, as described in section 6.1. Increases in luminal carbonate and phosphate concentration will occur on ingestion of food which contains both anions, food which increases duodenal acidity resulting in profuse secretion of bicarbonate-rich pancreatic juice, and food which contains carbohydrates, fats and proteins which either increase luminal bicarbonate concentrations by increasing bile secretion or increase luminal phosphate levels by stimulating ATP hydrolysis at the plasma membrane. As a result, a portion of lead may be rendered unavailable for

transport either by being precipitated as lead carbonate or phosphate, or by forming the soluble lead carbonate or lead phosphate ion pair.

3. Luminal pH

Hilburn *et al.* (1980), demonstrated that, under increasingly acidic conditions, lead transport to the serosal compartment was increased in all regions of the intestine, the increase being significant in the jejunum and ileum at pH 4.4. Although the increased hydrogen concentration may have reduced the ionic charge in the extracellular channels, permitting more cation movement as suggested by Hilburn *et al.* (1980), the dissociation of the lead carbonate ion pair that occurs at acid pH (O'Shea and Mancy, 1978; Jackson and Sheiham, 1980) which would lead to increased concentrations of the transportable hydrated lead cation, may also have played a part in the increased transport. It is unlikely that increased lead transport *in vivo* will occur due to a fall in luminal pH to a value as low as 4.4 as normally, any fall in duodenal pH is compensated for by profuse secretion of bicarbonate-rich pancreatic juice (Hardy, 1981). However, metabolizable hexoses (Blair *et al.*, 1982) and other metabolizable substrates or substances which stimulate the breakdown of adenosine triphosphate (ATP) by mucosal ATPase, may indirectly increase lead transport by producing hydrogen ions in sufficient quantities to maintain or reduce further the pH of the region of high acidity between apical cell membrane and luminal contents existing in the glycocalyx (the acid microclimate (Blair and Matty, 1974), see section 1.5). This would allow dissociation of the non-transportable lead carbonate ion pair into the readily transportable hydrated cation.

6.5 Summary

The chemical form of lead in the gut is of great importance when determining its bioavailability. To date, much of the published work has regarded the absorption of lead as being dependent simply on the solubility of lead salts in the gut lumen. However the soluble lead species present in the gut are also important in controlling the absorption of lead. The hydrated lead cation appears to be transported in preference to soluble inorganic lead ion pair species. When present in the gut lumen as an ion pair, lead transport will only occur on dissociation of the complex, the transport of lead being limited by the rate of dissociation of the ion pair. The factors influencing the formation of soluble lead ion pairs in the gut lumen have been discussed.

Chapter 7

DISCUSSION

Lead is a ubiquitous metal whose deleterious effects on man has been recognised for centuries. The majority of lead absorbed by the body enters via the gastrointestinal tract and it is therefore necessary to determine not only how ingested lead transverses the gut wall, but also the factors which increase or decrease absorption.

7.1 Assessment of the suitability of the rat as an experimental model for humans

In these studies the rat was used as an experimental model for humans and consequently the limitations of such a model should be taken into account before discussing the experimental data. The major limitations in relating an animal model to the human condition were discussed in detail by Porter (1982). Discrete morphological differences exist between the gastrointestinal tract of rats and humans (summarisd in table 7.1) which may affect the transport of lead across the gut wall. Indeed, substantially less lead is absorbed from the intestine of rats compared to humans (on average approximately 1% compared to 10% (Kostial and Kello, 1979)). Such differences may not, however, be due solely to the differences in gut morphology between rat and humans, but also to differences in the diet. Studies by Kostial and Kello (1979) demonstrated that rats fed human diets absorbed similar amounts of lead compared to humans and it is therefore possible that the rat model is more useful when studying the effect of individual components of the human diet, rather than the effect of total diet. In doing this, however, there is the potential to over simplify the experimental system (Porter, 1982). Studying the effect of various dietary components in isolation allows determination of their individual effect on lead absorption, but provides limited information on the component when administered as part of a mixed diet when its effect on lead absorption may be increased or decreased by other dietary constituents. It is important that this be borne in mind when considering the experimental data.

7.2 The transport of lead across the intestine

Clinically evident lead poisoning often results from the absorption of lead across the gastrointestinal tract (Hilburn, 1979). To understand how food or other agents can alter the absorption of lead, it is necessary to consider the ways in which lead is transported across the intestinal wall. Studies by Blair *et al.* (1979) suggest that in the rat,

Table 7.1 Differences between the morphology of the intestine of rat and human (Porter, 1982).

1. The laboratory rat does not posess Kerkring folds which, in humans, increase the surface area three fold.
2. The villi of the human small intestine are longer than those of the rat.
3. The number of microvilli/surface area is greater in the rat (approximately $75/\mu^2$) compared with humans (approximately $50/\mu^2$)
4. The diameter of the lumen of the human small intestine is much greater than in the rat (2.5-4cms compared with 6-8mm respectively).
5. The rat has no gall bladder. The release of bile into the intestine is controlled by a sphincter at the doudenal end of the duct.*

Although the dimension differences are proportional to animal size, they may result in a different percentage of lead being absorbed by the two species.

* Saunders, J.T., Manton, S.M., (1969). A Manual of Practical Vertebrate Morphology. Revised by Manton, J.T., Brown, M.E., 4th edition. Clarendon Press, Oxford.

lead is transported into the serosal compartment by a process of passive diffusion, occurring at the same rate in all regions of the small intestine. However, Barton (1984) produced experimental data which suggested that lead is actively transported in the duodenum, but not in the jejunum or ileum of rats. The criterion for active transport was the movement of lead from the mucosal to serosal compartments against a concentration gradient, to give a [S]:[M] ratio significantly greater than unity. The existence of an active transport mechanism which is confined to a single region of the small intestine seems unlikely, so the work described by Barton (1984), which involved the incubation of viable everted duodenal sacs with equimolar concentrations of lead in the mucosal and serosal compartments, was repeated, his method being followed as closely as possible. No evidence for the active transport of lead was found. Barton reported [S]:[M] ratios of 2.56 and 6.06 after 60 and 120 minute incubations respectively, but in this study, sacs incubated for periods of up to 120 minutes showed no increase in [S]:[M] ratios above unity A number of reasons for the discrepancy between the results of Barton and those in this study are discussed in detail in section 2.4.2, but the primary reason for the inability to obtain similar results may have been due to inaccuracies in Barton's work, caused by binding of lead to the tissue. It is possible that [S]:[M] ratios greater than unity, may occur simply as a result of a fall in mucosal lead concentration due to tissue binding and a constant serosal lead concentration. It is clear that despite the suitability of the everted sac to study lead transport (section 2.4.4), the use of [S]:[M] ratios as a criterion for active transport of lead and other substances which bind to the intestinal tissue, is unsatisfactory.

The data in chapter 2 provides further evidence for the simple diffusion of lead across the small intestine and confirms the work of Blair *et al.* (1979). However, there is a discrepancy between this work and that of Aungst and Fung (1981b), who found that there was an active component involved in lead transport. It is possible that their results were confounded by gassing what were initially carbonate-free buffers with a $95\%O_2/5\%CO_2$ mixture, thereby inducing the formation of lead carbonate, a lead salt which is insoluble above a concentration of approximately $5x10^{-6}M$ (Coogan, 1982), and therefore untransportable .

7.3 Tissue distribution of lead in fed and fasted rats

The protective effect of food on lead absorption has been well documented both in humans (Heard and Chamberlain, 1982; Blake *et al.*, 1983; James *et al.*, 1985), and in rats (Heaven, 1985; Partridge, 1986), and was confirmed by the data in chapter 3. In the fed rat, the percentage of the dose administered entering the body (% T.D.A.) was 9% compared to 30% in the fasted rat. A greater percentage of the lead administered was found in the blood and within the tissue of the internal organs of fasted compared to fed rats, except the heart and lung, probably as these organs are thought not to accumulate lead. The main aim of the investigation was, however, to examine the tissue distribution of lead 4 hours after the administration of a lead dose, by taking into account the lead present in the blood in an attempt to improve the recovery of orally administered lead. Although there was a greater percentage of lead in the blood of both the fed and fasted animals than in any of the internal organs, a proportion of lead remained unaccounted for. It is likely that improved recoveries would have been achieved if the lead in bone and muscle had been accounted for, but, as described earlier (section 3.1), the quantity of lead in these potential reservoirs is difficult to assess. By determining the blood volumes of the organs, the amount of lead in each could be split into that present within the organ tissue itself, and that present in the blood within the organ. It was demonstrated that a large proportion of lead in the internal organs was due to the lead present in the blood within each organ. It is essential that during assessment of the potential of an organ to accumulate lead, or any other substance which is transported round the body by the blood system, the amount of substance present in the blood in the organ be taken into account.

7.4 The importance of competition and lead speciation on the transport of lead across the intestine

The whole body studies, discussed in section 7.3, demonstrated that the presence of food in the gut reduces lead absorption, but provides little information as to how this reduction is brought about. To date there have been a number of hypotheses:

1. Dietary status may cause alterations in the transport properties of the intestinal membrane

Aungst and Fung (1981a), demonstrated that lead flux across everted duodenum and proximal jejunum sacs from rats allowed food *ad libitum* was similar to

that of fasted rats, and concluded that dietary status does not affect the intrinsic transport properties of lead across the small intestine.

2. Feeding may reduce lead absorption by reducing gastric emptying and gastrointestinal motility (Aungst and Fung, 1981a; Coogan, 1982)

Coogan (1982) suggested that the stimulation of peristalsis following feeding may be responsible for reducing lead absorption by increasing the transit time of a lead dose thereby shortening the length of time the lead is in contact with the gut wall. Decreased peristalsis in the fasted rat would, he suggested, reduce transit time and consequently increase lead absorption. The experimental evidence in this chapter and that obtained by Heaven (1985), demonstrates that transit time is decreased following feeding, which, according to Coogan (1982), would suggest that intestinal lead absorption should be increased. It is not. It is likely that the reduction in lead transport observed following feeding is a result of the interaction of lead with components of food, resulting in a decrease in lead bioavailability either by competition with other cations for transport, or the formation of various non-transportable species.

In contrast to Coogan (1982), Aungst and Fung (1981a), suggested that the reduction in the rate of gastric emptying and gastrointestinal motility following feeding may contribute to the reduction of lead absorption seen in the fed compared to the fasted state. Propantheline, a drug which has an effect on the intestine consistent with that observed following the ingestion of food in that it causes a substantial reduction in gastric emptying and gastrointestinal motility, was administered to fasted rats. Less of an oral lead dose was absorbed in the treated compared to the untreated groups and led to the conclusion that in the fasted group, more lead may have precipitated in the less acidic intestinal fluids because of the delay in gastric emptying. The primary cause for reduced lead absorption was, therefore, the change in the chemical nature of the lead from a transportable to a non-transportable species.

3. The effect of competition and the formation of various non-transportable species on lead absorption

Both the diet and intestinal secretions provide many cations with which lead may compete for transport, and anions and ligands with which lead can complex. The formation of various lead species may enhance or reduce absorption by forming more or less soluble lead complexes, by increasing or decreasing the suitability of soluble lead for

transport by passive mechanisms, or possibly by increasing the amounts of lead able to be transported by active mechanisms. The factors which increase the absorption of lead are beyond the scope of this thesis, emphasis being placed on the factors which reduce lead absorption.

i. Competition

As stated in section 1.9.1, lead may compete with other cations such as calcium and iron for their respective sites on intestinal transport proteins (Barton *et al.*, 1978; Flanagan *et al.*, 1979). However, the absence of saturatable transport mechanisms suggests that lead transport is passive, and it is likely that competition occurs at the tight junction between epithelial cells. Ions known to be transported solely by the paracellular route are thought to reduce lead absorption by competing for anionic sites within the tight junctions, or competing for entry into the tight junctions from the bathing medium, or possibly a combination of both (Coogan, 1982). Similarly, the depressive effect of iron and calcium on lead transport may also occur at the tight junction in situations where the carriers for both cations are saturated, forcing transport of the ion by the paracellular route (Coogan, 1982).

Various cations may also compete with lead for ligands. A cation which forms a more stable species with a ligand than lead, will complex with that ligand in preference to lead. If the ligand is originally complexed with lead, the cation may cause dissociation of the species, drawing the ligand into a complex with itself. The competition between lead and certain cations may therefore affect lead speciation and may result in either increased or reduced lead bioavailability. Although the importance of competition in reducing lead absorption is recognised, it is not within the scope of this thesis, and will not be considered further.

ii. Precipitation

To be available for transport the lead cation requires to be in solution. Therefore, anything which reduces cation solubility reduces lead absorption. Lead binds readily with sulphur donors and phosphate, carboxyl and amino groups on biomolecules. Lead also forms stable complexes with carbonate and phosphate anions, found in high concentrations in the gut lumen. Phosphate ions are derived from the glycocalyx where they are formed from the enzymic hydrolysis of ATP (Koenig and Vial, 1970). Carbonate ions are derived either from bicarbonate ions secreted in the pancreatic juice

and bile (Keele and Neil, 1975), are formed at the surface of the intestine by the enzymic activity of carbonic anhydrase, or may appear as a result of simple diffusion from plasma to lumen (Partridge, 1986). Both anions are also prevalent in the diet. Lead carbonate, oxides, sulphides and organic complexes have been identified in the insoluble phase of gut washings of fed and fasted rats (Partridge, 1986), and it is the formation of these insoluble complexes in greater amounts in the fed compared to the fasted rat, which is thought to be mainly responsible for the dramatic decrease in lead absorption following feeding. Although the majority of insoluble lead in both fed and fasted rats was found to be present as the carbonate (Partridge, 1986), there appears to be no direct evidence as to the effect of the formation of this insoluble lead species on lead absorption. However, it has been suggested that the formation of insoluble lead phosphate was responsible for the decrease in lead absorption when the cation was administered to rats together with either high phosphate diets (Barton and Conrad, 1981), or prior to the administration of various phosphate-containing products (Aungst and Fung, 1983)

iii. Co-precipitation

Co-precipitation occurs when a substance in solution becomes insoluble through a chemical reaction and precipitates out of solution (forming the macrocomponent), carrying other substances, present in trace amounts, with it (the microcomponent). There are two types of co-precipitation that may occur. The first occurs when the trace substance has a similar charge and size to a constituent of the macrocomponent, thereby enabling it to enter its crystal lattice. The second type of co-precipitation involves ion-exchange or other chemical or physical adsorption of the microcomponent onto the surface of the precipitated macrocomponent. Lead co-precipitated by incorporation into the crystal lattice formation of the macrocomponent is rendered permanently unavailable for transport unless the macrocomponent itself is resolubilised. Conversely, lead co-precipitated by adsorption onto the surface of the macrocomponent is potentially available for exchange with the intestinal lumen, and may therefore be important in lead absorption.

Heard and Chamberlain (1982) suggested that co-precipitation of lead with calcium phosphate was responsible for reducing lead absorption by a factor of six, following simultaneous administration of calcium and phosphate salts, even though, when administered separately, they only reduced absorption by factors of 1.3 and 1.2

respectively. Indeed, the reduction in lead uptake observed with a calcium, phosphate and phytate-free glucose and alcoholic drink, and a low-mineral low-phytate meal was thought to be due to co-precipitation of lead with calcium phosphate formed from the calcium and phosphate salts secreted in saliva and gastric juice (James *et al.*, 1985) .

Co-precipitation of lead with calcium phytate is also thought to be an important determinant of lead absorption. Both calcium and phytate are important constituents of human vegetarian and cereal-based diets, although there is an average of only 0.9g phytic acid/person/day in the U.K. diet (Wise and Gilburt, 1981). Below pH 4, calcium phytate is soluble and therefore lead in the stomach would be in solution. However, *in vitro*, sodium phytate precipitates lead from calcium-containing solutions at pH 7 (Wise and Gilburt, 1981), a pH similar to that in the small intestine. It is this co-precipitation which probably accounts for the larger decrease in the deposition of an oral dose of lead in the tissues of rats administered both phytate and calcium, compared to that observed after calcium and phytate are administered separately (Rose and Quarterman, 1984).

At any one time, there may be a number of potential ligands present in the gut lumen, derived from the diet, saliva and gastric juice. Exactly which lead-ligand complexes are formed depends on the concentration of the various ligands, and the stability constants for the complexes. The characterization of the lead complexes formed in the small intestine is fundamental to the understanding of the various factors that affect lead absorption. To date, little data is available.

The various lead species formed in the insoluble phase of the gut washings of fed and fasted rats have been elucidated by Partridge (1986), as described earlier. However, the identification of the species present in the soluble phase of the gut washings is probably more important, since the lead is in solution and is potentially available for transport.

7.4.1 The identification of the various lead species formed in the soluble phase of gut washings from fed and fasted rats

Partridge (1986) used gel filtration chromatography to separate the lead species present in the soluble phase of the gut washings from either fed or fasted rats following an oral lead dose. The elution profile for a fed rat gave a single lead peak,

whilst that for a fasted rat also gave a second minor lead peak. He suggested that the large peak (named "Pb-Y") was a mixture of the lead cation and various lead-ligand complexes such as lead-histidine and lead-bile salts. The minor peak (named "Pb-X"), remained unidentified, but as it was transported less rapidly than the hydrated lead cation, it was suggested that it may play a protective role (Partridge, 1986).

Further investigation into the possible identity of the soluble lead species formed in fed and fasted rats was conducted using a method in which gut washing supernatants were incubated with lead *in vitro* before analysis by gel filtration chromatography on Sephadex G-15. The chromatographs obtained following incubations performed *in vitro*, compared favourably with those obtained *in vivo* (Partridge, 1986), thereby validating the *in vitro* technique. However it must be remembered that *in vivo*, the presence of food in the lumen of the fed rats will result in strong competition for ionic lead, and hence the actual concentration of lead available to form soluble lead species will be reduced. Of particular interest in this study was the elucidation of a possible identity for "Pb-X". Elution of the species at a position which indicated that its molecular weight was greater than 1,500, led to the suggestion that it may be a protein. A number of simple chemical tests were used to determine its identity.

1. The Lowry assay (Lowry *et al.*, 1951)

The Lowry assay demonstrated that the "Pb-X" peak contained a protein derived from the diet and gut wall in fed rats ("Pb-X" was identified in both incubations of stomach and gut washing supernatants from fed rats), and the gut wall only in fasted rats ("Pb-X" was identified only in the gut washing supernatant of fasted rats). There is no direct evidence to prove that the protein and lead in the first peak are bound, but considering both the high binding capacity of lead to proteins compared to other components of the gut wall whose molecular weights are in excess of 1,500, it is unlikely that there is another potential lead ligand eluted at this position. Peptides and amino acids were also detected in the "Pb-Y" peak, suggesting elution of lead-peptide or lead-amino acid complexes at this position.

2. Heating

Gut washing supernatants from fed and fasted rats were heated to 100°C for 2 minutes prior to analysis by gel filtration chromatography, to determine how strongly the lead and ligand in the "Pb-X" peak were complexed. The lead-protein complex from

fed rats dissociated on heating, whereas that from the fasted rat remained stable even after heating to a similar temperature for 10 minutes. A lead-protein complex also known to remain stable despite heating to 100°C is lead-metallothionein. Heating also caused a significant fall in the "Pb-Y" peak, and since there was no conclusive effect of heating on the protein profiles, it was assumed that the changes in "Pb-Y", were due to changes in inorganic lead chemistry. On heating, a lead-containing precipitate was formed. Soluble lead carbonate, which would be eluted in "Pb-Y", is known to be converted to insoluble basic lead carbonate when heated to 100°C, the removal of this species from solution by precipitation resulting in the reduction in the "Pb-Y" peak. Soluble lead phosphate is also insoluble in hot water, and although not eluted in "Pb-Y" (lead phosphate is insoluble in acetic acid and will therefore precipitate on the column), the removal of this species is thought to have caused the increase in column recoveries following heat treatment of the gut washing supernatants.

3. Addition of trichloroacetic acid (T.C.A.)

Metallothionein is resistant to precipitation by T.C.A. (a property exploited in the assay for metallothionein described by Piotrowski *et al.*, 1973). Since heating suggested that lead-metallothionein may be present in the gut washing supernatant of the fasted rat only, T.C.A. was only added to fasted rat gut washing supernatants prior to analysis by gel filtration chromatography. The T.C.A. caused a reduction in the amount of lead and protein eluted in the "Pb-X" peak, but did not remove the complex altogether. This provides further evidence to suggest that a lead-protein complex eluted in "Pb-X" is possibly lead-metallothionein.

Synthesis of the lead-metallothionein complex *in vitro*, by incubation of lead with metallothionein, allowed the complex's heat stability, stability on the gel filtration column and the elution position to be verified.

The molecular weights of the proteins eluted in the "Pb-X" peak were determined by re-chromatography on a Sephadex -75 column. The length of the isolation procedure for "Pb-X" meant that the lead associated with the protein in this peak had decayed, and it was not possible, therefore, to determine to which protein the lead had bound. Nevertheless, both before and after heating, proteins both with molecular weights both in excess of 80,000, and in the range 3,000 to 13,000 were eluted. One of the proteins in the second group could have been metallothionein, whose actual molecular

weight is approximately 6,600, although when determined by gel filtration chromatography, it is approximately 10,000.

The observations outlined here, and detailed in chapter 4, suggest that besides the main soluble inorganic lead species, namely lead carbonate and lead phosphate, lead is also present as a number of soluble lead-protein, lead-peptide and lead-amino acid complexes in both fed and fasted animals. In the fed animal, the identity of the lead-protein complex(es) remains unknown. In fasted animals, one of the protein ligands is thought to be metallothionein, which binds to lead after its release from the gut wall following desquamation of epithelial cells, or secretion in bile. The lack of lead-metallothionein in the fed rat does not necessarily suggest a lack of metallothionein in the gut lumen. In the fed rat, there may be competition between lead and dietary ions for binding sites on the protein, or competition with dietary proteins as potential ligands for lead.

7.4.2 The effect of the presence, in the gut lumen, of the various lead species elucidated by gel filtration chromatography, on the transport of lead across the gut wall

Having determined which lead species may be present in the soluble phase of gut washings from fed and fasted rats, the effect of their formation on the transport of lead across the gut wall was studied in the living animal.

1. Lead-metallothionein

The effect of the formation of lead-metallothionein species was studied by comparing its absorption with that of a comparable lead dose. Lead dosed with metallothionein appeared to be absorbed less readily than lead dosed as the cation, although the difference was not significant. It was estimated from chromatographic evidence, however, that only approximately 12-26% of lead administered in the test dose was actually bound to metallothionein, and the lead absorbed by this group of animals could be accounted for by the 74-88% of lead not bound to metallothionein. Conditions which increase the amount of lead-metallothionein in the small intestine, such as a reduction of stomach acidity (thereby preventing the dissociation of the complex that is known to occur at acid pH), chronic administration of lead-metallothionein or the administration of metallothionein to which larger amounts of lead than in this study have

bound, may therefore cause a more substantial reduction in lead absorption. It is likely, therefore, that lead-metallothionein formed intrinsically in the gut lumen may have a potent effect on lead absorption.

2. Soluble lead carbonate and lead phosphate ion pairs

The presence of large amounts of carbonate and phosphate ions in the gut lumen (section 7.4), and the great affinity of these potential ligands for lead (section 6.1), suggests that a proportion of the lead in the gut may be present as lead carbonate and lead phosphate ion pairs. Indeed, the presence of soluble lead carbonate and lead phosphate ion pairs was demonstrated during the speciation studies (section 7.4.1). The effect of the presence of these ion pairs in the gut lumen was studied by comparing the transport of lead from a Tris buffer, in which the hydrated lead cation was the predominant lead species, with that from Krebs-Henseleit buffer in which the predominant lead species were the lead carbonate and lead phosphate ion pairs. The *in vivo* perfusion technique of Morton (1982) was used throughout these studies. The presence of lead as the soluble ion pair species reduced lead absorption. Originally, it was thought that this reduction may occur because the ion pairs are not transported. However, since the tight junctions are cation specific, the soluble lead cation in equilibrium with the soluble ion pairs, would be transported, resulting in disruption of the equilibrium and dissociation of the ion pair. It appears likely that the reduced lead transport observed when in the presence of the soluble lead carbonate and lead phosphate ion pair is a result of the slow dissociation of these ion pair complexes. The dissociation of the ion pair complexes may be the rate limiting step in lead absorption.

Further evidence for the transport of lead as the hydrated cation, came from studies by Hilburn *et al.* (1980). They demonstrated that, under increasingly acidic conditions, lead transport to the serosal compartment was increased in all regions of the intestine, the increase being significant in the jejunum and ileum at pH 4.4. Although the increased hydrogen concentration may have reduced the ionic charge in the extracellular channels, permitting more cation movement as suggested by Hilburn *et al.* (1980), the dissociation of the lead carbonate ion pair that occurs at acid pH (O'Shea and Mancy, 1978; Jackson and Sheiham, 1980) which would lead to increased concentrations of the transportable hydrated lead cation, may also have played a part in the increased transport.

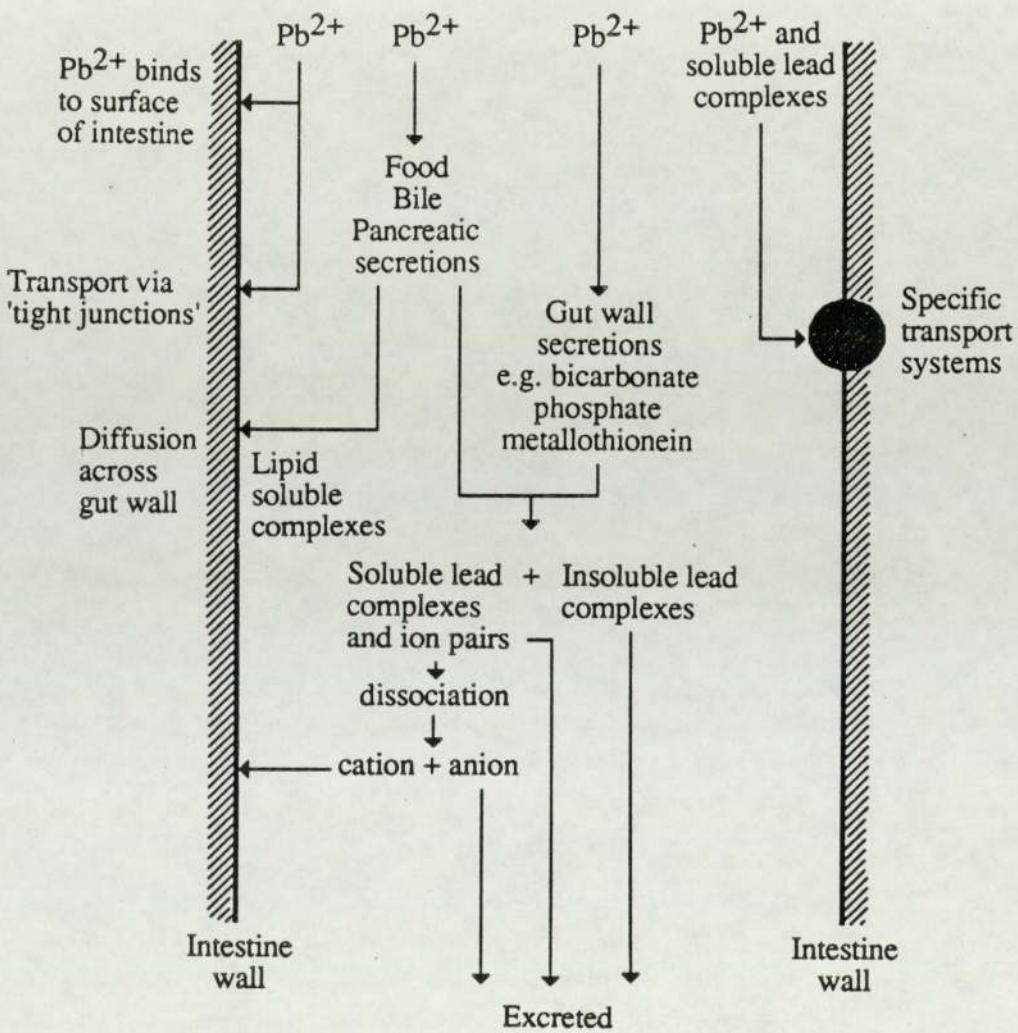
7.5 A proposed model for the speciation of lead in the small intestine

Lead may enter the intestine either as insoluble complexes, or soluble lead species such as lead-protein complexes or soluble ion pairs. A proportion of ingested lead will also enter the gut as the soluble hydrated cation, $Pb(H_2O)_4^{2+}$. A number of soluble lead species either survive the acidic pH of the stomach, or dissociate in the stomach and recombine in the intestine, and exert an effect on lead transport (Cherian, 1979; Holt *et al.*, 1987). However, the lead complexes ingested may not necessarily be those present in the gut lumen.

The lead species formed in the gut, following oral administration of a lead dose, will depend on the relative concentrations of the various ligands, the stability constants for the various species and the volume of gut fluids. On ingestion, weakly associated lead-ligand complexes may dissociate to form more strongly associated complexes. Strongly associated complexes may dissociate into cation and ligand, and insoluble lead complexes may be resolubilised especially if the volume of gut fluids is large. The effect of the presence of lead as various soluble species on lead absorption, demonstrated during this study, reinforces the importance of determining the identity of the soluble lead species present in the gut lumen.

A simplified view of the possible fate of lead on entering the gut lumen, is represented diagramatically in figure 7.1. A portion of lead binds covalently to the intestinal wall on entering the gut lumen, as has been shown by the everted sac experiments of Coleman (1979) and Coogan, (1982). This binding may reduce lead absorption by reducing luminal lead concentrations. However, some of the bound lead may be released from the gut wall when luminal lead concentrations fall and it appears, therefore, that the gut wall may act, to a certain extent, as a lead reservoir. A portion of ingested lead has also been shown to bind to the calcium and iron transport proteins (Barton *et al.*, 1978; Flanagan *et al.*, 1979). A model for the transport of lead, which includes both a carrier-mediated component and a minor passive component, has been suggested by Aungst and Fung (1981b), and the active lead transport in the duodenum has been suggested by Barton (1984). However, the experimental evidence does appear to suffer from a number of flaws as discussed in section 7.2, and the majority of evidence suggests that in the rat, lead is transported by a process of passive diffusion occurring through the tight junctions between epithelial cells (Blair *et al.*, 1979; Hilburn *et al.*,

Figure 7.1 The possible speciation and fate of lead in the small intestine



1980). The evidence presented in this thesis supports the view that lead is transported across the gut by a passive process. To date, little work has been done to investigate the possibility that lead may be actively transported when complexed to actively transported ligands such as amino acids, ascorbate and citrate (Coleman *et al.*, 1982).

A certain proportion of lead in the gut lumen forms insoluble and soluble lead species with ligands derived from the gut wall and the diet. The identity of the insoluble lead species in the gut was determined by Partridge (1986). He found the largest proportion of insoluble lead was present as lead carbonate, although significant amounts of lead phosphate and lead oxide were also present. These lead species are not transported and are excreted. The soluble lead species present in the gut lumen were determined in this study (chapter 4). Besides lead carbonate and lead phosphate ion pairs, lead-protein (including lead-metallothionein in the fasted rat), lead-peptide and lead-amino acid complexes were identified. The effect on lead absorption of the presence of soluble lead phosphate and lead carbonate ion pairs or the soluble lead-metallothionein complex in the gut lumen, suggested that these species are not transported themselves. Evidence that the transport of lead occurs through the cation specific tight junctions between epithelial cells (Coogan, 1982), suggested that the majority of lead is transported as the cation species. The data presented in chapter 6 led to the conclusion that the absorption and subsequent decrease in concentration of the hydrated lead cation caused dissociation of the soluble ion pair complexes into cation and anion, the rate of this dissociation being the limiting step in lead absorption. A similar explanation would account for the slight reduction in lead absorption observed when lead is administered as the stable lead-metallothionein complex rather than as the cation (chapter 5). In contrast, some lead complexes, such as lead-NTA lead-EDTA and lead-DTPA, may be lipid soluble and traverse the gut wall transcellularly (Coleman *et al.*, 1982).

In conclusion, although the presence of ligands affects lead transport by altering lead solubility, it appears from the *in vivo* absorption studies that various ligands may also affect lead absorption by forming different soluble lead-ligand complexes. Since lead transport occurs through cation specific tight junctions, any ligand reducing the concentration of the free hydrated cation in the gut lumen, whether by precipitation, co-precipitation or by forming stable, soluble complexes which do not readily dissociate into cation and ligand, will decrease lead absorption.

7.6 Further work

1. The identity of the soluble lead species formed in the gut washings of fed and fasted rats requires further work. Inorganic lead species may be identified by anodic stripping voltametry or an ion selective electrode, the lead protein species, by further isolation by gel filtration chromatography or by gel electrophoresis, and the amino acids, by H.P.L.C. or various appropriate spectrophotometric assays.

2. Improvements in the techniques for determining the lead species in rats may be extended to allow the lead species formed in the gut lumen of humans to be determined, possibly forming the species by *in vitro* incubation of gut washings with lead.

3. The identification of various lead species would allow their transport to be determined, initially in the rat, either by *in vivo* dosing, or *in vivo* perfusion techniques. In humans, the absorption of the various species could be determined by performing balance studies, in which the various species could be administered individually or as part of a mixed diet. Alteration of the concentration of the various lead ligands present in the human diet may give a clearer picture as to how the various ligands interact.

4. The distribution of lead throughout the body should be studied following its administration as a specific complex. When absorbed as a certain species, lead may target a specific organ in a similar manner to cadmium, which when present as the cation, targets the liver, but when present complexed to metallothionein, targets the kidney.

5. The effect of natural dietary components on lead absorption could be investigated so that ultimately, a diet or dietary supplement could be recommended whose constituents would reduce lead absorption, as a result of competition or the formation of non-transportable complexes. Such a diet or supplement could be used by sections of the population especially at risk from lead poisoning, such as children and workers in the lead industry.

APPENDICES

Appendix 1

Physiological buffers

The chemicals required for the buffers were obtained as in section 2.2.1. Gases were supplied as in section 2.2.1.

A.1.1 Buffers used by Barton (1984)

1. Washing solution (pH 7.4)

   sodium chloride, 0.146M; potassium chloride, 0.004M.

2. Incubation medium (pH 7.4)

   sodium chloride, 0.145M; calcium chloride, 0.1mM; D-mannose, 0.04M; Tris (Tris-(hydroxymethyl)-aminomethane), 4.0mM; lead chloride, $1x10^{-6}M$

The solution was adjusted to pH 7.4 with 1M hydrochloric acid.

Enough lead-203 was added to the buffer to give approximately 50,000 c.p.m./ml.

A.1.2 Krebs Henseleit bicarbonate buffer (Krebs and Henseleit, 1932)

The buffer refered to as K.H.B. contained:

sodium chloride, 112mM; potassium chloride, 4.5mM; calcium chloride, 2.4mM; potassium dihydrogen phosphate, 1.12mM; magnesium sulphate, 1.12mM; D-glucose, 20mM (when required).

The solution was adjusted to pH 7.4 with sodium bicarbonate (2.4mM), saturated with carbon dioxide until acidic to prevent the formation and precipitation of calcium carbonate. Incubations carried out with K.H.B. were gassed with a 95% oxygen 5% carbon dioxide mixture.

A.1.3 Tris buffer (Coogan, 1982)

The buffer refered to as the Tris buffer contained:

sodium chloride, 112mM; potassium chloride, 4.5mM; calcium chloride, 2.4mM; magnesium sulphate 1.12mM; Tris, 5.9mM; D-glucose, 20mM (when required).

The solution was adjusted to pH 7.4 with 1M hydrochloric acid. Incubations carried out with Tris buffer were gassed with 100% oxygen.

Appendix 2

The viability of the everted sac preparation

A.2.1 Introduction

Before being used for experimental work, it was necessary to assess the viability of the everted sac preparation throughout the experimental period. This was done by examining a number of normal tissue functions detailed by Armstrong (1987) and summarised below.

A.2.1.1 Water transport

Present evidence suggests that the movement of water across the intestine is a passive process linked to the active transport of sodium ions (Diamond and Bossert, 1967). These ions move into the mucosal epithelial cell down a concentration gradient, the intracellular sodium ions being actively pumped across the basolateral membrane into the interstitial space, causing an osmotic imbalance across the microvilli of the brush border. This is corrected by the flow of water through the cell membranes or through the tight junctions between adjacent cells (Diamond and Bossert, 1967; Levine *et al.*, 1970). A consequence of water absorption is an elevation in the hydrostatic pressure, which results in further transport of solutes and water into the serosal compartment. An everted sac showing water movement across to the serosal compartment comparable to previously published values (Coleman, 1979; Coogan, 1982) can therefore be considered as viable.

A.2.1.2 Glucose transport

The uptake of glucose is an active process requiring energy and it is believed that this energy is provided by a linked sodium ion transport system (Crane, 1968; Faust, 1975). It is thought that the glucose carrier has sites for the glucose molecules and the sodium ion, both of which must be filled before the transport of either can take place (Crane *et al.*, 1965). The energy required for the movement of the glucose molecule into the cell against its concentration gradient is provided by the movement of sodium into the epithelial cells down its concentration gradient (Crane *et al.*, 1965). Such a system relies on the structural integrity of the epithelial cells and hence, if it can be shown that glucose can be transported across the intestine against a concentration gradient to give a [S]:[M] ratio significantly different to unity, the sac preparation can be regarded as viable.

A.2.1.3 Transmural potential difference measurement

The presence of an electrical potential difference across the walls of the everted sac, as first demonstrated by Barry *et al.* (1961), is known to be due to an imbalance in ionic concentrations across the intestinal wall, a consequence of the active pumping of ions from the mucosal to the serosal compartment. Such a potential difference is especially associated with sodium ion transport and is stimulated by the presence of glucose (Barry *et al.*, 1964; Coleman, 1979; Coogan, 1982). The maintenance of a steady transmural potential difference which is comparable to previously published values (Coleman, 1979; Coogan, 1982) is therefore regarded as an indirect measurement of active ionic flow through the gut wall and hence is an indication of a viable preparation.

A.2.2 Materials and methods

A.2.2.1 Chemicals

All chemicals required throughout this investigation were supplied as detailed previously (section 2.2.1). Gases were supplied as detailed in section 2.2.1. "Inactin" (5-sec butyl-5-ethyl-2-thiobarbituric acid) was supplied by Promonta U.K..

A.2.2.2 Animals

As section 3.2.3.

A.2.2.3 Physiological buffer solution

All sacs were incubated in Krebs-Henseleit bicarbonate buffer (K.H.B.) containing 20mM glucose. The composition of this buffer is detailed in appendix A.1.2.

A.2.2.4 Preparation and incubation of the everted sac

Rats were anaesthetised by an intraperitoneal injection of "Inactin" in 0.9% saline to give a dose of 100mg/kg body weight. The abdomen was opened by a mid-line incision and the duodenum isolated by cutting just distal to the pyloric sphincter and at the ligature of Trietz. The section was flushed out with ice-cold oxygenated glucose free K.H.B. to remove debris. The rat was killed by cutting into the thoracic cavity.

A sac 6cm in length was made from the mid-duodenum, as described by

Wilson and Wiseman (1954). The section was everted over a glass rod, 3mm in diameter, tied at one end and weighed (w_1) on a torsion balance (White Electrical Instrument Co. Ltd., U.K.). Approximately 0.5mls of oxygenated K.H.B. containing 20mM glucose, was injected through the open end of the sac using a blunt needle attached to a hypodermic syringe and this end was then fastened with a cotton ligature. The sac was gently blotted on filter paper moistened with 0.9% saline and reweighed (w_2). This procedure took approximately two or three minutes. All sacs were incubated in 10mls of continuously oxygenated K.H.B., containing 20mM glucose, in a Tecam shaking water bath (Jennings Lab. Suppliers, Nottingham, U.K.) at 37°C and shaking at 80 oscillations/minute.

After incubation for between 10 and 60 minutes, the sac was again carefully blotted, weighed (w_3), emptied, drained, reblotted and reweighed (w_4). Fluid movement was calculated from the following equations:

$w_3 - w_2$ = total fluid uptake

$w_4 - w_1$ = fluid uptake by the tissue

$(w_3 - w_2) - (w_4 - w_1)$ = fluid transfer to the serosal compartment

Thread weights were found to be negligible compared to the sac weights.

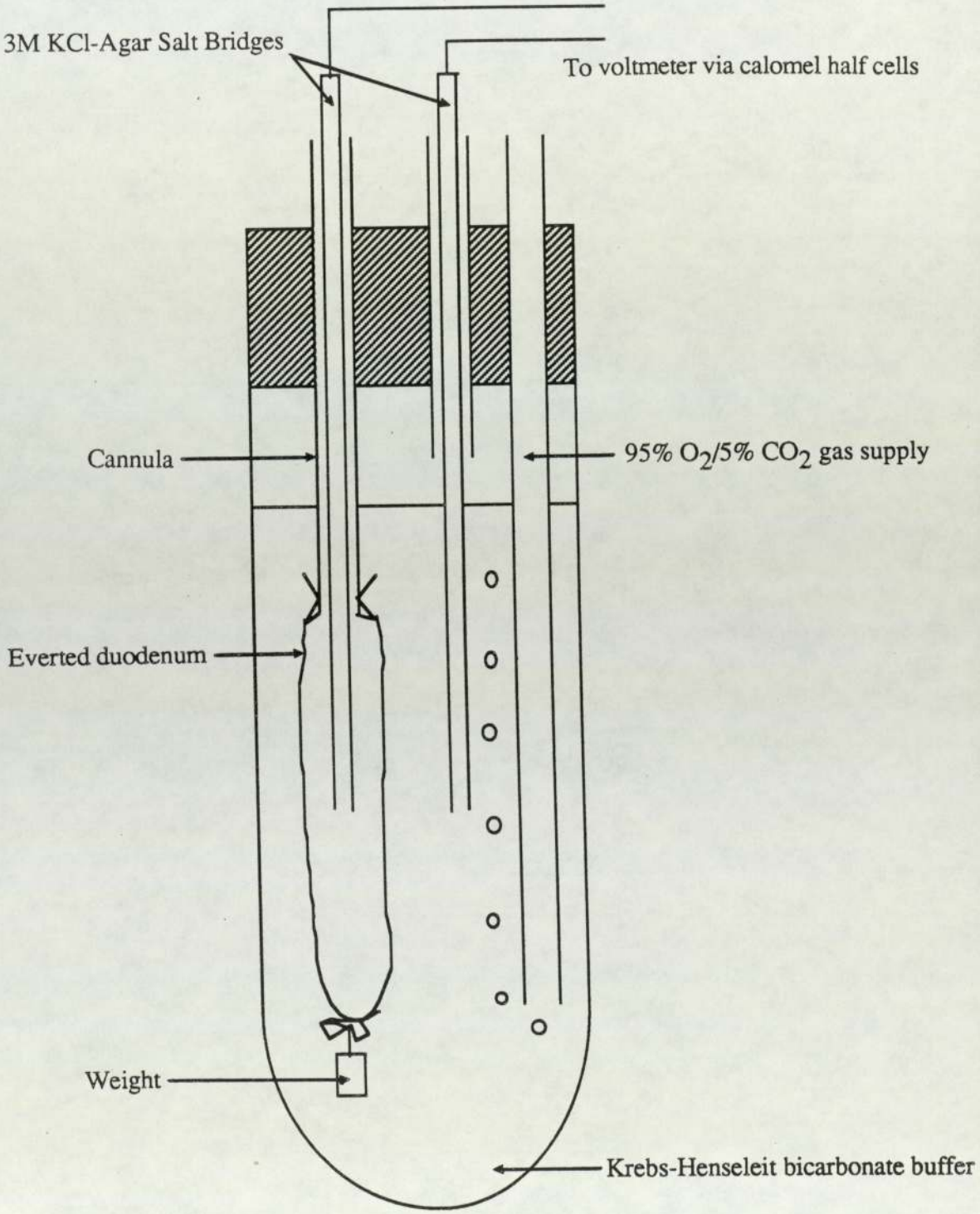
A.2.2.5 Determination of glucose in mucosal and serosal fluid

Mucosal and serosal fluid from sacs prepared as detailed in section A.2.2.4 and incubated for 60 minutes, were analysed for glucose using the assay described in appendix A.3.2.

A.2.2.6 Measurement of transmural potential difference

The method used to measure the potential difference across the intestinal wall was essentially that of Barry *et al.* (1964). A length of everted duodenum was ligated at one end and weighted with glass beads. A fluted glass cannula, 3mm in diameter was inserted into the other end and tied in place. The sac was filled with oxygenated K.H.B. containing 20mM glucose, via the cannula, (care being taken to exclude air bubbles from the system) and the sac was carefully lowered into 30mls of continuously oxygenating

Figure A.2.1 Apparatus for the measurement of transmural potential difference (Coleman, 1979).



K.H.B. containing 20mM glucose at 37°C (figure A.2.1).

A salt bridge was positioned on the serosal side of the sac via the cannula, and one on the mucosal side so both ends were level. The other ends of the bridges were placed in separate beakers containing saturated potassium chloride solution and calomel half cells. The potential difference was measured on a Pye Unicam Digital Voltmeter, readings being taken every 10 minutes for one hour.

The salt bridges consisted of polythene tubing filled with 5% Agar-Agar which had been dissolved in 3M potassium chloride solution at 60°C. The bridges were checked for air bubbles and tested between the calomel electrodes to ensure the reading across them was 0 ± 0.5 mV.

A.2.3 Results

A.2.3.1 Water transport

Over a 10-60 minute period, the rate at which fluid was removed from the mucosal buffer by the sac was linear (rate, 810µl/g wet weight/hour, figure A.2.2).

Fluid taken up by the tissue itself (figure A.2.3) was very rapid over the first 10 minutes of incubation but was slower over the period 10-60 minutes (linear rate, 312µl/g wet weight/hour). The initial rapid uptake is probably due to the entry of water into either the intracellular compartment (Jackson and Cassidy, 1970), or the extracellular space (Barry and Smyth, 1960).

Movement of fluid into the serosal compartment was not observed until the sac had been incubating for approximately 20 minutes (figure A.2.4). During the initial 20 minutes, fluid was lost from the serosal compartment of the sac, presumably taken up by the tissue. After 20 minutes, serosal transport proceeded linearly at a rate of 486µl/g wet weight/hour, a value approximately twice that of 200µl/g wet weight/hour observed by Coleman (1979), and substantially larger than that of 300µl/g wet weight/hour observed by Zavareh'ee (1979).

A.2.3.2 Glucose transport

After a 60 minute incubation, the sac had accumulated glucose on its serosal side against a concentration gradient as seen by a ratio of glucose in the serosal compartment to that in the mucosal compartment ([S]:[M]) of 1.23 ± 0.11 (n=8). This

Table A.2.1 Transport of water in the everted duodenal sac.

Incubation time (minutes)	n	Total fluid uptake	Fluid uptake by tissue	Transfer of fluid to serosal side
		(µl water/g wet tissue)		
10	3	155 ± 20	256 ± 34	-101 ± 30
20	7	305 ± 18	363 ± 18	-58 ± 7
30	3	499 ± 59	377 ± 46	122 ± 17
45	4	575 ± 63	443 ± 52	132 ± 16
60	4	838 ± 61	538 ± 44	300 ± 21

Water transport in the everted duodenum of the rat after incubation for various times in K.H.B. containing 20mM glucose (pH 7.4) at 37°C. Results are expressed as means ± standard error of means.

Figure A.2.2 Total fluid uptake by the everted duodenum of the rat, incubated for various times in K.H.B. (pH 7.4) at 37°C.

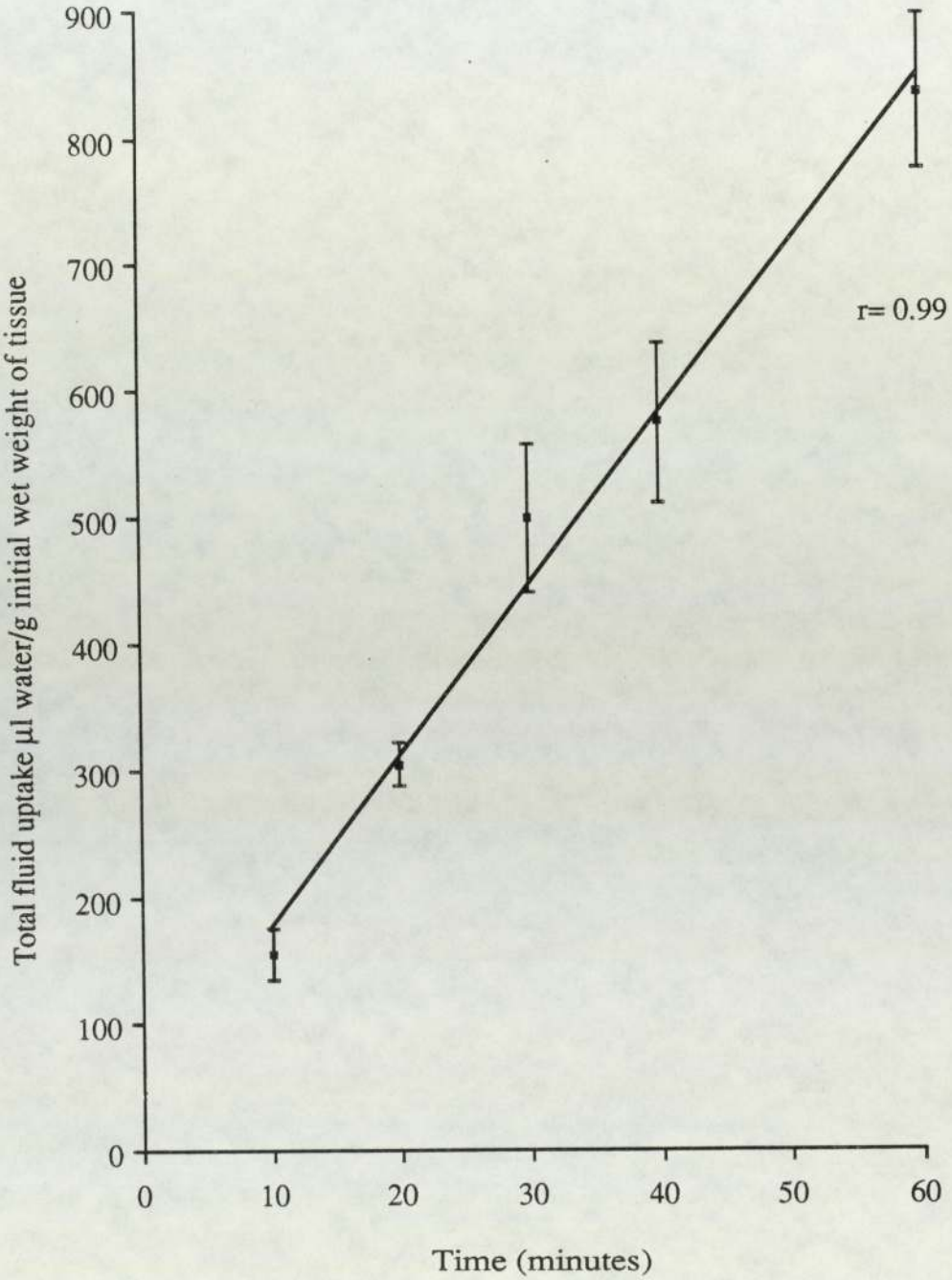


Figure A.2.3 Fluid uptake by the tissue of the everted duodenum of the rat, incubated for various times in K.H.B. (pH 7.4) at 37°C.

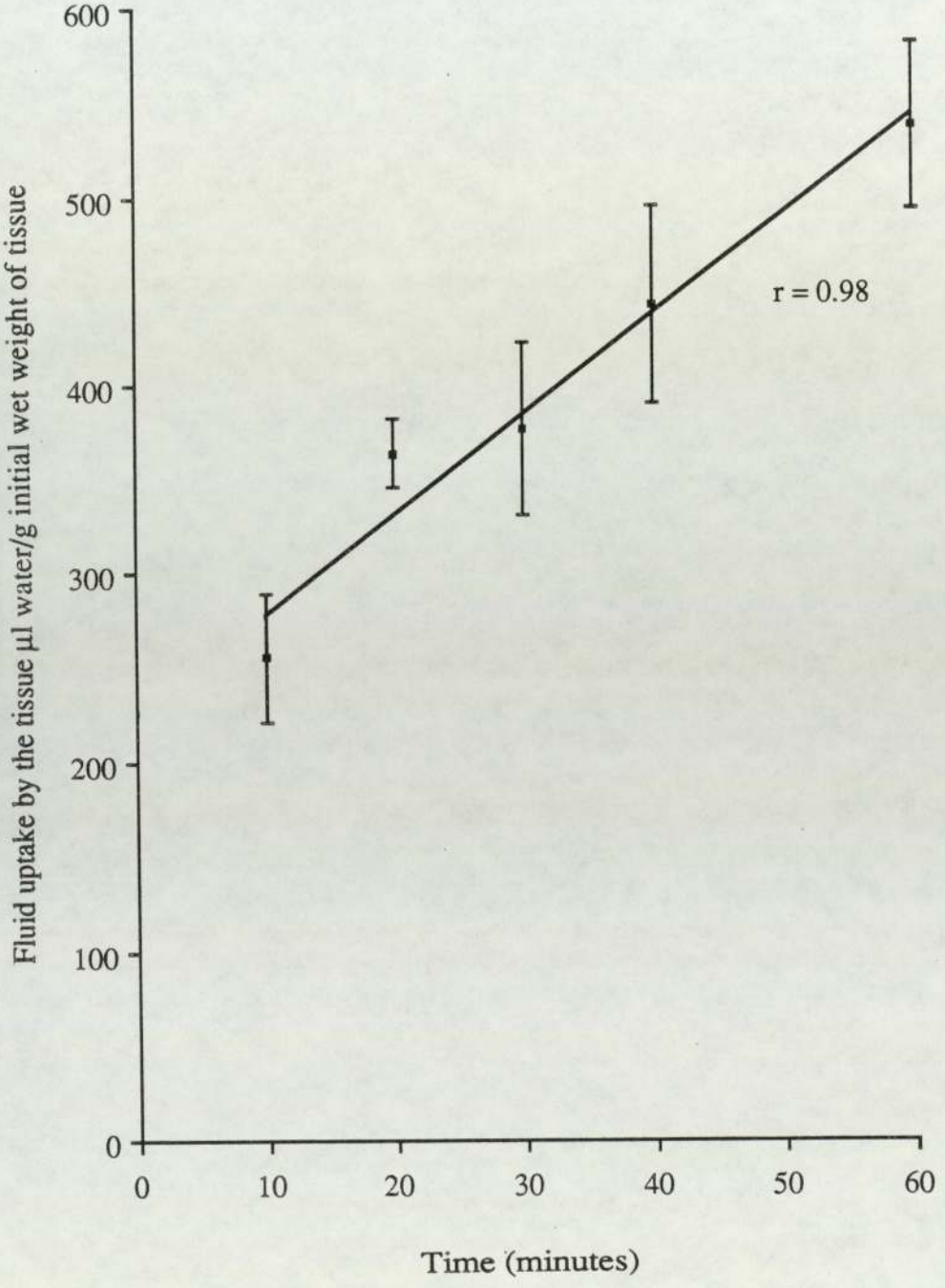
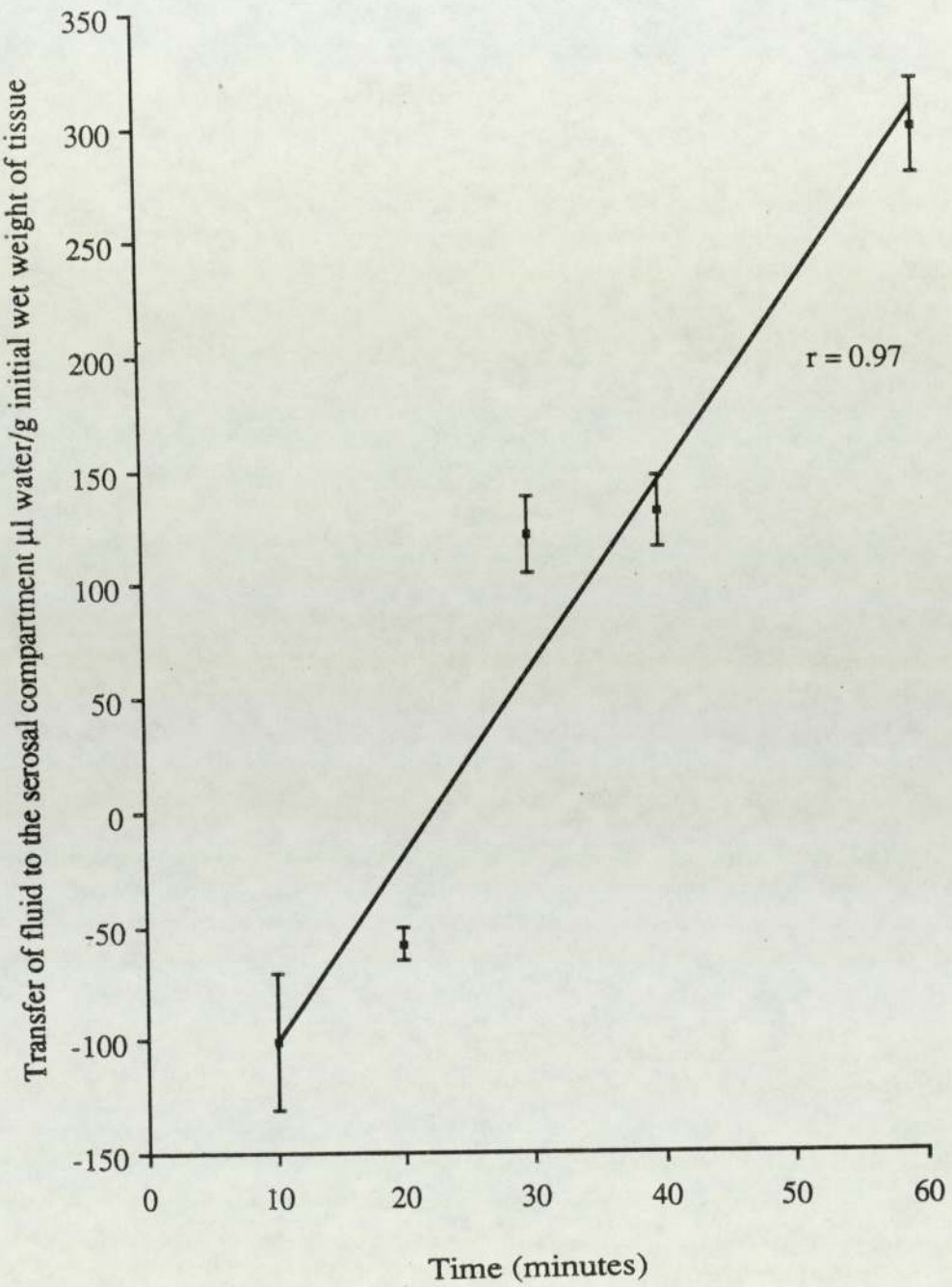


Figure A.2.4 Transfer of fluid to the serosal compartment of the everted duodenum of the rat, incubated for various times in K.H.B. (pH 7.4) at 37°C.



value is significantly different from one ($p<0.05$), and compared favourably with the values of 1.27 ± 0.04 and 1.1 ± 0.1 quoted by Coleman (1979) and Coogan (1982) respectively.

A.2.3.3 Transmural potential difference

Over the 60 minute incubation, the potential difference remained steady, there being no significant difference between the initial and final voltage (figure A.2.5). The mean voltage over the 60 minutes was 4.64 ± 0.08 (n=36), the serosal surface being positive with respect to the mucosal surface. This value compared favourably with those of 4.40 ± 0.33 and 4.4 ± 0.5 quoted by Coleman (1979) and Coogan (1982) respectively.

A.2.4 Discussion

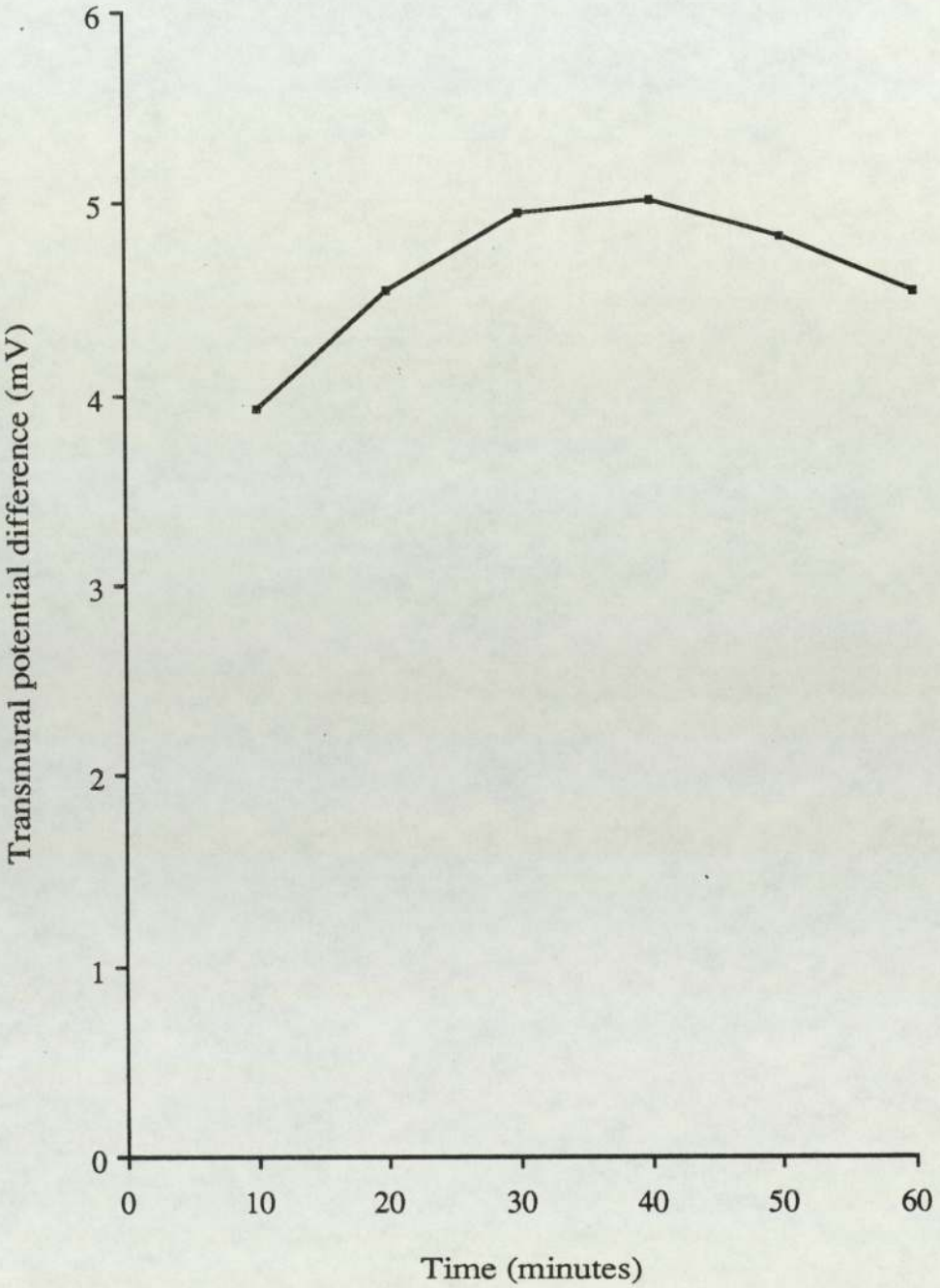
The everted sacs prepared were shown to fill the requirements for tissue viability detailed in section A.2.1. Over the 60 minute incubation period, water transport was linear, glucose was transported against a concentration gradient and a relatively steady transmural potential difference was observed. Values obtained for glucose transport and the transmural potential difference were comparable to those obtained previously (Coleman, 1979; Coogan, 1982).

The transport of water by everted sacs showed a similar trend to the sacs prepared by Coleman (1979) and Zavareh'ee (1979), although in this study the rate of water transport was greater over the 60 minute period. Differences in the rate of water transport may be due to differences in the hydrostatic pressure in the serosal compartment of the sac. A greatly distended sac will show reduced water transport to one which is only moderately distended, and it has been shown by Wilson and Wiseman (1954) that once a critical degree of distention is reached, fluid will start to move from serosal to mucosal compartment.

A.2.5 Conclusion

It has been demonstrated that sacs prepared by the method described in section A.2.2.4 remain physiologically viable for periods of up to 60 minutes and can therefore be used for the study of intestinal absorption.

Figure A.2.5 Change in transmural difference across the mid duodenum of the rat with time.



Appendix 3

Spectrophotometric assays

All absorption measurements were made at room temperature using either a Pye Unicam PU8800 UV/VIS Spectrophotometer, a Unicam SP1700 UV Spectrophotometer or a Shimadzu UV 240 Recording Spectrophotometer. Samples were contained in plastic cuvettes with a path length of 1cm.

All chemicals required for the assay reagents were obtained as detailed in section 2.2.1. Folin and Ciocalteu's phenol reagent was obtained from B.D.H. Ltd., Poole, U.K.. Enzymes used in the glucose assay were peroxidase type II (E.C. 1.11.1.7.) and glucose oxidase type VII (E.C. 1.1.3.4) and were supplied by Sigma Company Ltd., Poole, U.K..

A.3.1 Protein estimation

The presence of protein in the fractions eluted from the gel filtration columns was determined using the method of Lowry *et al.* (1951). To 0.4mls of the sample (containing up to 80µg of protein), was added 2mls of freshly prepared reagent. The reagent consisted of 50mls of 2% sodium bicarbonate in 0.1M sodium hydroxide plus 1ml of 0.5% copper sulphate in 1% sodium potassium tartrate, or multiples thereof. The sample and reagent were mixed and incubated at room temperature. After 10 minutes, 0.2mls of Folin and Ciocalteu's phenol reagent (diluted 1:1 with distilled water) was added to each sample which was mixed immediately and incubated at room temperature for a further 30 minutes. A reagent blank was prepared containing the assay reagents but substituting the protein sample with an identical volume of distilled water.

The optical density of the sample was measured at 750nm against a water blank. The extinction value obtained from the reagent blank was subtracted from those of the samples and standards. The protein was estimated by comparison of the absorbance of the samples with those of standard protein solutions of bovine serum albumin, made up and diluted in the appropriate buffer and containing up to 80µg of protein/0.4mls. A plot of absorbance against respective protein concentrations gave a calibration graph with a very shallow curve, but which passed through the origin.

A white precipitate formed following addition of the assay reagents to samples in a lead-containing buffer. As both reagents contain carbonate and phosphate

ions the precipitate was probably due to the formation of insoluble lead carbonate and lead phosphate. Such samples were cleared by centrifugation at top speed in a bench centrifuge for 5 minutes. The formation of the precipitate had no effect on the ability of the assay to accurately determine protein concentrations, the calibration curves comparing favourably with those prepared with lead-free buffers.

A.3.2 Glucose estimation

The assay for glucose determination was essentially that of Bergmeyer and Bernt (1974), modified by Morton (1982).

Exactly 2.5mls of freshly prepared reagent containing Tris-HCl buffer pH 7.0, 500mM; glucose oxidase, 70mg/l; peroxidase, 15mg/l and A.B.T.S. (2,2'-azino di-(3 ethyl benzthiazoline sulphonic acid)),1.14g/l, was added to 50µl of sample containing up to 25nmol of glucose. Sample and reagent were mixed and incubated for one hour at room temperature and the extinction read at 436nm using water as a reference. A reagent blank was prepared, containing assay reagents but substituting the glucose sample with an identical volume of water, and its extinction value subtracted from those of the samples and standards. A linear calibration curve, passing through the origin, related absorbance to glucose concentration for standards containing up to 500nmol glucose/ml.

REFERENCES

Albergoni, V., Piccinni, E., (1983).
Biological response to trace metals and their biochemical effects.
Trace element speciation in surface waters and its ecological implications. Ed., Leppard, G.G. NATO Conference series 1: Ecology, vol. 6, 159-175.
Plenum Press, New York, London.

Andrews, P., (1964).
Estimation of the molecular weights of proteins by Sephadex gel filtration.
Biochem. J., 91, 222-232

Armstrong, W.M., (1987).
Cellular mechanisms of ion transport in the small intestine.
Physiology of the Gastrointestinal Tract. 2nd edition Ed. Johnson, L.R., vol 2, 1251-1265.
Raven Press, New York.

Ashraf, M.A., Fosmire, G.J., (1985).
Effects of marginal zinc deficiency on subclinical lead toxicity in the neonate rat.
J. Nutr., 115, 334-346.

Aungst, B.J., Fung, H.L., (1981a).
Intestinal lead absorption in rats: effects of circadian rhythm, food, undernourishment and drugs which alter gastric emptying and gastrointestinal motility.
Res. Commu. Chem. Pathol. Pharmacol., 34, (3), 515-530.

Aungst, B.J., Fung, H.L., (1981b).
Kinetic characterization of *in vitro* lead transport across the rat small intestine. Mechanism of intestinal lead transport.
Toxicol. Appl. Pharmacol., 61, 39-47.

Aungst, B.J., Fung, H.L., (1983).
Inhibition of oral lead absorption in rats by phosphate-containing products.
J. Pharm. Sci., 72, (4), 345-348.

Aungst, B.J., Fung, H.L., (1985).
The effects of dietary calcium in lead absorption, distribution and elimination kinetics in rats.
J. Toxicol. Environ. Health, 16, (1), 147-159.

Aust, J.B., Chou, S.N., Marvin, J.F., Brackney, E.L., Moore, G.E., (1951).
A rapid method for clinical total blood volume determination using radioactive iodinated human serum albumin (RIHSA).
Proc. Soc. Exp. Biol. Med., 77, 514-518.

Baloh, R., (1974).
Laboratory diagnosis of increased lead absorption.
Arch. Environ. Health, 28, 198-208.

Barltrop, D., Khoo, H.E., (1976).
The influence of dietary minerals and fat on the absorption of lead.
Sci. Total Environ., 6, 265-273.

Barry, P.S.I., (1975).
A comparison of concentrations of lead in human tissues.
Br. J. Ind. Med., 32, 119-139.

Barry, R.J.C., Dikstein, S., Matthews, J., Smyth, D.H., (1961).
Electrical potentials in the isolated intestine.
J. Physiol., 155, 17-18.

Barry, R.J.C., Dikstein, S., Matthews, J., Smyth, D.H., Wright, E.M., (1964).
Electrical potentials associated with intestinal sugar transfer.
J. Physiol., 171, 316-338.

Barry, R.J.C., Smyth, D.H., (1960).
Transfer of short-chain fatty acids by the intestine.
J. Physiol. London, 152, 48-66.

Barton, J.C., (1984).
Active transport of lead-210 by everted segments of rat duodenum.
Am. J. Physiol.(Gastrointest. Liver Physiol. 10), 247, G193-G198.

Barton, J.C., Conrad, M.E., (1981).
Effect of phosphate on the absorption and retention of lead in the rat.
Am J. Clin. Nutr., 34, 2192-2198.

Barton, J.C., Conrad, M.E., Harrison, L., Nuby, S., (1978).
Effects of calcium on the absorption and retention of lead.
J. Lab. Clin. Med., 91, (3), 366-376.

Barton, J.C., Conrad, M.E., Harrison, L., Nuby, S., (1980).
Effects of vitamin D on the absorption and retention of lead.
Am. J. of Physiol. (Gastrointest., Liver Physiol.1), 238, G124-G130.

Beach, J.R., Henning, S.J., (1988).
The distribution of lead in milk and the fate of milk lead in the gastrointestinal tract of suckling rats.
Ped. Res., 23, (1), 58-62.

Bergmeyer, H.U., Bernt, E., (1974).
D-Glucose. Determination with glucose oxidase and peroxidase.
Methods of Enzymatic Analysis. Ed., Bergmeyer, H.U., vol. 3, 1205-1215.
2nd edition.
Verlag Chemie Weinheim (Academic Press Inc.), New York, San Fransisco, London.

Blair, J.A., Coleman, I.P.L., Hilburn, M.E., (1979).
The transport of the lead cation across the intestinal membrane.
J. Physiol., 286, 343-350.

Blair, J.A., Hilburn, M.E., Lucas, M.L., Said, H.M., (1982).
The effects of various metabolic inhibitors and substrates on the maintenance of surface pH *in vitro* of rat proximal jejunum.
Biochem. Soc. Trans., 10, 470-471.

Blair, J.A., Matty, A.J., (1974).
Acid microclimate in intestinal absorption.
Clinics in Gastroenterology, 3, (1), 183-197.

Blake, K.C.H., Barbezat, G.O., Mann, M., (1983).
Effect of dietary constituents on the gastrointestinal absorption of ^{203}Pb in man.
Environ. Res., 30, 182-187.

Blake, K.C.H., Mann, M., (1983).
Effect of calcium and phosphorus on the gastrointestinal absorption of ^{203}Pb in man.
Environ. Res., 30, 188-194.

Boeckx, R.L., (1986).
Lead poisoning in children.
Anal. Chem., 58, (2), 247A-287A.

Bratton, G.R., Zmudzki, J., Bell, M.C., Warnock, L.G., (1981).
Thiamin (vitamin B_1): effects on lead intoxication and deposition of lead in tissues: theraputic potential.
Toxicol. Appl. Pharmacol., 59, 164-172.

Bremner, I., (1987a).
Interactions between metallothionein and trace elements.
Prog. Food Nutr. Sci., 11, 1-37.

Bremner, I., (1987b).
Involvement of metallothionein in the hepatic metabolism of copper.
J. Nutr., 117, 19-29.

Bremner, I., Davies, N.T., (1975).
The induction of metallothionein in rat liver by zinc injection and restriction of food intake.
Biochem. J., 149, 733-738.

Bremner, I., Mehra, R.K., Morrison, J.N., Wood, A.M., (1986).
Effects of dietary copper suplementation of rats on the occurance of metallothionein-1 in liver and its secretion into blood, bile and urine.
Biochem. J., 235, 735-739.

Brewer, G.J., Hill, G.M., Dick, R.D., Prasad, A.S., Cossack, Z.T., (1985).
Interactions of trace elements: clinical significance.
J. Am. College Nutr., 4, 33-38.

Castellino, N., Aloj, S., (1964).
Kinetics of the distribution and excretion of lead in the rat.
Br. J. Ind. Med., 21, 308-314.

Cerklewski, F.L., (1984).
Postabsorptive effects of increased dietary zinc on toxicity and removal of tissue lead in rats.
J. Nutr., 114, 550-554.

Cerklewski, F.L., Forbes, R.M., (1976).
Influence of dietary zinc on lead toxicity in the rat.
J. Nutr., 106, (5), 689-696.

Chamberlain, A.C., (1985).
Prediction of response of blood lead to airborne and dietary lead from volunteer experiments with lead isotopes.
Proc. Royal Soc. London, B224, 149-182.

Chamberlain, A.C., Heard, M.J., Little, P., Newton, D., Wells, A.C., Wiffen, R.D., (1978)
Investigations into lead from motor vehicles.
A.E.R.E. - R9198, U.K. Atomic Energy Authority, Harwell, 101-110.
H.M.S.O., London.

Cherian, M.G., (1979).
Metabolism of orally administered cadmium-metallothionein in mice.
Environ. Health Perspect., 28, 127-130.

Cherian, M.G., Goyer, R.A., (1978).
Metallothioneins and their role in the metabolism and toxicity of metals.
Life Sciences, 23, (1), 1-10.

Cherian, M.G., Goyer, R.A., Valberg, L.S., (1978).
Gastrointestinal absorption and organ distribution of oral cadmium chloride and cadmium-metallothionein in mice.
J. Toxicol. Environ. Health, 4, 861-868.

Cikrt, M., (1970).
The uptake of Hg^{203}, Cu^{64}, Mn^{52} and Pb^{212} by the intestinal wall of the duodenal and ileal segment *in vitro*.
Int. J. Clin. Pharmacol., 4, 351-357.

Cikrt, M., Tichý, M., (1975).
Role of bile in intestinal absorption of ^{203}Pb in rats.
Experientia, 31, (11), 1320-1321.

Coleman, I.P.L., (1979).
The gastrointestinal absorption of lead.
PhD Thesis, University of Aston in Birmingham.

Coleman, I.P.L., Blair, J.A., Hilburn, M.E., (1982).
Effect of dietary and synthetic chelating agents on the intestinal absorption of lead.
Int. J. Environ. Stud., 18, 187-191.

Coleman, I.P.L., Blair, J.A., Hilburn, M.E., (1983).
A mechanistic approach to lead absorption studies.
Biochem. Ed., 11, (1), 18-22.

Conrad, M.E., Barton, J.C., (1978).
Factors affecting the absorption and excretion of lead in the rat.
Gastroenterology, 74, 731-740.

Coogan, M.J., (1982).
Analysis of a model to describe lead transport by the small intestine.
PhD Thesis, University of Aston in Birmingham.

Cousins, R.J., (1979).
Regulatory aspects of zinc metabolism in liver and intestine.
Nutr. Rev., 37, (4), 97-103.

Cousins, R.J., (1985).
Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin.
Physiol. Rev., 65, (2), 238-309.

Crane, R.K., (1968).
Absorption of sugars.
Handbook of Physiology. A Critical Comprehensive Presentation of Physiological Knowledge and Concepts. Ed., Code, C.F., Section 6, vol. 3, 1323-1351.
American Physiological Society, Washington D.C..

Crane, R.K., Forstner, G., Eicholz, A., (1965).
Studies on the mechanism of the intestinal absorption of sugars X. An effect of Na^+ concentration on the apparent Michaelis constants for intestinal sugar transport *in vitro*.
Biochem. Biophys. Acta., 109, 467-477.

Damstra, T., (1977).
Toxicological properties of lead.
Environ. Health Perspect., 19 , 297-307.

DeMichele, S.J., (1984).
Nutrition of lead.
Comp. Biochem. Physiol., 78A, (3), 401-408.

Department of the Environment, (1977).
Lead in drinking water - a survey in Great Britain 1975-1976.
Pollution Paper no. 12.
H.M.S.O., London.

Diamond, J.M., Bossert, W.H., (1967).
Standing-gradient osmotic flow. A mechanism for coupling of water and solute transport in epithelia.
J. Gen. Physiol., 50, 2061-2083.

Dunn, M.A., Blalock, T.L., Cousins, R.J., (1987).
Metallothionein (42525A).
Proc. Soc. Exp. Biol. Med., 185, 107-119.

Eaton, D.L., (1985).
Effects of various trace metals on the binding of cadmium to rat hepatic metallothionein determined by the Cd/haemoglobin affinity assay.
Toxicol. Appl. Pharmacol. 78, 158-162.

Farrar, G., (1988).
Personal communication.

Faust, R.G., (1975).
The intestinal brush border as an organelle
Intestinal Absorption and Malabsorption. Ed., Csaky, T.Z., 155-175.
Raven Press Publishers, New York.

Flanagan, P.R., Chamberlain, M.J., Valburg, L.S., (1982).
The relationship between iron and lead absorption in humans.
Am. J. Clin. Nutr., 36, 823-829.

Flanagan, P.R., Hamilton, D.L., Haist, J., Valberg, L.S., (1979).
Interrelationships between iron and lead absorption in iron-deficient mice.
Gastroenterology, 77, 1074-1081.

Foulkes, E.C., McMullen, D.M., (1986).
Endogenous metallothionein as determinant of intestinal cadmium absorption: a reevaluation.
Toxicology, 38, 285-291.

Freifelder, D., (1982).
Physical Biochemistry. Applications to Biochemistry and Molecular Biology.
2nd edition.
W.H. Freeman and Company, San Fransisco.

Garber, B.T., Wei, E., (1974).
Influence of dietary factors on the gastrointestinal absorption of lead.
Toxicol. Appl. Pharmacol., 27, 685-691.

Gerber, G.B., Deroo, J., (1975).
Absorption of radioactive lead (^{210}Pb) by different parts of the intestine in young and adult rats.
Environ. Physiol. Biochem., 5, 314-318.

Goyer, R.A., (1988).
Lead.
Handbook on Toxicity of Inorganic Compounds. Eds., Seiler, H.G., Sigel, H., 359-382.
Marcel Dekker Inc. New York, Basel.

Goyer, R.A., Rhyne, B.C., (1973).
Pathological effects of lead.
International Review of Experimental Pathology. Eds., Richter, G., Epstein, M.A., vol. 12, 1-77.
Academic Press, New York.

Gregersen, M.I., Rawson, R.A., (1959).
Blood volume.
Physiol. Rev., 39, 307-342.

Greninger, D., Kollonitsch, V., Kline, C.H., (1975).
Lead Chemicals.
International Lead Zinc Research Organization Inc., New York.

Grunden, N., Stantić, M., (1975).
Transfer of lead through the rat's intestinal wall.
Sci. Total Environ., 3, 288-292.

Guyton, A.C., (1982).
Human Physiology and Mechanisms of Disease.
3rd edition.
W.B. Saunders Co., Philadelphia, London, Toronto, Mexico City, Sidney, Tokyo.

Hamer, D.H., (1986).
Metallothionein.
Ann. Rev. Biochem., 55, 913-951.

Hamilton, W.F., (1962).
Handbook of Physiology, section 2, vol. 1, Circulation.
American Physiological Society, Washington D.C..

Hardy, R.N., (1981).
Endocrine Physiology.
Edward Arnold (Publishers) Ltd., London.

Harrison, R.M., Laxen, D.P.H., (1981).
Lead Pollution, Causes and Control.
Chapman and Hall, London, New York.

Heard, M.J., Chamberlain, A.C., (1982).
Effect of minerals and food on uptake of lead from the gastrointestinal tract in humans.
Human Toxicol., 1, 411-415.

Heaven, J.A., (1985).
The absorption of the lead cation from the intestine of the rat.
PhD Thesis, University of Aston in Birmingham.

Heilmaier, H.E., Schramel, P., Drasch, G.A., Kretschmer, E., Summer, K.H., (1987).
Speciation of trace elements in human tissues: role of metallothionein.
Trace-Element Analytical Chemistry in Medicine and Biology, Proceedings of the 4th International Workshop, Neuherburg. Eds., Brätter, P., Schramel, P., vol 4, 495-500.
Walter de Gruyter and Co., Berlin, New York.

Hejtmancik Jr., M.R., Dawson, E.B., Williams, B.J., (1982).
Tissue distribution of lead in rat pups nourished by lead-poisoned mothers.
J. Toxicol. Environ. Health, 9, 77-86.

Henning, S.J., Leeper, L.L., (1984).
Duodenal uptake of lead by suckling and weanling rats.
Biol. Neonate, 46, 27-35.

Hernberg, S., Nurminen, M., Hasan, J., (1967).
Nonrandom shortening of red cell survival times in men exposed to lead.
Environ. Res., 1, 247-261.

Hilburn, M.E., (1979).
Environmental lead in perspective.
Chem. Soc. Rev., 8, (1), 63-84.

Hilburn, M.E., Coleman, I.P.L., Blair, J.A., (1980).
Factors influencing the transport of lead across the small intestine of the rat.
Environ. Res., 23, 301-308.

Hileman, B., (1987).
Lead levels in water raise concerns anew.
Chemical and Engineereng News, 65, (51), 5.

Holt, A.C., Partridge, S., Morton, A.P., Blair, J.A., (1987).
The intestinal absorption of lead: the importance of lead speciation.
Proceedings of the 6th International Conference on Heavy Metals in the Environment, New Orleans. Eds., Lindberg, S.E., Hutchinson, T.C., vol. 2, 375-377.
C.E.P. Consultants Ltd., Edinburgh.

Horlock, P.L., Thakur, M.L., Watson, I.A., (1975).
Cyclotron produced lead-203.
Postgrad. Med. J., 51, 751-754.

Huang, K.C., Bondurant, J.H., (1956).
Simultaneous estimation of plasma volume, red cell volume and thiocyanate space in unanaesthetised normal and spleenectomized rats.
Am. J. Physiol., 185, 441-445.

Ikebuchi, H., Teshima, R., Suzuki, K., Terao, T., Yamane, Y., (1986).
Simultaneous induction of Pb-metallothionein-like protein and Zn-thionein in the liver of rats given lead acetate.
Biochem. J., 233, 541-546.

Jackson, M.J., Cassidy, M.M., (1970).
Studies on intestinal fluid transport II. The location of fluid accumulated in the wall of rat jejunum during incubation *in vitro*.
Biochim. Biophys. Acta., 211, 436-447.

Jackson, P.J., Sheiham, I., (1980).
Calculation of lead solubility in water.
Water Research Centre Technical Report TR152.
Water Research Centre Environmental Protection, Medmenham, Bucks..

James, H.M., Hilburn, M.E., Blair, J.A., (1985).
Effects of meals and meal times on uptake of lead from the gastrointestinal tract in humans.
Human Toxicol., 4, (4), 401-407.

Kägi, J.H.R., Himmelhoch, S.R., Whanger, P.D., Bethune, J.L., Vallee, B.L., (1974).
Equine hepatic and renal metallothioneins. Purification, molecular weight, amino acid composition and metal content.
J. Biol. Chem., 249, (11), 3537-3542.

Kägi, J.H.R., Vallee, B.L., (1961).
Metallothionein: a cadmium and zinc-containing protein from equine renal cortex. II Physicochemical properties.
J. Biol. Chem., 236, (9), 2435-2442.

Kägi, J.H.R., Vasak, M., Lerch, K., Gilg, D.E.O., Hunziker, P., Bernhard, W.R., Good, M., (1984).
Structure of mammalian metallothionein.
Environ. Health Perspect., 54, 93-103.

Karhausen, L., (1973).
Intestinal lead absorption.
Proceedings International Symposium: Environmental Health Aspects of Lead, Amsterdam (Netherlands), 427-440
The Commision of the European Communities Directorate General for Dissemination of Knowledge Centre for Information and Documentation - C.I.D., Luxembourg.

Keele, C.A., Neil, E., (1975).
Samson Wright's Applied Physiology.
12th edition.
Oxford University Press, London.

Kehoe, R.A., (1976).
Pharmacology and toxicology of heavy metals: lead.
Pharmacol. Ther., Part A, 1, 161-188.

Kello, D., Sugawara, N., Voner, C., Foulkes, E.C., (1979).
On the role of metallothionein in cadmium absorption by rat jejunum *in situ.*
Toxicology, 14, 199-208.

Klein, D., Greim, H., Summer, K.H., (1986).
Stability of metallothionein in gastric juice.
Toxicology, 41, 121-129.

Koenig, C.S., Vial, J.D., (1970).
A histochemical study of adenosine triphosphate in the toad (*Bufo spinulosus*) gastric mucosa.
J. Histochem. Cytochem., 18, 340-353.

Kostial, K., Kello, D., (1979).
Bioavailability of lead in rats fed "human" diets.
Bull. Environ. Contam. Toxicol., 21, 312-314.

Kotsonis, F.N., Klaassen, C.D., (1978).
The relationship of metallothionein to the toxicity of cadmium after prolonged oral administration to rats.
Toxicol. Appl. Pharmacol., 46, 39-54.

Kotsonis, F.N., Klaassen, C.D., (1981).
Metallothionein and its interactions with cadmium.
Cadmium in the Environment. Part 2, Health Effects. Ed., Nriagu, J.O., 595-616.
John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore.

Krebs, H.A., Henseleit, K., (1932).
Untersuchungen über die harnstoffbildung in tierkorper.
Hoppe-Seyler's Z Physiol. Chem., 210, 33-66.

Lawther, P., (1980).
Lead and health - report of a working party on lead in the environment.
H.M.S.O., London.

Levin, R., (1987).
Reducing lead in drinking water: a benefit analysis.
U.S. Environmental Protection Agency, E.P.A.-230-09-86-019.

Levine, R.R., McNary, W.F., Kornguth, P.J., LeBlanc, R., (1970).
Histological reevaluation of everted gut technique for studying intestinal absorption.
Europ. J. Pharmacol., 9, 211-219.

Lherminier, M., Alvarado, F., (1981).
Virtual elimination of the interference of unstirred water layers on intestinal sugar transport kinetics by use of the tissue accumulation method at appropriate shaking rates.
Pflügers Archiv., 389, 155-158.

Lilienthal, H., Winneke, G., Lenaerts, C., Hennekes, R., (1987).
Alterations of peripheral and central sensory potentials in lead exposed monkeys.
Proceedings of the 6th International Conference on Heavy Metals in the Environment, New Orleans. Eds., Lindberg, S.E., Hutchinson, T.C., vol. 1, 217-220.
C.E.P. Consultants Ltd., Edinburgh.

Little, P., Fleming, R.G., Heard, M.J., (1981).
Uptake of lead by vegetable foodstuffs during cooking.
Sci. Total Environ., 17, 111-131.

Louis-Ferdinand, R.T., Glass, L., Sharp, R., Beuthin, F.C., (1982).
Altered lead distribution in thiamine-treated rodents.
Toxicologist, 2, 82 (abs.).

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., (1951).
Protein measurement with the folin phenol reagent.
J. Biol. Chem., 193, 265-275.

Mahaffey, K.R., (1981).
Nutritional factors in lead poisoning.
Nutr. Rev., 39, (10), 353-362.

Maitani, T., Watahiki, A., Suzuki, K.T., (1986).
Induction of metallothionein after lead administration by three injection routes in mice.
Toxicol. Appl. Pharmacol., 83, 211-217.

Massey, R.C., Crews, H., Ebdon, L., Dean, J.R., (1988).
The effects of cooking and simulated gastro-intestinal digestion on the speciation of cadmium in pig kidney.
Trace-Element Analytical Chemistry in Medicine and Biology, Proceedings of the 5th International Workshop, Munich. Eds., Bratter, P., Schramel, P., vol 5.
Walter de Gruyter and Co., Berlin, New York. In Press.

Miller, G.D., Massaro, T.F., Granlund, R.W., Massaro, E.J., (1983).
Tissue distribution of lead in the neonatal rat exposed to multiple doses of lead acetate.
J. Toxicol. Environ. Health, 11, 121-128.

Monero, J.H., Diamond, J.M., (1975)
Nitrogenous cations as probes of permiation channels
J. Memb. Biol., 21, 197-259.

Moore, M.R., Hughes, M.A., Goldberg, D.J., (1979).
Lead absorption in man from dietary sources. The effect of cooking upon lead concentrations of certain foods and beverages.
Int. Arch. Occup. Environ. Health, 44, 81-90.

Morton, A.P., (1982).
Transport and metabolism in the gastrointestinal tract of diabetic animals: a study involving lean and (ob/ob) mice.
PhD Thesis, University of Aston in Birmingham.

Muller, L., Abel, J., Ohnesorge, F.K., (1986).
Absorption and distribution of cadmium (Cd), copper and zinc following oral subchronic low level administration to rats of different binding forms of cadmium (Cd-acetate, Cd-metallothionein, Cd-glutathione).
Toxicology, 39, 187-195.

Mykkänen, H.M., Dikerson, J.W.T., Lancaster, M.C., (1979).
Effect of age on the tissue distribution of lead in the rat.
Toxicol. Appl. Pharmacol., 51, 447-454.

Mylroie, A.A., Moore, L., Erogbogbo, U., (1977).
Influence of dietary factors on blood and tissue lead concentrations and lead toxicity.
Toxicol. Appl. Pharmacol., 41, 361-367.

Nutrition Reviews, (1981).
Nutritional Influences on lead absorption in man.
Nutr. Rev., 39, (10), 363-365.

Onoska, S., Cherian, M.G., (1981).
The induced synthesis of metallothionein in various tissues of rat in response to metals.
1. Effect of repeated injection of cadmium salts.
Toxicology, 22, 91-101.

O'Shea, T.A., Mancy, K.H., (1978).
The effect of pH and hardness metal ions on the competitive interaction between trace metal ions and inorganic and organic complexing agents found in natural waters.
Water Res., 12, 703-711.

Partridge, S., (1986).
The gastrointestinal absorption of lead.
PhD Thesis, Aston University.

Petering, H.G., (1980).
The influence of dietary zinc and copper on the biologic effects of orally ingested lead in the rat.
Ann. N.Y. Acad. Sci., 355, 298-308.

Petrucci, S., (1971).
Statistical thermodynamics of ionic association and complexation in dilute solutions of electrolytes.
Ionic Interactions: From Dilute Solutions to Fused Salts, vol. 1. Equilibrium and Mass Transport. Ed., Pettrucci, S., 117-177
Academic Press, New York, London.

Piotrowski, J.K., Bolanowska, W., Sapota, A., (1973).
Evaluation of metallothionein content in animal tissues.
Acta Biochim. Polonica, 20, (3), 207-215.

Pocock, S.J., (1980).
Factors influencing household water lead: a British national survey.
Arch. Environ. Health, 35, (1), 45-51.

Porter, J.L., (1982).
The intestinal absorption of the cadmium cation.
PhD Thesis, University of Aston in Birmingham.

Rabinowitz, M.B., Kopple, J.D., Wetherill, G.W., (1980).
Effect of food intake and fasting on gastrointestinal lead absorption in humans.
Am. J. Clin. Nutr., 33, 1784-1788.

Remington, R.D., Schork, M.A., (1970).
Statistics With Applications to the Biological and Health Sciences.
Prentice-Hall Inc., Englewood Cliffs, New Jersey.

Richards, M.P., Cousins, R.J., (1976).
Metallothionein and its relationship to the metabolism of dietary zinc in rats.
J. Nutr., 106, 1591-1599.

Rickard, D.T., Nriagu, J.O., (1978).
Aqueous environmental chemistry of lead.
The Biogeochemistry of Lead in the Environment. Ed., Nriagu, J.O. 119-283.
Elsevier/North-Holland Biomedical Press, Amsterdam.

Rieke, W.O., Everett, N.B., (1957).
Effect of pentobarbital anesthesia on the blood values of rat organs and tissues.
Am. J. Physiol., 188, 403-408.

Robinson, R.A., Stokes, R.H., (1968).
Electrolyte Solutions: The Measurement and Interpretation of Conductance, Chemical Potential and Diffusion in Solutions of Simple Electrolytes.
2nd edition (revised).
Butterworth and Co. (Publishers) Ltd., London.

Rose, H., Quarterman, J., (1984).
Effect of dietary phytic acid on lead and cadmium uptake and depletion in rats.
Environ. Res., 35, 482-489.

Rose, J., (1983).
Trace Elements in Health - A Review of Current Issues.
Butterworth and Co. (Publishers) Ltd., London.

Rutter, M., (1983).
Low level lead exposure: sources, effects and implications.
Lead Versus Health. Eds., Rutter, M., Jones, R.R., 333-370.
John Wiley and Sons Ltd., Chichester, New York, Brisbane, Toronto, Singapore.

Sasser, L.B., Hall, G.G., Bratton, G.R., Zmudzki, J., (1984).
Absorption and tissue distribution of lead in thiamin-replete and thiamin-deficient rats.
J. Nutr., 114, 1816-1825.

Sato, M., Bremner, I., (1984).
Biliary excretion of metallothionein and a possible degradation product in rats injected with copper and zinc.
Biochem. J., 223, 475-479.

Scheuhammer, A.M., Cherian, M.G., (1986).
Quantification of metallothioneins by a silver-saturation method.
Toxicol. Appl. Pharmacol., 82, 417-425.

Schroeder, H.A., Tipton, I.H., (1968).
The human body burden of lead.
Arch. Environ. Health, 17, 965-978.

Schwartz, J., Otto, D., (1987).
Blood lead and minor hearing impairment.
Proceedings of the 6th International Conference on Heavy Metals in the Environment, New Orleans. Eds., Lindberg, S.E., Hutchinson, T.C., vol. 1, 220-222.
C.E.P. Consultants Ltd., Edinburgh.

Sigma Chemical Company Ltd., (1986).
Gel filtration molecular markers.
Technical Bulletin GF-3.

Sigma Chemical Company Ltd., (1988).
Personal communication.

Six, K.M., Goyer, R.A., (1970).
Experimental enhancement of lead toxicity by low dietary calcium.
J. Lab. Clin. Med., 76, 933-942.

Smith, C.M., DeLuca, H.F., Tanaka, Y., Mahaffey, K.R., (1978).
Stimulation of lead absorption by vitamin D administration.
J. Nutr., 108, (5), 843-847.

Snedecor, G.W., Cochran, W.G., (1980).
Statistical Methods.
7th edition.
Iowa State University Press, U.S.A..

Snoeyink, V.L., Jenkins, D., (1980).
Water Chemistry.
John Wiley and Sons, New York, Chichester, Brisbane, Toronto.

Song, M.K., Adham, N.F., Ament, M.E., (1984).
Metabolism of zinc-binding ligands in rat small intestine.
Biol. Trace Element Res., 6, 181-193.

Southwood, T.R.E., (1983).
Royal Commission on Environmental Pollution, 9th Report - Lead in the Environment.
H.M.S.O., London.

Spickett, J.T., Bell, R.R., Stawell, J., Polan, S., (1984).
The influence of dietary citrate on the absorption and retention of orally ingested lead.
Agents and Actions, 15, (3/4), 459-462.

Squibb, K.S., Cousins, R.J., Silbon, B.L., Levin, S., (1976).
Liver and intestinal metallothionein: function in acute cadmium toxicity.
Exp. Mol. Pathol., 25, 163-171.

Steel, L., McMaster, D., Love, A.H.G., (1981).
A vascular perfusion technique for the study of zinc absorption.
Proceedings of the 4th International Symposium on Trace Element Metabolism in Man and Animals (TEMA-4), Perth, W. Australia. Eds., Gawthorne, J.M., Howell, J.McC., White,C.L., 117-120.
Springer-Verlag, Berlin, Heidelberg, New York.

Triplett, J.W., Hayden, T.L., McWhorter, L.K., Gautam, S.R., Kim, E.E., Bourne, D.W.A., (1985).
Determination of gallium concentration in "blood-free" tissue using a radiolabelled blood marker.
J. Pharmaceu. Sci., 74, (9), !007-1009.

Valberg, L.S., Haist, J., Cherian, M.G., Delaquerriere-Richardson, L., Goyer, R.A., (1977).
Cadmium-induced enteropathy: Comparative toxicity of cadmium chloride and cadmium-thionein.
J. Toxicol. Environ. Health, 2, 963-975.

Valberg, L.S., Sorbie, J., Hamilton, D.L., (1976).
Gastrointestinal metabolism of cadmium in experimental iron deficiency.
Am. J. Physiol., 231, (2), 462-467.

Vašák, M., Armitage, I., (1986).
Discussion summary. Nomenclature and possible evolutionary pathways of metallothionein and related proteins.
Environ. Health Perspect., 65, 215-216.

Waalkes, M.P., Harvey, M.J., Klaassen, C.D., (1984).
Relative *in vitro* affinity of hepatic metallothionein for metals
Toxicol. Letters, 20, 33-39.

Waldron, H.A., (1980).
Lead.
Metals in the Environment. Ed., Waldron, H.A., 155-197.
Academic Press Inc., (London), Ltd..

Wang, L., (1959).
Plasma volume, cell volume, total blood volume and F_{cells} factor in the normal and spleenectomized Sherman rat.
Am. J. Physiol., 196, (1), 188-192.

Webb, M., Cain, K., (1982).
Functions of metallothionein.
Biochem. Pharmacol., 31, (2), 137-142.

Whitaker, J.R., (1963).
Determination of molecular weights of proteins by gel filtration on Sephadex.
Anal. Chem., 35, (12), 1950-1953.

Wilson, T.H., Wiseman, G., (1954).
The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface.
J. Physiol., 123, 116-125.

Wise, A., Gilburt, D.J., (1981).
Binding of cadmium and lead to the calcium-phytate complex *in vitro*.
Toxicol. Letters, 9, 45-50.

Wiseman, R., Irving, C.C., (1963).
A rapid micro blood volume method in laboratory animals.
Proc. Soc. Exp. Biol. Med., 114, 728-731.

World Health Organisation, (1977).
Environmental Health Criteria 3: lead.
World Health Organisation, Geneva.

Zavareh'ee, A., (1979).
Absorption of chromium across the rat small intestine.
PhD Thesis, University of Aston in Birmingham.

Zelazowski, A.J., Piotrowski, J.K., (1977).
The levels of metallothionein-like proteins in animal tissues.
Experientia, 33, (12), 1624-1625.